Caracterización del mecanismo molecular por el cual la vía WNT/Bcatenina especifica el eje anteroposterior y controla la organogénesis en las planarias

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CARACTERIZACIÓN DEL MECANISMO MOLECULAR POR EL CUAL LA VÍA WNT/βCATENINA ESPECIFICA EL EJE ANTEROPOSTERIOR Y CONTROLA LA ORGANOGÉNESIS EN LAS PLANARIAS

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Imagen de portada y contraportada: Inmunotinción de un embrión de planaria en estadio 4, previo a la metamorfosis. βcatenina-1 (verde), Tubulina (rojo) marca los blastómeros, DAPI (azul) marca los nucleos.

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INTRODUCCIÓN

1. Información posicional, gradientes y organizadores

Uno de los problemas centrales en la biología del desarrollo es cómo se forman los patrones corporales. Uno de los conceptos que se ha utilizado para abordar este problema es el de información posicional, que plantea diferentes mecanismos para dar explicación a la formación de estos patrones corporales. El concepto de información posicional propone que las células adquieren identidades posicionales en un sistema coordinado. Luego interpretan sus posiciones para dar lugar a patrones espaciales (Wolpert, 2011). Probablemente, la forma más asumida de originar información posicional es mediante gradientes de señalización (Crick, 1970; Wolpert, 2011). Las primeras evidencias sobre la información posicional y los gradientes provienen principalmente de trabajos hechos en la extremidad del embrión de pollo, las hidras y la extremidad de insectos (French et al., 1976; Summerbell et al., 1973; Tickle et al., 1975; Wolpert et al., 1971).

Una forma sencilla de generar información posicional a través de un gradiente de señalización es mediante una única fuente de secreción. Esta fuente secreta una señal que se perderá por un sumidero que está lejos de la fuente. De esta manera se formará un gradiente exponencial de esta señal. La concentración de la señal es alta cerca de la fuente y baja cerca del sumidero. Así, se pueden originar diferentes niveles de señal (información posicional) que las células pueden interpretar (Figura1) (Meinhardt, 2009; Wolpert, 2011). Esta señal o señales que forman los gradientes se conocen como morfógenos (término acuñado por A. Turing).

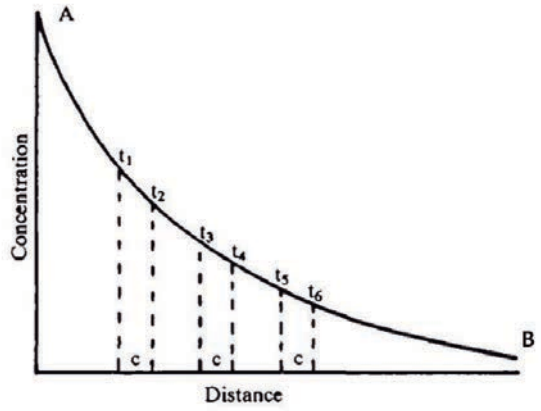


Figura 1. Diagrama que ilustra el mecanismo de información posicional que especifica tres regiones. El punto A representa la fuente y el punto B el sumidero. Las regiones especificadas (c) están delimitadas por 6 umbrales (t1-t6). [Adaptado de Wolpert, 1989].

Hace unos años se vio que los morfógenos son proteínas que difunden y que son secretadas al medio. Por tanto, mediante la difusión de estas proteínas se genera una diferencia de concentración que origina la información posicional (Wolpert, 2011). De hecho, se han descrito diferentes mecanismos para explicar cómo difunden estos morfógenos por el medio. Por ejemplo, a través de su secreción al medio de forma libre, mediante vesículas e incluso a través de citonemas (Langton et al., 2016). Recientemente, se ha demostrado que también se puede originar información posicional sin necesidad de secretar morfógenos (Alexandre et al., 2014).

La fuente que secreta estos morfógenos se denomina organizador o centro de señalización y se encarga de organizar un espacio. Mediante la secreción de un morfógeno, el organizador forma un gradiente, originando una información posicional que se traduce en un patrón espacial.

1.1 Formación de patrones y gradientes

En base a observaciones de formas de vida y relieves de la naturaleza, se han propuesto diferentes mecanismos sobre cómo se pueden generar estos patrones corporales o espaciales. Los gradientes de morfógeno se utilizan para explicar diferentes modelos/mecanismos sobre cómo se forman estos patrones corporales o espaciales, como por ejemplo el establecimiento de un eje corporal (Meinhardt, 2009). Algunos de los modelos se propusieron incluso antes de conocerse las moléculas que generaban estos gradientes. Alan Turing en los años 50 formuló un modelo de reacción-difusión con el cual podía explicar algunos patrones observados en la naturaleza, especialmente los patrones espaciales repetitivos, como los tentáculos de una hidra (Turing, 1952). Alan Turing propuso que substancias químicas (a las que denominó morfógenos) que reaccionan y difunden en un medio pueden volverse inestables dando lugar a pequeñas fluctuaciones espaciales. Si estas fluctuaciones se amplifican, pueden originar patrones espaciales de estas substancias químicas. Sobre estos patrones espaciales se podría dar el desarrollo de formas o estructuras de diferentes organismos. Actualmente su modelo se utiliza para explicar la formación de diferentes patrones corporales (Kondo and Miura, 2010), por ejemplo la formación de los dedos en tetrápodos (Sheth et al., 2012) o la pigmentación de la piel de diversos tipos de peces (Kondo and Asai, 1995; Nakamasu et al., 2009).

Diferentes autores han postulado mecanismos basados en gradientes de morfógenos, como es el caso de Gierer y Meinhardt. Estos autores propusieron un modelo basado en el de Alan Turing, según el cual un activador de corto alcance se retroalimenta promoviendo su producción. Además, el activador también "activaría" la producción de su antagonista, un inhibidor, el cual sería de largo alcance (Gierer and Meinhardt, 1972). De esta forma partiendo de un equilibrio se llegaría a formar un gradiente donde el activador estaría reprimido a largo alcance pero no a corto (Figura 2). Este mecanismo también se utiliza para explicar cómo se pueden generar fronteras entre tejidos (Meinhardt, 2009). Además, se han propuesto otros mecanismos más simples basados en gradientes de morfógeno. Por ejemplo, un modelo basado en una sola molécula en el cual el morfógeno estimula su propia degradación (Eldar et al., 2003).

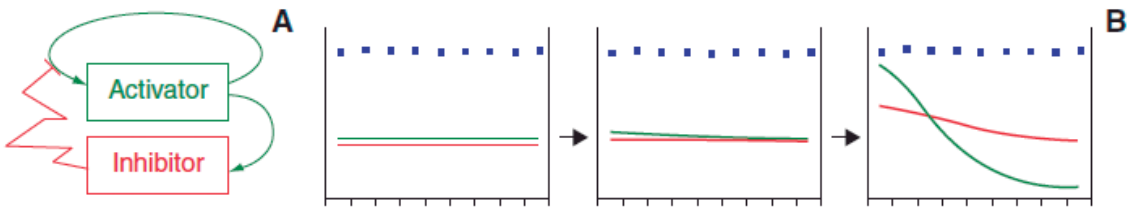


Figura 2. Modelo del activador de corto alcance - inhibidor de largo alcance. A) Esquema de la reacción. El activador promueve su propia producción y la de su inhibidor. El inhibidor tiene una difusión rápida. B) Partiendo de una distribución homogénea del activador (línea verde) y el inhibidor (línea roja), las fluctuaciones producidas de forma aleatoria (cuadrados azules) conducen a una diferencia de distribución de ambas moléculas. [Adaptado de Meinhardt, 2009].

Muchos de estos mecanismos/modelos explican cómo se forman diferentes estructuras corporales en base a estos gradientes, aunque la mayoría son aún teóricos y no se ha demostrado cómo sucede a nivel molecular. Una de las primeras evidencias de que una molécula se encuentra en forma de gradiente y que esto se traduce en una información posicional fue en el huevo de *Drosophila*. Esa molécula que actúa como morfógeno es la proteína Bicoid (Nüsslein-Volhard and Roth, 1989). Mediante diferentes experimentos se demostró que su incorrecto funcionamiento produce la pérdida de estructuras anteriores y medias del embrión de *Drosophila*. Estos datos relacionados con la localización de la proteína en forma de gradiente llevaron a proponer que en función de la cantidad de proteína Bicoid (morfógeno) se forman unas estructuras/identidades diferentes (Nüsslein-Volhard and Roth, 1989). En el caso del embrión de *Drosophila*, una alta concentración especifica segmentos anteriores. En cambio una concentración media/baja especifica segmentos centrales. El modelo que se usó para explicar este fenómeno fue el teorizado por Lewis Wolpert ("The French Flag Problem", Figura 3). Este modelo se propuso para explicar cómo se forman los dedos de la extremidad del pollo (Wolpert, 1969).

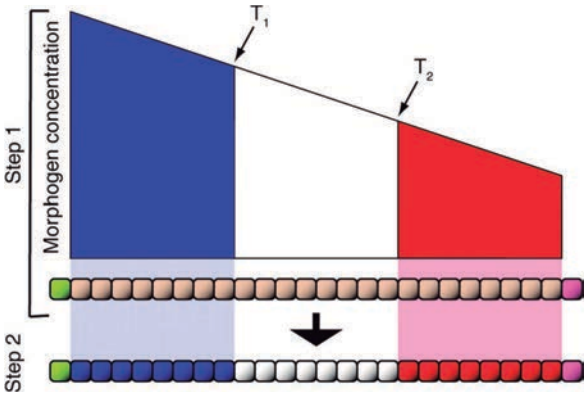


Figura 3. Modelo de la bandera francesa. Step 1: un morfógeno difunde produciendo un gradiente linear. Step 2: las células indiferenciadas detectan la concentración del morfógeno y se diferencian en función del umbral (T1 y T2) detectado. [Adaptado de Jaeger et al., 2008].

Sin embargo la realidad es más complicada, ya que hay que tener en cuenta diferentes variables, por ejemplo la densidad del medio. A día de hoy, ningún modelo puede reproducir cómo se forma el gradiente de Bicoid y cómo se origina la información posicional (Grimm et al., 2010). Si aún no existe un modelo claro para explicar cómo funciona el gradiente de Bicoid en una única célula, uno puede imaginarse lo difícil que puede ser explicar un gradiente en un tejido, donde encontramos diferentes obstáculos.

En el caso de la formación de un patrón en un tejido compuesto por más de una célula, el morfógeno debe viajar por el medio hidrofílico intercelular. Pero la mayoría de proteínas que se consideran morfógenos son proteínas unidas a un lípido, y esto hace más difícil explicar cómo viajan a través de un medio hidrofílico. Además, en muchos casos se desconoce como viajan o se secretan estos morfógenos. Por ejemplo, en el caso de los ligandos Wnt, uno de los morfógenos mas estudiados, no existe una respuesta única (Langton et al., 2016).

Debido a los diferentes obstáculos que un morfógeno secretable se encuentra en el espacio extracelular, se han propuesto diferentes mecanismos que originan gradientes sin necesidad de secretar nada al medio. Por ejemplo, a través de canales entre células o uniones de tipo Gap. Mediante estos canales, el morfógeno podría difundir fácilmente

(Levin, 2007). También podemos encontrar otros mecanismos basados en el tiempo en que aparece o desaparece una molécula, como el modelo del reloj y el frente de onda (en inglés, "Clock and wavefront model") (Cooke and Zeeman, 1976). Este modelo se ha aplicado para explicar la somitogénesis en vertebrados, tras observar que algunos genes como *c-hairy* oscilan de forma rítmica en el tiempo (Figura 4) (Palmeirim et al., 1997).

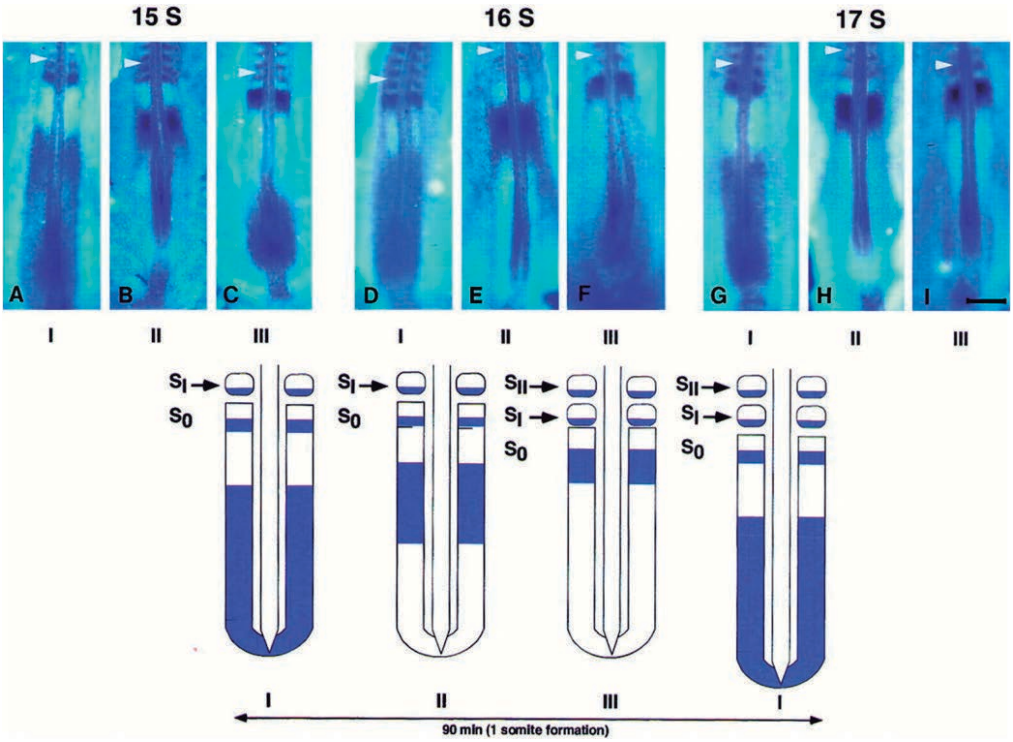


Figura 4. Oscilación periódica del gen *hairy* en el embrión de pollo que ayudó a proponer el modelo del reloj y el frente de onda. (Arriba) Hibridación *in situ* del gen *hairy* en embriones de estadio 15 (A-C), 16 (D-E) y 17 (G-I) de somitas. (Abajo) Representación esquemática de la correlación entre la expresión de *hairy* en el mesodermo presomítico con la progresión durante la formación de los somitas. S0 indica somita en formación. SI y SII indica somita formado 1 y 2 respectivamente. [Adaptado de Palmeirim et al., 1997].

1.2 Organizadores

En muchos casos, la información posicional y el patrón corporal se originan a partir de un organizador o centro de señalización. Un organizador, como su propio nombre indica, organiza regiones o tejidos, es decir, es capaz de controlar e inducir una respuesta en un conjunto de células dándoles una identidad específica. Hans Spemann y Hilde Mangold fueron los primeros en utilizar la palabra organizador para referirse a una pequeña zona del embrión de anfibios, en el labio dorsal del blastoporo. Cuando esta zona era trasplantada a otra región del embrión (incluso en un embrión de otra especie de anfibio) era capaz de reclutar las células del huésped y cambiar su destino celular. Como resultado se obtiene una duplicación del eje DV que se traduce en la formación de una imagen especular del embrión (Figura 5) (Spemann and Mangold, 1924).

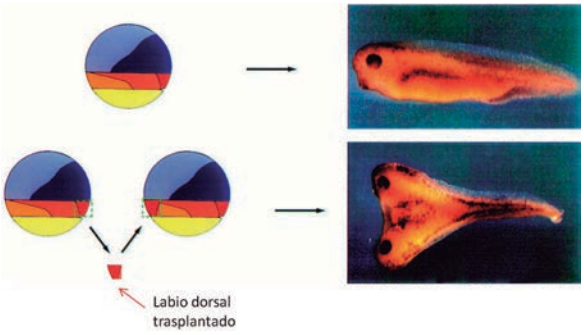


Figura 5. Reproducción del experimento de Spemann-Mangold. El trasplante del labio dorsal a la región ventral de un embrión provoca la inducción de un nuevo eje, causando la formación de un siamés. [Adaptado de Hemmati-Brivanlou y Melton, 1997].

A raíz de este descubrimiento se empezaron a describir regiones organizadoras en diferentes organismos, especialmente en embriones de vertebrados. Algunos ejemplos son el nódulo de Hensen en el embrión de pollo (Waddington, 1932; Waddington, 1933), el nódulo en el embrión de ratón (Beddington, 1994) o el escudo en el embrión de pez cebra (Oppenheimer, 1936; Shih and Fraser, 1996). Todos ellos comparten las mismas características: son regiones que al ser trasplantadas a otro lugar del embrión, incluso de especies diferentes, son capaces de reclutar y patronear a las células adyacentes (propias o del huésped) (Figura 6). La mayoría de estos organizadores son transitorios, es decir, aparecen en un determinado momento y más tarde en el desarrollo pierden su actividad.

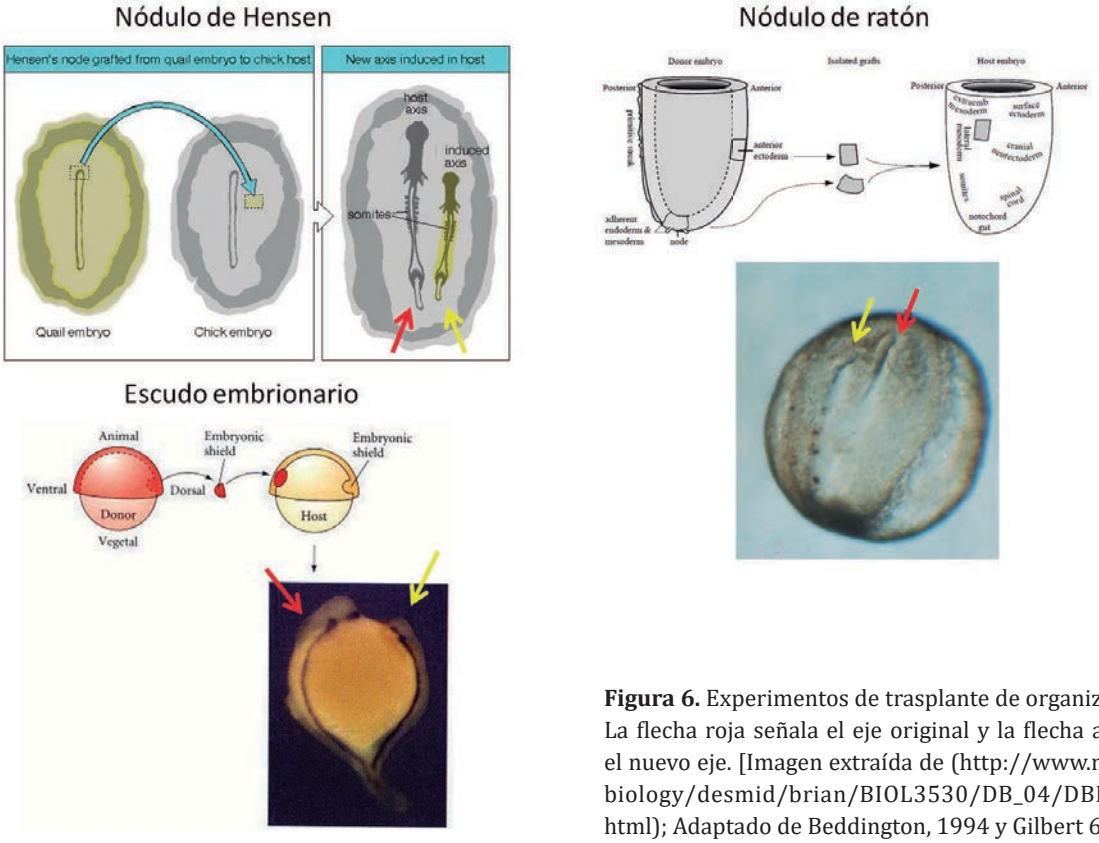


Figura 6. Experimentos de trasplante de organizadores. La flecha roja señala el eje original y la flecha amarilla el nuevo eje. [Imagen extraída de (http://www.mun.ca/biology/desmid/brian/BIOL3530/DB_04/DBNVert2.html); Adaptado de Beddington, 1994 y Gilbert 6ª ed.].

No siempre se conoce cómo se originan los organizadores, aunque existen diferentes teorías y modelos. Algunos teóricos como Meinhardt proponen un modelo de fronteras, en el cual al confrontarse dos o más regiones, dan lugar en la zona de confluencia a un organizador (Figura 7) (Meinhardt, 2009).

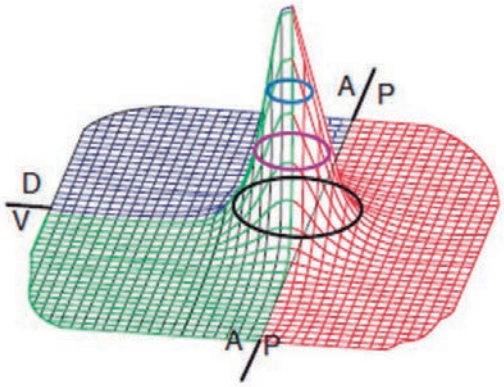


Figura 7. Modelo de confrontación de fronteras. La colaboración entre los diferentes compartimentos crea un cono que puede dar lugar a la aparición de un nuevo organizador. [Adaptado de Meinhardt, 2009].

Sin embargo, sí que se ha podido documentar y demostrar cómo los organizadores ejercen su función en diferentes organismos y se ha observado que los morfógenos juegan un papel esencial. Por ejemplo, los morfógenos secretados por el organizador de Spemann (o centro de señalización dorsal) son indispensables para el correcto desarrollo del embrión. Sorprendentemente, la mayoría de morfógenos que secreta este organizador son inhibidores o represores de otras proteínas secretadas por otro centro de señalización opuesto, el centro de señalización ventral. El centro de señalización ventral secreta principalmente BMP (inductor de identidad ventral) y Wnt (inductor de identidad posterior). Desde el organizador de Spemann o centro dorsal se secretan sus antagonistas: Chordin en el caso de BMP y Dickkopf en el caso de Wnt. Esta actividad antagonista protege la zona dorsal anterior de la influencia de las señales ventrales posteriores. Entre estas moléculas y otras se crean diferentes gradiente de morfógeno y relaciones de retroalimentación positiva/negativa que dan lugar a un embrión con sus diferentes ejes anterior-posterior y dorsal-ventral (Figura 8) (De Robertis, 2009; Dosch et al., 1997; Reversade and De Robertis, 2005; Thisse and Thisse, 2015).

Los organizadores más conocidos son los implicados en la gastrulación. Además encontramos regiones organizadoras en embriones durante la formación del sistema nervioso o durante la formación de las extremidades (Anderson and Stern, 2016). Pero aunque la mayoría de organizadores se han descrito en embriones, no son exclusivos de ellos. También los encontramos en organismos adultos (Vogg et al., 2016). El ejemplo más claro de organizador en adultos es en el caso de *Hydra*, la cual posee un organizador en el hipostoma o cabeza (región oral) y otro en el pie (región aboral) (Bode, 2009). Por otro lado también se ha propuesto que los blastemas en animales adultos que pueden regenerar poseen regiones organizadoras para patronear las estructuras perdidas. El caso de la regeneración de las extremidades en anfibios o equinodermos; o animales completos como en el caso de las planarias son solo algunos ejemplos de blastemas con una posible actividad organizadora (Vogg et al., 2016).

A continuación se exponen dos ejemplos representativos de organizador y gradiente en adulto y en embrión.

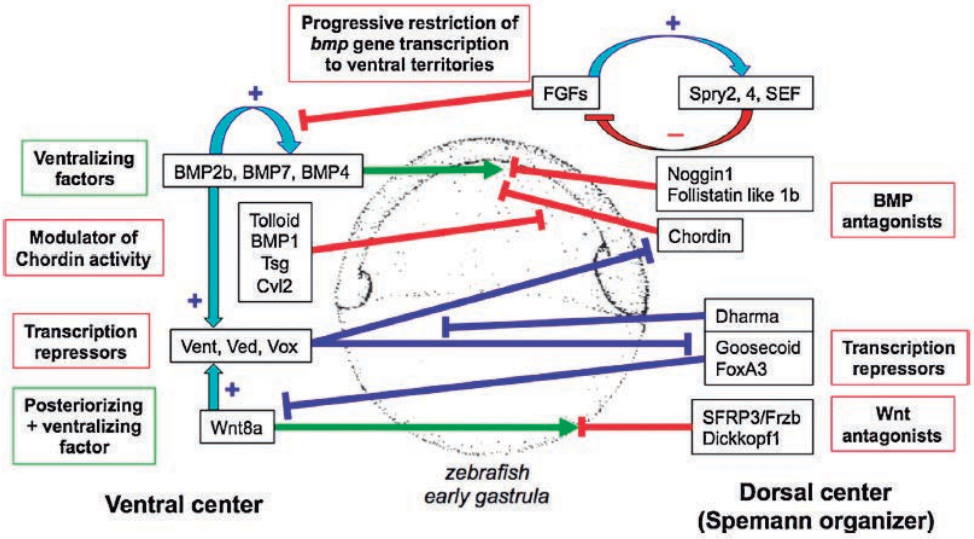


Figura 8. Interacciones principales entre el organizador de Spemann y el centro de señalización ventral en el embrión de pez cebra. Las flechas verdes indican la acción de los morfógenos del centro ventral. Las flechas rojas indican la acción negativa entre proteínas. Las flechas azules indican represión transcripcional. [Adaptado de Thisse y Thisse, 2015].

1.3 Gradientes y organizadores en adultos: el caso de *Hydra*.

La hidra es un organismo que presenta una simetría radial en la cual distinguimos un eje oral/aboral. Podemos dividir el cuerpo de la hidra en tres regiones diferentes: la cabeza, el cuerpo columnar y el pie. La cabeza se subregionaliza en el hipostoma y los tentáculos (Figura 9). La hidra posee una dinámica celular muy activa ya que constantemente pierde células y produce nuevas. Este recambio continuo de células implica que el animal debe disponer de información posicional adecuada para que las células puedan interpretar su ubicación. De este modo las células pueden adquirir la identidad correcta (Bode, 2009).

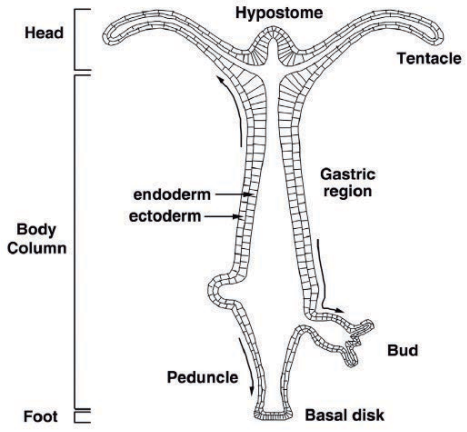


Figura 9. Estructura corporal de la hidra. [Adaptado de Bode, 2012].

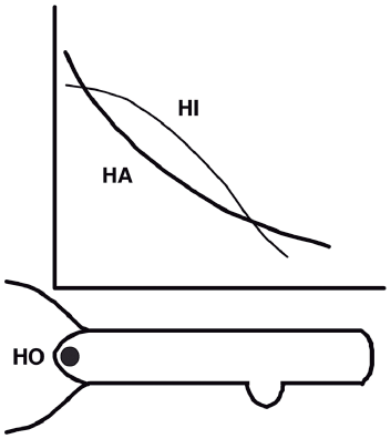


Figura 10. Formación del patrón oral-aboral de la Hydra. El organizador de cabeza (HO) secreta el activador de cabeza (HA) y su propio inhibidor (HI). [Adaptado de Bode, 2012].

El organizador de la cabeza (HO) de la hydra origina la información posicional que es interpretada por las células. El HO presenta las características básicas de los organizadores que encontramos en embriones. Se ha propuesto que el HO origina la información posicional a través de la secreción de dos tipos de señales, una activadora de la cabeza (HA) y otra inhibidora de la cabeza (HI). Estas señales difundirían por el cuerpo de la hidra formando dos gradientes distintos (Figura 10) (Bode, 2012). El modelo de reacción-difusión propuesto por Gierer y Meinhardt explica como estos dos gradientes establecen el patrón oral/aboral de la hidra (Gierer and Meinhardt, 1972; Meinhardt, 2009).

Diferentes experimentos indican que las señales que forman los gradientes de activación e inhibición de la cabeza son moléculas difusibles de vida media diferente (MacWilliams, 1983a; MacWilliams, 1983b). Sin embargo, no se conocen las bases moleculares de ambos gradientes (Bode, 2009; Bode, 2012). Se han descrito diferentes patrones de expresión de genes en forma de gradiente (Vogg et al., 2016), pero no se ha propuesto cuál o cuáles son esas señales activadoras e inhibidoras.

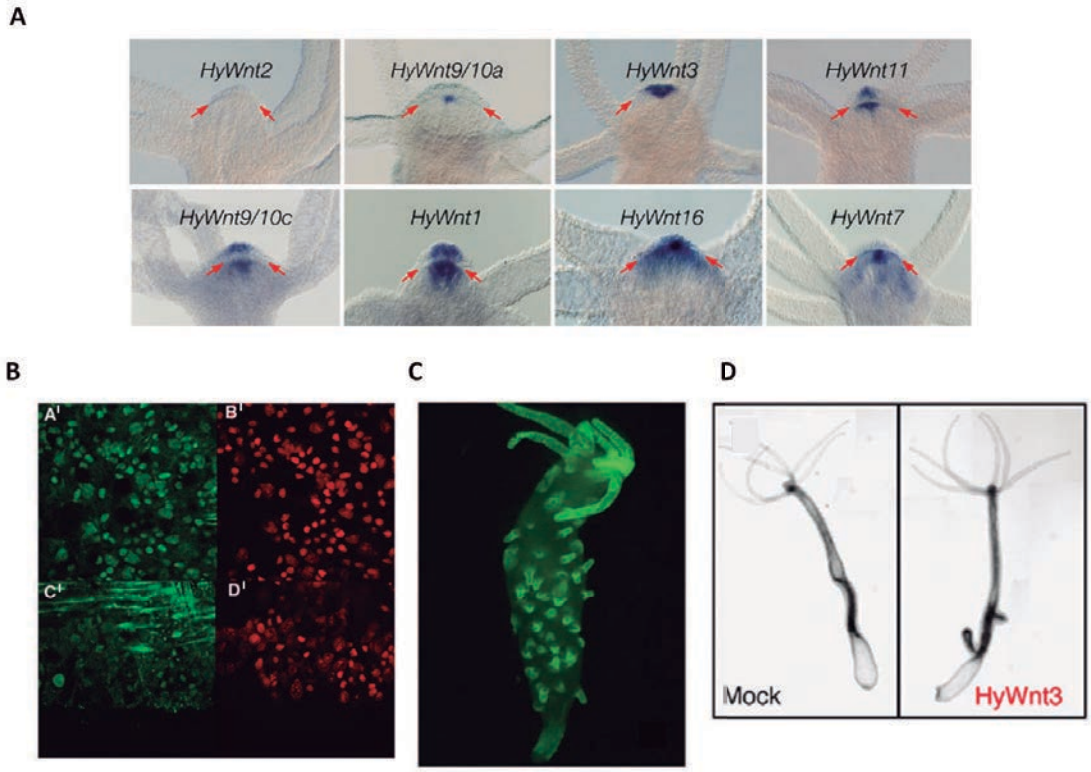


Figura 11. La vía Wnt/βcatenina y el organizador de cabeza. A) Patrón de expresión de diferentes ligandos *wnt* en el hipostoma/cabeza. B) La región del hipostoma (A'-B') posee una alta concentración de βcatenina nuclear (verde) comparado con la región columnar (C'-D'). En rojo se observan los núcleos. C) Hidra con múltiples tentáculos (verde) alrededor del cuerpo después del tratamiento con el fármaco alsterpaulona. D) Las Hydras tratadas con HyWnt3 recombinante forman ejes secundarios comparadas con el control (Mock). [Adaptado de Lengfeld et al., 2009 y Broun et al., 2005].

A pesar de no conocer las señales de los gradientes, si que se han descrito las bases moleculares del organizador de la cabeza. Debido a la homeostasis que sufre la hidra, continuamente perdiendo células y generando nuevas que las reemplacen, el organizador también debe estar continuamente renovándose (Bode, 2012). Diferentes trabajos

apuntan a que la vía Wnt canónica es la responsable de mantener y restablecer el organizador de la cabeza. Por ejemplo, distintos elementos de la vía Wnt se encuentran expresados en la región de la cabeza, como son los ligandos *wnt* (Figura 11 A) (Lengfeld et al., 2009). Por otra parte, aunque la distribución del ARNm de la *βcatenina* sea ubicuo por todo el cuerpo, solo se encuentra nuclearizada de forma específica en la región del organizador (Figura 11 B) (Broun et al., 2005). Además, la sobreactivación de βcatenina mediante fármacos o usando transgénicos produce la formación de cabezas ectópicas por todo el cuerpo del animal (Figura 11 C) (Broun et al., 2005; Gee et al., 2010). Incluso la formación de una fuente ectópica de Wnt3 recombinante es capaz de originar un nuevo organizador de cabeza (Figura 11 D) (Lengfeld et al., 2009).

Todos estos datos apoyan que la vía Wnt canónica juega un papel primordial en la formación, mantenimiento y restablecimiento del organizador de cabeza en la hidra.

1.4 Gradientes y organizadores durante el desarrollo embrionario: el caso de la extremidad de vertebrados

La extremidad de los vertebrados es uno de los órganos más estudiados y utilizados para comprender cómo se forman y establecen los patrones corporales. Encontramos tres ejes en la extremidad: el próximo-distal (PD), el anteroposterior (AP) y el dorsoventral (DV). En este apartado nos centraremos en el patrón AP, y de forma más concreta en el implicado en la formación de la mano y sus dedos.

Tickle y colaboradores observaron que al trasplantar una región del primordio de la extremidad, llamada ZPA (zona de actividad polarizadora), a una región opuesta del primordio, se forma una imagen especular de los dedos de la mano (Figura 12 A) (Tickle et al., 1975). Más adelante se demostró que esta región (ZPA) es la responsable de secretar un morfógeno, la proteína Sonic Hedgehog (SHH), ya que generar una fuente de SHH produce el mismo efecto que trasplantar la ZPA (Riddle et al., 1993). El gradiente de SHH se traduce en un gradiente inverso de otra proteína, Gli3 represor, que es el factor de transcripción de la vía Hedgehog. Por tanto, la función de SHH es prevenir la formación de Gli3 represor (Figura 12 B) (Wang et al., 2000). Estos resultados sugieren que la información posicional generada por el gradiente de SHH establece la identidad de los diferentes dedos. Así, una concentración alta de SHH especifica dedo 5, y a medida que el gradiente de SHH decae se forman los siguientes dedos (4, 3, 2, 1) (Bastida and Ros, 2008).

Sin embargo, esta visión clásica del establecimiento del patrón AP de la mano a través del gradiente de SHH es actualmente muy discutida (Delgado and Torres, 2016). Diferentes evidencias experimentales apuntan hacia otros modelos o mecanismos. Además dependiendo del modelo de estudio utilizado (pollo o ratón) se sugiere un modelo u otro.

Recientemente se ha propuesto una versión integrativa de diferentes modelos descritos hasta ahora en la que se identifican tres fases (Figura 13) (Delgado and Torres, 2016; Lopez-Rios, 2016).

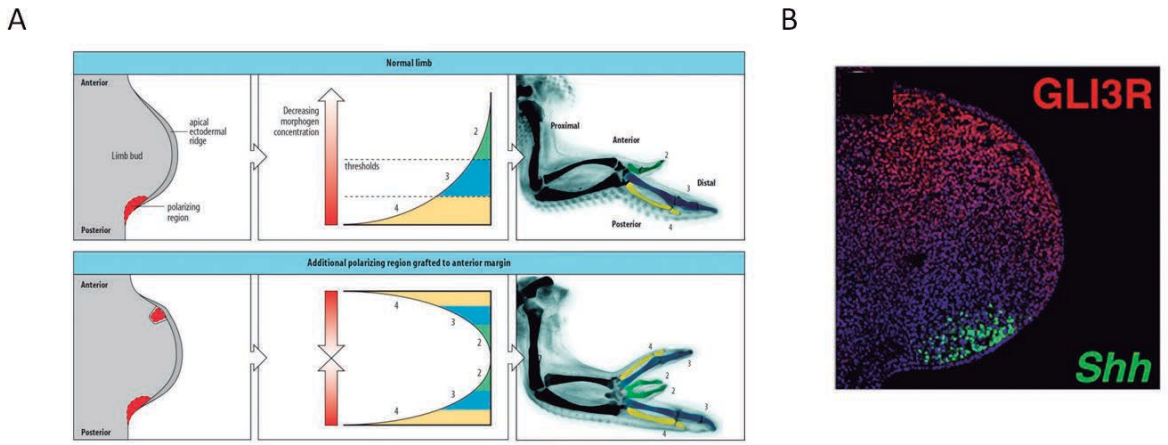


Figura 12. La región ZPA. A) Situación normal (arriba) e imagen especular (abajo) del ala de pollo tras trasplantar la ZPA a una región opuesta. B) Patrón de expresión de *shh* (verde) y de la proteína GLI3R (rojo). [Adaptado de http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_11/devo_11.html (A) y Lopez-Rios, 2016 (B)].

1. La primera fase es de pre-patrón, y culmina con la activación de *shh* en la ZPA.

2. Durante la segunda fase se establece el gradiente de SHH que controla el crecimiento y la identidad AP de los dedos. Sin embargo, experimentos en ratón no apoyan que el gradiente de SHH controle la identidad de los dedos (Zhu et al., 2008).

3. La tercera fase, en la cual se especifica la región dedo-interdedo, está controlada por un mecanismo de tipo Turing de reacción-difusión (Sheth et al., 2012). Evidencias moleculares apoyan este modelo de tipo Turing, en el cual los Hox, la vía Wnt canoníca y la vía FGF tienen un papel primordial (Raspopovic et al., 2014; Sheth et al., 2012).

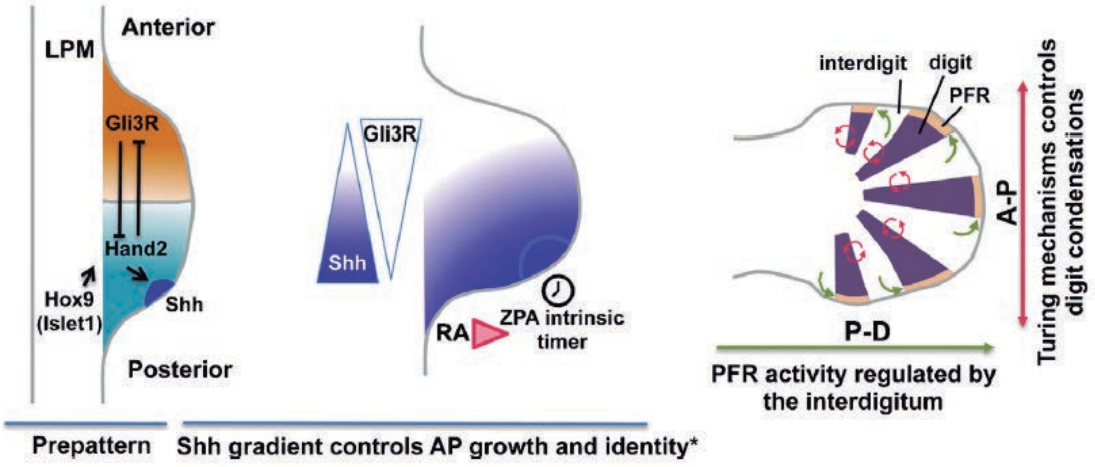


Figura 13. Modelo integrativo del patrón anteroposterior (AP) de la extremidad. El prepatrón AP sucede en el primordio temprano de la extremidad cuando los Hox9 (o *islet1*) de la LPM (placa lateral del mesodermo) induce la expresión de *Hand2*, que es restringido en la región posterior por GLI3R. Hand2 induce la expresión de *shh* en la ZPA estableciendo un gradiente de Shh a lo largo del eje AP que determina el crecimiento y la identidad* AP. Además la duración de la expresión de Shh depende de un reloj intrínseco. En estadios tardíos un mecanismo Turing de reacción-difusión especifica las regiones dedo e interdedo generando el patrón AP de condensación de los dedos. El numero de dedos depende del tamaño de la paleta de la mano. El numero de falanges de cada dedo se determina por la actividad PFR (región formadora de falanges), cuya duración depende de las señales provenientes de la región interdedo posterior adyacente. * El rol de Shh en la identidad de dedo no está apoyado por los experimentos en ratón [Adaptado de Delgado y Torres, 2016].

2. La vía Wnt

Las vías de señalización son de vital importancia para poder establecer una comunicación intercelular. Intervienen en una gran variedad de procesos, desde coordinar el desarrollo embrionario, especificar el destino celular, patronear estructuras y un largo etcétera. De igual forma, también están implicadas en un gran número de enfermedades debido a su incorrecto funcionamiento. Por lo tanto conocerlas a fondo es crucial, no solo para entender el desarrollo, sino también el campo de la medicina. De hecho, una de estas vías de señalización se identificó a raíz de un estudio que buscaba genes implicados en cáncer mamario utilizando como modelo ratones. Al gen descrito e iniciador de esta vía se le llamó *int1* (*integration site 1*) (Nusse and Varmus, 1982). Unos años antes, gracias al screening de Heidelberg se describieron una gran cantidad de genes implicados en la segmentación del embrión de *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980), entre los cuales se identificó *wingless*, el cual recibe su nombre por la mutación que provoca en el adulto (Sharma and Chopra, 1976). En realidad estos dos genes, *wingless* e *int1*, eran el mismo gen y se rebautizó como *wnt* (*Wingless-related integration site*). Además, el gen *wnt* dio nombre a una de las vías de señalización actualmente más estudiada y funcionalmente más conservada a lo largo de la evolución (Nusse and Varmus, 2012). Dentro de la vía Wnt encontramos dos tipos de vías, la canónica y la no canónica.

2.1 Vía Wnt canónica o dependiente de βcatenina

La vía Wnt canónica es la más conocida de las diferentes ramas de la vía Wnt. Esta vía se caracteriza principalmente por regular la actividad del factor de transcripción βcatenina.

Las vías de señalización se utilizan como una forma de comunicación entre células. Mediante la secreción de un ligando y la recepción del mismo se puede establecer una comunicación entre dos células, la que secreta y la que recibe. En el caso de la vía Wnt canónica (Figura 14), el ligando secretado es la proteína Wnt, que se unirá a su receptor y co-receptor (Frizzled y LRP respectivamente). Esta unión provoca que los receptores cambien de conformación dentro de la célula y activen a otra proteína, Dishevelled. Cuando Dishevelled es activa, se encarga del desacoplamiento del complejo de destrucción de la βcatenina y permite la entrada de la βcatenina al núcleo. Dentro del núcleo βcatenina se une al factor de transcripción TCF/LEF, activando la transcripción de diferentes genes. En ausencia del ligando Wnt, la vía está desactivada y el complejo de destrucción de la βcatenina fosforila e inhibe a βcatenina, provocando su degradación por el proteasoma.

Es importante mencionar que βcatenina no actúa solo como factor de transcripción. También podemos encontrarla participando en las uniones adherentes independientemente de si la vía está activa o no (Clevers and Nusse, 2012; Nusse, 2012).

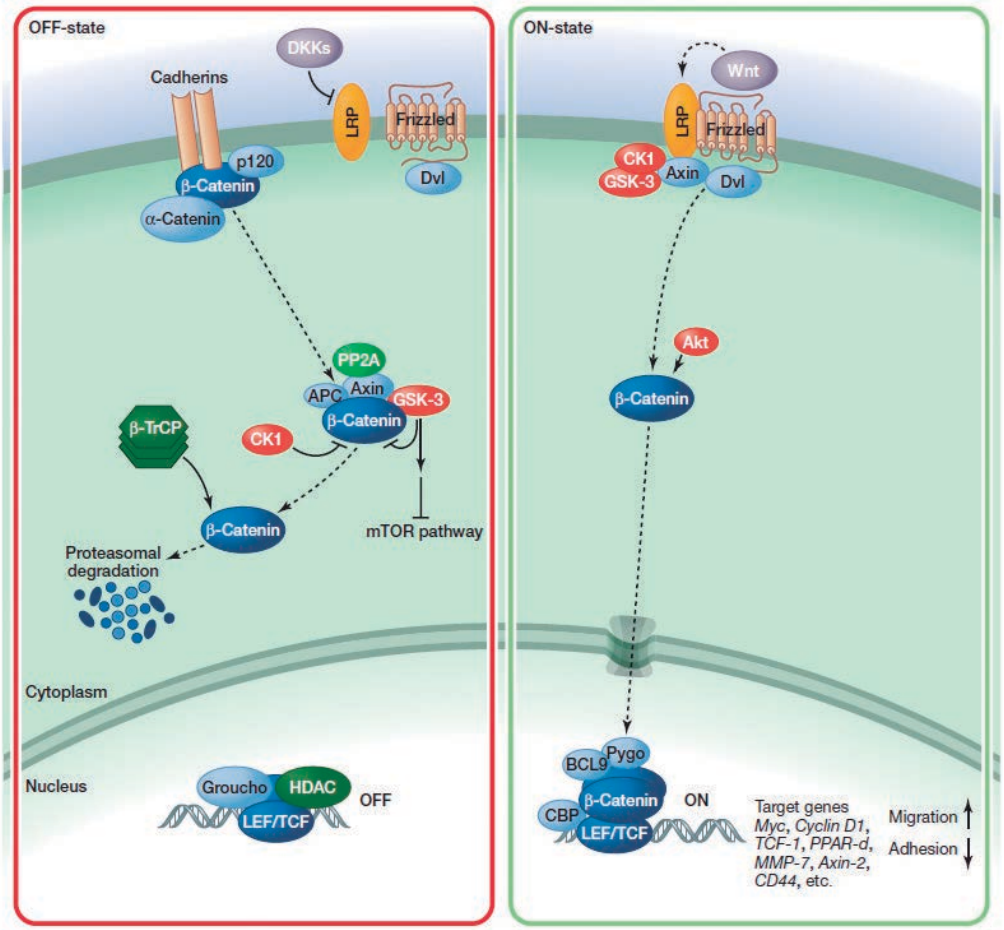


Figura 14. La vía Wnt canónica en ausencia (OFF-state) y en presencia (ON-state) del ligando Wnt. Cuando la vía esta inactiva (OFF-state) el complejo de destrucción de la βcatenina fosforila y provoca la degradación de la βcatenina por el proteasoma. En cambio si la vía se activa (ON-state) por la unión del ligando Wnt a sus receptores, el complejo de destrucción de la βcatenina es secuestrado permitiendo la nuclearización de βcatenina y la activación transcripcional de diferentes genes. [Adaptado de Nusse, 2012].

2.1.1 Elementos principales de la vía Wnt canónica

- Ligandos Wnt

Los ligandos Wnts son proteínas pequeñas, de unos 40 kDa. Se han descrito hasta 13 familias distintas de Wnts, conservadas desde *Hydra* hasta humanos. Poseen varias cisteínas conservadas que les confieren su capacidad de unión al receptor. Además, los Wnts son modificados postranscripcionalmente mediante la adición de un lípido, el ácido palmitoleico, a una serina también conservada. Este lípido es necesario para su correcto funcionamiento, y la proteína Porcupine es la responsable de este proceso (Willert and Nusse, 2012).

Los Wnts son proteínas secretables y la proteína Wntless es necesaria para este proceso (Figura 15) (Bartscherer et al., 2006). El hecho de que los Wnts posean un lípido hace

que no sean solubles en un medio acuoso. Diferentes estudios han propuesto y demostrado distintas formas de secreción y transporte extracelular (Langton et al., 2016):

- Mediante citonemas/filopodios, por lo tanto no viajarían fuera de la célula.

- A través de la unión a la proteína Swim, que actuaria de escudo tapando el lípido.

- Por medio de partículas de lipoproteínas, que también enmascararían el lípido haciéndolo soluble.

- Mediante exosomas, microvesículas desprendidas de las células.

En relación al rango de dispersión de los Wnts, existe controversia. Aunque se ha demostrado que pueden influir a largas distancias (Zecca et al., 1996), estudios recientes demuestran que el ligando Wnt no viaja lejos de la fuente de secreción (Alexandre et al., 2014; Farin et al., 2016).

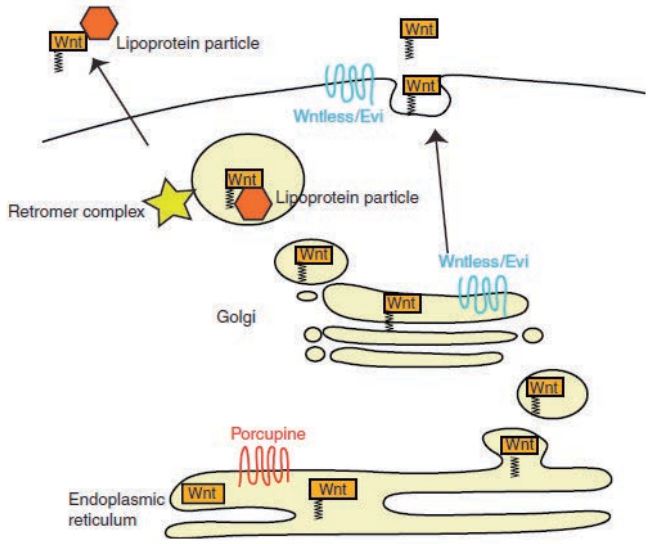


Figura 15. Función de la proteína Porcupine y de la proteína Wntless/Evi en el procesamiento y la secreción de los Wnt, respectivamente. En el retículo endoplasmático Porcupine añade el ácido palmitoleico al ligando Wnt. Después la secreción del ligando está controlada por Wntless/Evi. [Adaptado de Mikels y Nusse, 2006].

- Receptores Frizzled y LRP5/6

Las proteínas Frizzled y LRP son los receptores que participan en la vía Wnt canónica. Solo hay una familia de LRP (o Arrow, como es conocido en *Drosophila*). En relación a los Frizzled, aunque no existe una filogenia clara, en mamíferos podemos encontrar hasta 10 genes diferentes subdivididos en cinco familias (Macdonald and He, 2012).

Cuando el ligando Wnt se une a los receptores Frizzled-LRP, estos heterodimerizan y se endocitan formando una vesícula llamada signalosoma. Esta vesícula se encarga de secuestrar al complejo de destrucción de la βcatenina para inhibirlo (Niehrs, 2012).

- El factor de transcripción βcatenina

El elemento clave de la vía canónica es la βcatenina. Curiosamente, su nombre proviene de la primera función descrita en las uniones adherentes. En estas estructuras, la βcatenina interacciona con la E-cadherina. Más tarde se descubrió que la βcatenina

también podía interaccionar con proteínas nucleares y activar la transcripción (Nusse and Varmus, 2012). Así pues, la βcatenina es una proteína bifuncional, con funciones estructurales (uniones adherentes) y señalizadoras (transcripción) (Valenta et al., 2012).

La βcatenina posee una región central donde encontramos una serie de dominios repetidos llamados repeticiones armadillo. Estas repeticiones sirven de anclaje a multitud de proteínas, sean factores de transcripción, proteínas inhibidoras, etc. Además posee un dominio N-terminal y uno C-terminal, este último de vital importancia. El dominio C-terminal es necesario para que la βcatenina sea transcripcionalmente activa, además de ser un lugar de unión de múltiples factores de remodelación de la cromatina. Es importante resaltar que la βcatenina no posee ningún dominio de unión al ADN, por lo que para ejercer su función transcripcional debe unirse a otra proteína que sí posea esta característica (Figura 16) (Valenta et al., 2012).

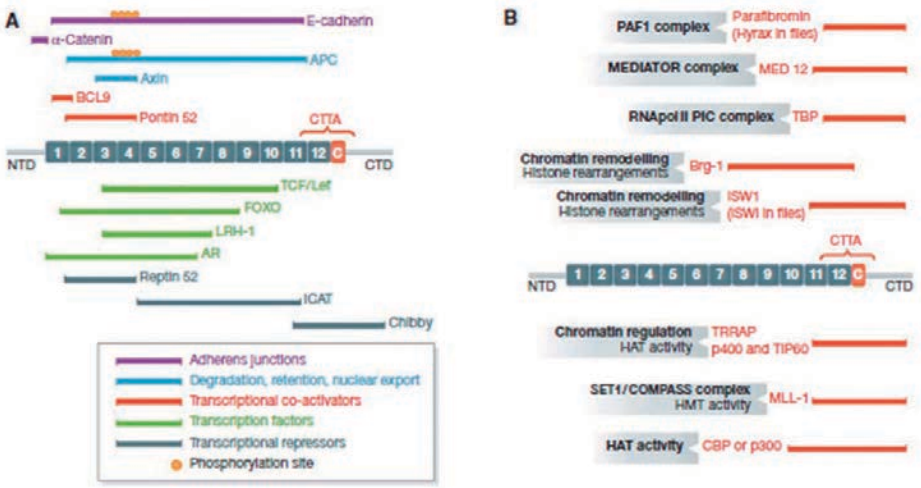


Figura 16. La βcatenina actúa como plataforma de unión de multitud de proteínas. A)La proteína βcatenina está formada por una región central compuesta por doce repeticiones Armadillo (cuadrado numerados), flanqueada por un dominio amino-terminal (NTD) y un dominio carboxi-terminal (CTD). Entre la última repetición Armadillo y la región flexible CTD se encuentra la hélice-C conservada (C, cuadrado naranja). Las líneas de color muestran los lugares de unión de diferentes proteínas a la βcatenina validadas experimentalmente. Código de colores: púrpura, componentes de las uniones adherentes; azul, miembros del complejo de destrucción de la βcatenina; rojo, coactivadores transcripcionales; verde, factores de transcripción que proporcionan el dominio de unión al ADN; gris, inhibidores transcripcionales. Activadores transcripcionales C-terminal (CTTA), el dominio crítico para su unión está marcado por el corchete. B) La región C-terminal de βcatenina sirve de lugar de unión para multitud de complejos que promueven la transcripción mediante βcatenina. Los motivos de unión para proteínas particulares validados experimentalmente están indicados con barras de color naranja. [Adaptado de Valenta et al., 2012].

Presente en la mayoría de metazoos, la βcatenina es un elemento altamente conservado (Wikramanayake et al., 2003). En la mayoría de organismos se encuentra una única βcatenina, aunque en diferentes animales se ha duplicado. En vertebrados la duplicación ha originado parálogos, como la Plakoglobina, cuya función principal es estructural (uniones adherentes y desmosomas) (Butz et al., 1992). En otros, la duplicación ha dado lugar a dos o más βcateninas que se han especializado en una de las dos funciones, debido a que la duplicacion afecta a alguno de los dominios de transactivación o adhesión. Existen diferentes ejemplos como en algunos insectos (Bao et al., 2012), *C. elegans* (el cual posee hasta 4 βcateninas diferentes) (Korswagen et al., 2000) o las planarias (dos βcateninas descritas al inicio de esta tesis, una implicada específicamente en transcripción y otra en adhesión) (Chai et al., 2010).

Cuando la vía Wnt canónica está activa, la βcatenina no es fosforilada y por tanto no es degradada por el proteasoma. Como consecuencia se acumula en el citoplasma y entra dentro del núcleo (Clevers and Nusse, 2012). Una vez dentro del núcleo, la βcatenina puede unirse al factor de transcripción TCF/LEF, el cual sí posee un dominio de unión al ADN, desplazando a su represor más conocido, Groucho. El binomio βcatenina-TCF provoca la activación de la transcripción de diferentes genes o programas génicos (Figura 17) (Valenta et al., 2012).

La βcatenina está implicada en multitud de procesos, desde mantener la pluripotencia a diferenciar una célula (Miyabayashi et al., 2007). Estas funciones opuestas se pueden dar debido a la interacción del complejo βcatenina/TCF con otros factores que pueden regularla más finamente (Valenta et al., 2012). Aunque se conocen muchas proteínas que pueden realizar estas funciones, el campo de la regulación nuclear de la βcatenina es poco conocido. Algunas de estas proteínas son activadoras, como Bcl9 y Pygopus (Figura 17), y otras inhibidoras, como ICAT o Reptin (Han and Wang, 2014). Es importante resaltar que aunque el cofactor por excelencia de la βcatenina para unirse al ADN es TCF/LEF, se ha demostrado que βcatenina puede unirse a otros factores de transcripción como son FOXO, Sox o Oct4 (Valenta et al., 2012).

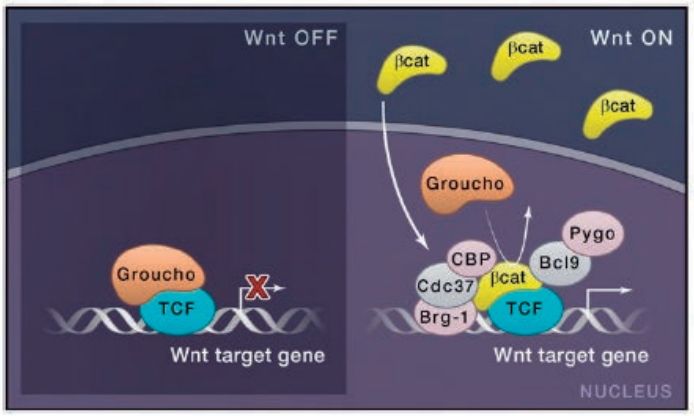


Figura 17. La vía Wnt en el núcleo, en ausencia (Wnt OFF) y en presencia (Wnt ON) del ligando Wnt. (Wnt OFF) En ausencia de señal Wnt, TCF reprime los genes target de la vía, ayudado por el represor transcripcional Groucho. (Wnt ON) Cuando la vía se activa, la βcatenina desplaza a Groucho del TCF y recluta co-activadores transcripcionales y modificadores de histonas como BRG1, CBP, etc. para conducir la expresión de los targets de la vía. [Adaptado de Clevers y Nusse, 2012].

- El factor de transcripción TCF/LEF

El factor de transcripción TCF/LEF es el responsable de que la βcatenina pueda ejercer su función. La mayoría de invertebrados presentan un único TCF/LEF, mientras que en vertebrados se ha expandido y se identifican 4 familias diferentes. TCF se caracteriza por poseer un dominio de unión a la βcatenina y un dominio de unión a Groucho, su principal represor. Además, presenta dos dominios de unión al ADN, uno de tipo HMG ("high-mobility group") y otro más desconocido llamado C clamp.

En ausencia de βcatenina, la proteína Groucho y otros represores menos conocidos, se unen a TCF/LEF, reprimiendo su actividad. La presencia de βcatenina en el núcleo provoca el desplazamiento de Groucho y por tanto la activación de la transcripción (Figura 17) (Cadigan and Waterman, 2012).

2.1.2 Reguladores principales de la vía Wnt canónica

- Reguladores extracelulares

Uno de los puntos más importantes de la vía es el momento de la unión ligando-receptor Wnt-Frizzled-LRP6. Es por eso que existen diferentes proteínas encargadas de regular esta unión (Langton et al., 2016).

Se han descrito varios mecanismos de regulación:

- Inhibición de la formación del heterodímero de receptores, mediante la proteína APCDDI (Shimomura et al., 2010).

- Modificación de la estructura del ligando: especialmente conocidas son las proteínas Notum y Tiki, las cuales modifican la estructura del ligando Wnt inactivándolo, bien sea porque cortan el lípido o algún fragmento proteico (Kakugawa et al., 2015; Zhang et al., 2012).

- Captura del ligando: las principales proteínas con esta función son WIF y sFRP (Figura 18) (Cruciat and Niehrs, 2013).

- Inhibición de la unión del ligando por competición: la proteína sFRP también puede unirse directamente al receptor Frizzled impidiendo la unión del ligando. Otras proteínas que inhiben la unión ligando-receptor son las proteínas Dickkopf, las cuales se unen al receptor LRP6 (Figura 18) (Cruciat and Niehrs, 2013).

No solo encontramos inhibidores, también hay agonistas de la unión ligando-receptor, como son los glipicanos, que actúan a nivel de membrana favoreciendo la unión ligando-receptor (Niehrs, 2012).

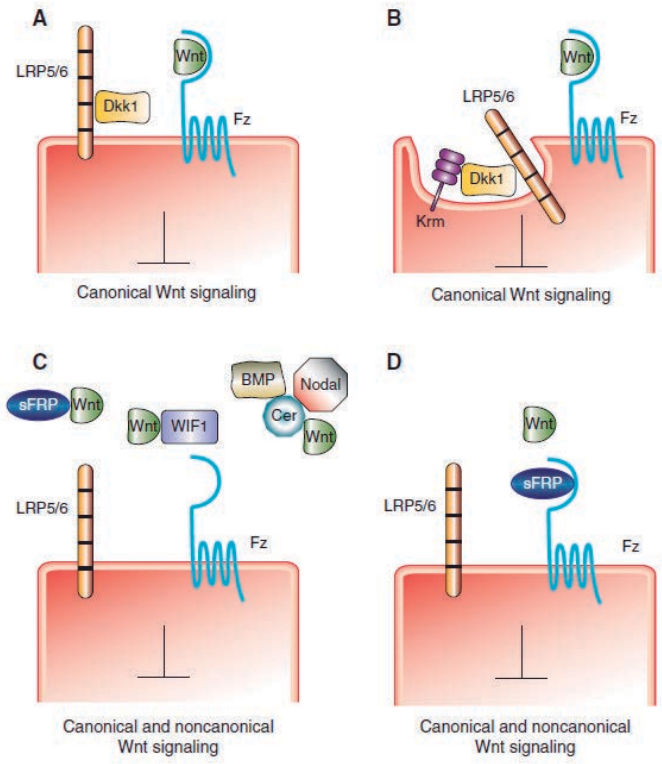


Figura 18. Modelos de inhibición de la señal Wnt. (A,B) Dkk1 se une a LRP6 disrupcionando el complejo Wnt-Fz-LRP6 (A) y/o induciendo la endocitosis de LRP6 en presencia del correceptor Kremen (B). (C) sFRP1, WIF-1 y Cerberus secuestran al ligando Wnt, inhibiendo la vía. (D) sFRP también puede inhibir la vía mediante la unión a Fz. [Adaptado de Cruciat y Niehrs, 2013].

- Activadores de la vía Wnt canónica

Existen otras proteínas que potencian la activación de la vía Wnt canónica pero no a través de favorecer la unión Wnt-Frizzled-LRP6, sino a través de potenciar su acción. La unión del ligando R-Spondin con su receptor LGR4/5 ayuda a incrementar la activación de la vía Wnt canónica. El mecanismo de actuación es poco conocido, pero se ha descrito un posible modelo. Una proteína de transmembrana del tipo E-3 ubiqüitín ligasa (ZNRF3) inhibe la vía Wnt promoviendo la degradación de los receptores LRP y Frizzled. Hao y colaboradores proponen que la unión R-Spondin-LGR4/5 secuestra a ZNRF3, permitiendo la acumulación de los receptores (Figura 19) (Hao et al., 2012).

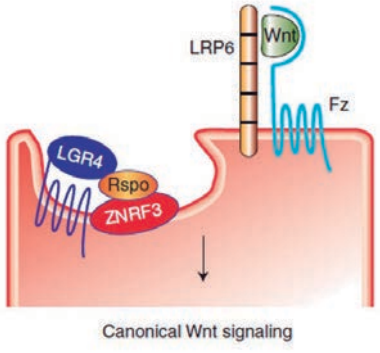


Figura 19. Mecanismo de acción de R-Spondin y LGR4/5. Rspo se une a ZNRF3 y LGR4 e induce la endocitosis del receptor. Si ZNRF3 se encuentra internalizado, no puede ubiqüitinizar los receptores de los Wnt para su degradación. Como consecuencia, Fz y LRP6 se acumulan en la membrana y transmiten la señal de la vía Wnt canónica [Adaptado de Cruciat y Niehrs, 2013].

Otra proteína que activa la vía Wnt canónica es Norrin. Esta proteína actúa como un Wnt, puede unirse a Frizzled y LRP6 y activar la vía canónica, sin la participación de ningún Wnt. Cabe destacar que Norrin solo puede unirse a un Frizzled de mamíferos, el Frizzled-4 (Cruciat and Niehrs, 2013).

- Reguladores intracelulares: el complejo de destrucción de la βcatenina

El regulador más conocido de la vía es el complejo de destrucción de la βcatenina, el cual está formado principalmente por Axina, APC, CK1, GSK3. Estas proteínas secuestran y fosforilan a la βcatenina cuando la vía Wnt canónica está inactiva. Esta serie de fosforilaciones son reconocidas por una E3-ligasa, llamada β-TrCP, la cual induce la degradación de la βcatenina por el proteosoma (Figura 20) (Stamos and Weis, 2013).

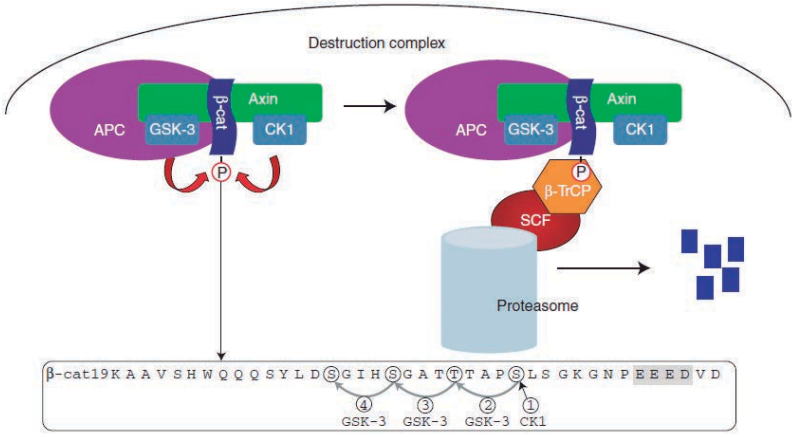


Figura 20. Esquema del complejo de destrucción de la βcatenina. Se muestra la secuencia amino terminal de βcatenina donde es fosforilada por GSK-3 y CK1. [Adaptado de Stamos y Weis, 2013].

Sin embargo, la activación de la vía Wnt canónica provoca la inhibición del complejo. Actualmente hay dos modelos propuestos que explican cómo se inhibe el complejo (Clevers and Nusse, 2012), pero no entraremos en detalle.

2.2 Vía Wnt no canónica o independiente de βcatenina

Tradicionalmente, todo lo que no es vía Wnt canónica (es decir, regular la actividad de la βcatenina) se considera vía Wnt no canónica o alternativa. En realidad no es una única vía, sino que existen varias ramas no canónicas o alternativas (van Amerongen, 2012).

Las principales vías alternativas son:

- La vía PCP ("Planar Cell Polarity") o de polaridad celular en un plano. Esta vía se encarga de alinear las células en un plano. En este proceso participan el receptor Frizzled y la proteína citoplasmática Dishevelled, además de otras proteínas de membrana como Flamingo o citoplasmáticas como Diego. Uno de los ejemplos mejor conocidos es el ala de *Drosophila*. En esta estructura, la vía PCP controla el correcto alineamiento de los pelos sensoriales en el ala (Mlodzik, 2002). Recientemente se ha demostrado que el Wnt4 está implicado en la vía PCP (Wu et al., 2013).

- Los movimientos de extensión convergentes o CE ("Convergent Extension"). Estos movimientos consisten en el alargamiento y estrechamiento simultaneo de un tejido como resultado de una intercalación direccional de células. Este proceso está implicado en la extensión del eje primario del cuerpo durante la gastrulación. Aunque la mayoría de componentes que participan en este proceso son los mismos que actúan en la vía PCP, los efectores son totalmente diferentes. Los ligandos Wnt11 y Wnt5 participan también en esta vía (Wallingford et al., 2002).

- El establecimiento del eje de simetría izquierda-derecha o el guiado de axones. Del proceso de guiado de axones también surge otra vía Wnt alternativa llamada vía Wnt del calcio, por la implicación del calcio en la vía.

- La vía Wnt/STOP o vía Wnt mitótica. En esta vía participan el binomio Wnt-Frizzled/LRP. Controla la estabilidad de diversas proteínas y regula el tamaño celular durante la fase G2/M mediante la inhibición de la quinasa GSK3 (Acebron et al., 2014).

Como se puede deducir de lo explicado anteriormente, los elementos de las diferentes vías alternativas no son exclusivos de la vía Wnt no canónica. Por ejemplo el ligando Wnt11 puede activar tanto la vía Wnt canónica como la no canónica (Tao et al., 2005). De igual forma los receptores Frizzled son conocidos por su alta promiscuidad, pudiendo unirse a diferentes ligandos Wnt, sean canónicos o no canónicos (Niehrs, 2012).

2.3. Procesos controlados por la vía Wnt canónica

La vía Wnt canónica controla una gran variedad de procesos durante el desarrollo embrionario, la homeostasis del adulto e incluso durante la regeneración de estructuras. Además su incorrecto funcionamiento en cualquiera de estos procesos puede llevar a producir diferentes malformaciones o enfermedades, como el cáncer o algunos tipos de

osteoporosis. Algunos de estos procesos que controla la vía Wnt canónica son:

- Establecimiento del eje anteroposterior (AP)

El establecimiento del eje AP es uno de los procesos más estudiados durante el desarrollo embrionario. En la gran mayoría de estos estudios se ha demostrado que la vía Wnt canónica tiene un rol principal en el establecimiento del eje AP, en concreto en especificar identidad posterior (Petersen and Reddien, 2009a).

La βcatenina tiene un papel conservado evolutivamente a la hora de generar asimetrias. Durante la gastrulación especifica el eje animal-vegetal en muchos organismos estudiados, como por ejemplo en el erizo de mar y en el gusano nemertino *C. lacteus*. En ambos casos la activación de la vía Wnt canónica especifica el destino celular vegetal en el embrión, siendo la βcatenina nuclearizada en esta región (Figura 21 A) (Henry et al., 2008; Wikramanayake et al., 1998).

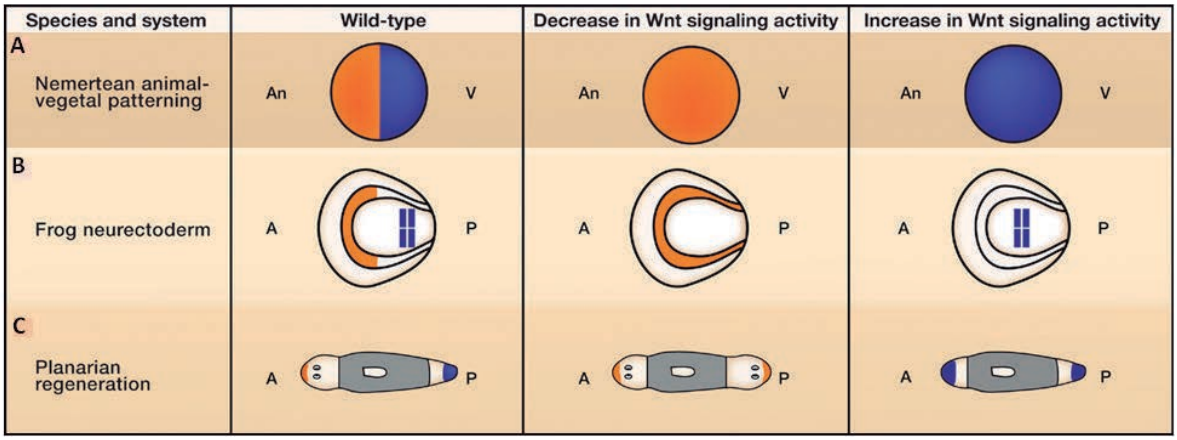


Figura 21. Defectos axiales tras perturbar la vía Wnt canónica. Los marcadores regionales o de identidad están representados en naranja y púrpura. La disminución de la actividad Wnt generalmente causa la expansión de marcadores anteriores y la pérdida de los posteriores. (A-C) Polo anterior (A), polo animal en bilaterales (An). (A) Izquierda, embrión de nemertino en estadio de blástula tardío. Marcadores de ectodermo (naranja) y endodermo (púrpura). Centro, silenciamiento de *βcatenina* mediante morfolino. Derecha, sobreexpresión del dominante negativo de GSK3. (B) Vista dorsal. Izquierda, estadio neurula de rana. Marcadores, *Bf1* (naranja, cerebro anterior) y *Krox20* (púrpura, cerebro posterior). Centro, sobreexpresión de *XDkk1* o *Xfrzb1*. Derecha, sobreexpresión de *Xwnt8*. (C) Vista dorsal. Izquierda, planaria después de 14 días de regeneración tras amputar la cabeza y la cola. Marcadores, *sFRP1* (naranja, polo anterior), *frizzled-4* (púrpura, polo posterior), tejido regenerante (blanco) y pre-existente (gris). Centro, silenciamiento de *βcatenina-1* mediante ARNi. Derecha, silenciamiento de *APC-1* mediante ARNi. [Adaptado Petersen y Reddien, 2009].

La βcatenina también especifica el eje AP en multitud de organismos. Por ejemplo, el patrón AP del neuroectodermo en vertebrados como *Xenopus* o el pez cebra. La sobreactivación de la vía en el neuroectodermo provoca la posteriorización de las regiones anteriores. Por el contrario la inhibición de la vía causa la anteriorización de las regiones posteriores del neuroectodermo (Figura 21 B). Además, los inhibidores de la vía se encuentran expresados en las regiones anteriores y los activadores en las posteriores (Kiecker and Niehrs, 2001; Shimizu et al., 2005).

La vía Wnt canónica no solo especifica el eje AP durante el desarrollo embrionario. También en algunos organismos adultos que poseen la capacidad de regenerar, la vía Wnt canónica participa a la hora de restablecer el patrón AP. Un ejemplo muy claro es el caso de las planarias. La inhibición de la vía Wnt canónica durante la regeneración de las regiones posteriores provoca la anteriorización de estos. Dicho de otro modo, al inducir la regeneración posterior debido a la amputación de la cola, si se inhibe la vía Wnt canónica, en lugar de regenerar una cola aparecerá una cabeza (Figura 21 C). Además, si se inhibe la vía Wnt canónica durante la homeostasis también se produce una anteriorización de las regiones (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008).

- Mantenimiento de las células madres

El mantenimiento de las células madre en estado indiferenciado es otra función de la vía Wnt canónica. El ejemplo más estudiado de este proceso son sin duda las células madre del intestino de los vertebrados. Estas células se encuentran en las criptas del intestino y las células Paneth se intercalan entre ellas. Estas células Paneth son las encargadas de secretar los ligandos Wnt hacia las células madre, manteniéndolas en estado indiferenciado. De esta forma, las células Paneth crean un nicho donde las células madre pueden mantenerse indiferenciadas. Por tanto si las células madre abandonan el nicho se diferenciarán (Beumer and Clevers, 2016).

Al mismo tiempo, la vía Wnt promueve la división asimétrica de las células madre. Al dividirse una célula madre, se diferenciará la célula hija más alejada de la fuente del ligando mientras que se mantendrá como célula madre la más cercana al ligando Wnt (Habib et al., 2013).

- Diferenciación celular

La vía Wnt no solo especifica destino celular mediante su silenciamiento, como en células madre, sino también mediante su activación, promoviendo la expresión de genes implicados en diferenciación. Un ejemplo es el desarrollo de la musculatura durante la formación de las extremidades. En este caso, la vía Wnt promueve la expresión de factores de transcripción como *Pax3* y *Myf5*, necesarios para iniciar el programa de diferenciación hacia músculo (Geetha-Loganathan et al., 2008).

Otro órgano en el que la vía Wnt canónica tiene también un papel importante durante la diferenciación es el hígado. Se ha descrito que la vía Wnt canónica es necesaria para la correcta diferenciación de los hepatoblastos en dos tipos celulares concretos: las células epiteliales biliares y los hepatocitos (Nejak-Bowen and Monga, 2008).

3. Las planarias como organismo modelo en biología del desarrollo

Las planarias son gusanos planos que poseen la capacidad de regenerar cualquier parte de su cuerpo. Después de una amputación, los dos fragmentos resultantes son capaces de generar un nuevo individuo cada uno. Esta gran plasticidad no solo se da durante la regeneración. Las planarias sufren un recambio celular continuo que les permite crecer y decrecer en función de la disponibilidad del alimento. Los responsables de esta plasticidad son los neoblastos, las células madre pluripotentes de las planarias (Baguñà, 2012; Saló, 2006).

Las planarias pueden ser utilizadas como organismo modelo para el estudio de infinidad de procesos debido a su plasticidad. La regeneración de tejidos (Roberts-Galbraith and Newmark, 2015), el establecimiento de los ejes anteroposterior (AP) (Iglesias et al., 2008) y dorsoventral (DV) (Molina et al., 2007), el crecimiento y decrecimiento del cuerpo (Miller and Newmark, 2012), el estudio de fármacos contra el cáncer (Isolani et al., 2012), o estudios ambientales (Wu and Li, 2015) y evolutivos (Alvarez-Presas et al., 2008) son solo algunos ejemplos donde las planarias se utilizan como modelo de estudio .

Las planarias están englobadas dentro del superfilo Lophocotrozoa (Figura 22), concretamente en el filo Platyhelminthes (gusanos planos), clase Turbellaria (Baguñà and Riutort, 2004). Se pueden encontrar planarias en casi cualquier hábitat, ya que existen planarias de agua dulce, marinas, cavernícolas y terrícolas (Alvarez-Presas et al., 2008). Por lo general son animales de vida libre, aunque existe alguna forma simbiótica o parásita. Las planarias se pueden clasificar en biotipos asexuales y sexuales, las cuales suelen ser hermafroditas.

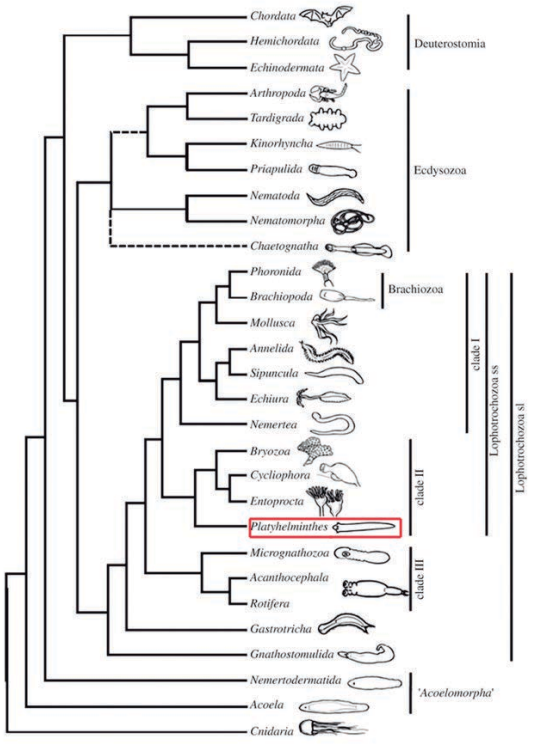


Figura 22. Árbol filogenético de los metazoos. El cuadro rojo marca los Platyhelminthes, filo al que pertenecen las planarias. [Adaptado de Paps et al., 2009]

En esta tesis doctoral nos hemos centrado en el estudio de dos especies de planarias pertenecientes a la familia Dugesiidae: *Schmidtea mediterránea*, el biotipo asexuado, y *Schmidtea polychroa*, que solo presenta biotipo sexuado (Figura 23). Son especies hermanas, morfológicamente casi idénticas (Alvarez-Presas et al., 2008). Generalmente, el biotipo asexuado de *S. mediterranea* es el más estudiado por su gran capacidad regenerativa. En cambio, *S. polychroa* se utiliza especialmente en el campo de la embriogénesis, ya que se encuentra con facilidad en nuestro entorno, es fácil de mantener en el laboratorio y tiene una ratio de reproducción muy alta comparada con el biotipo sexuado de *S. mediterranea*. Esta última característica también convierte a *S. polychroa* en un buen modelo para estudiar la reproducción sexual.

Figura 23. Planarias modelo utilizadas, *Schmidtea mediterranea* y *Schmidtea polychroa*. [Adaptado de Planmine, Brandl et al., 2016].

3.1 Anatomía

[La información relativa a la anatomía que se describe en los siguientes apartados proviene principalmente de los siguientes libros y artículos científicos: Chandebois, 1976; Hyman, 1951; Rieger et al., 1991; Saló, 2006]

Las planarias son organismos bilaterales. Podemos distinguir un eje AP y un eje DV. Las planarias son planas dorsoventralmente y alargadas. Su tamaño varía enormemente (desde unos pocos milímetros hasta medio metro) en función de la especie, las condiciones ambientales y del alimento disponible. Las planarias utilizadas en esta tesis, *S.mediterránea* y *S. polychroa*, presentan un tamaño medio entre 0,1-1 cm.

Las planarias son organismos acelomados, es decir, poseen una masa celular compacta entre la epidermis y los órganos conocida como parénquima. Poseen sistema nervioso, digestivo, excretor y muscular, y carecen de sistema circulatorio, respiratorio y esquelético. La función de estos sistemas se suple mediante la presencia de una extensa red de ramas digestivas, una respiración por difusión a través del epitelio externo y diferentes capas musculares. Además, los biotipos sexuales presentan un sistema reproductor. A continuación se explicará de forma breve las características de cada sistema.

3.1.1 Sistema nervioso y sensorial

El sistema nervioso de las planarias se divide en central y periférico. El sistema nervioso central se compone de dos ganglios cefálicos ventrales conectados por una comisura. Cada ganglio presenta proyecciones nerviosas laterales externas o ramas nerviosas. Además, unido a cada ganglio aparece un cordón nervioso ventral que recorre todo el cuerpo a lo largo del eje AP. Los cordones nerviosos están formados por pequeños ganglios que proyectan axones hacia otros órganos formando el sistema nervioso periférico. El sistema nervioso periférico está formado por diferentes plexos nerviosos, como el plexo muscular, el plexo epitelial, el plexo de la faringe y el plexo digestivo (Figura 24 A).

Aunque la morfología del sistema nervioso de las planarias es simple, está compuesto por numerosos tipos neuronales homólogos a los encontrados en vertebrados, como son neuronas GABAérgicas, serotoninérgicas, dopaminérgicas, octopaminérgicas, etc. (Cebrià, 2007; Cebrià, 2008; Fraguas et al., 2012). Además, recientes estudios han demostrado que las planarias poseen células gliales (Roberts-Galbraith et al., 2016; Wang et al., 2016), lo que sugiere que este sistema es más complejo de lo que se pensaba.

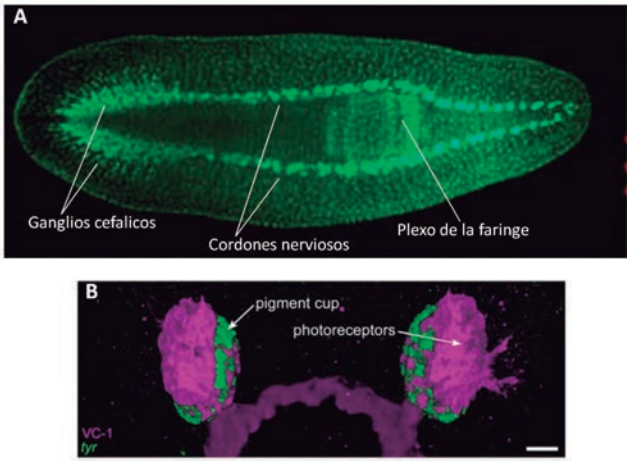


Figura 24. Sistema nervioso y visual de las planarias. A) Inmunotinción del sistema nervioso utilizando el anticuerpo 3C11 que marca la sinapsina. B) Sistema visual: las células pigmentarias (verde) han sido marcadas mediante la hibridación *in situ* del gen *tirosina*; las células fotoreceptoras (púrpura) han sido marcadas mediante el anticuerpo VC-1. [Adaptado de Cebrià, 2007 (A); adaptado de Lambrus et al., 2015 (B)].

El sistema sensorial se localiza principalmente en la zona de la cabeza. Está formado por un sistema visual, un conjunto de quimioreceptores para localizar el alimento, y un conjunto de mecanoreceptores, como los reoreceptores que se utilizan para detectar la corriente de agua. El sistema sensorial más estudiado es el visual. Está compuesto por dos ojos en la parte anterior, que presentan dos tipos celulares: las células fotoreceptoras y las pigmentarias (Figura 24 B). Las células fotoreceptoras del tipo rabdomérico proyectan axones hacia ambos ganglios cefálicos formando un quiasma óptico (Okamoto et al., 2005). Las células pigmentarias están distribuidas en forma de copa alrededor de las fotoreceptoras para protegerlas del exceso de luz. Debido a su importancia en el presente trabajo, explicaremos más adelante el desarrollo y formación del sistema visual.

3.1.2 El sistema digestivo y excretor

El sistema digestivo de las planarias (Figura 25) se caracteriza por ser un sistema ciego, de una sola apertura o boca por la cual se evagina una faringe. Posee tres ramas digestivas, una anterior y dos posteriores, muy ramificadas para repartir los nutrientes por todo el cuerpo del animal. Estas tres ramas convergen en la faringe, situada en la parte central. La faringe es un órgano musculado e inervado por el cual entra el alimento. Además, se encarga de expulsar los desechos del animal (Forsthoefel et al., 2011).

El sistema excretor se compone principalmente por protonefridios, la función de los cuales es la regulación osmótica del animal mediante filtración. Estos protonefridios se encuentran repartidos por todo el animal (Rink et al., 2011).

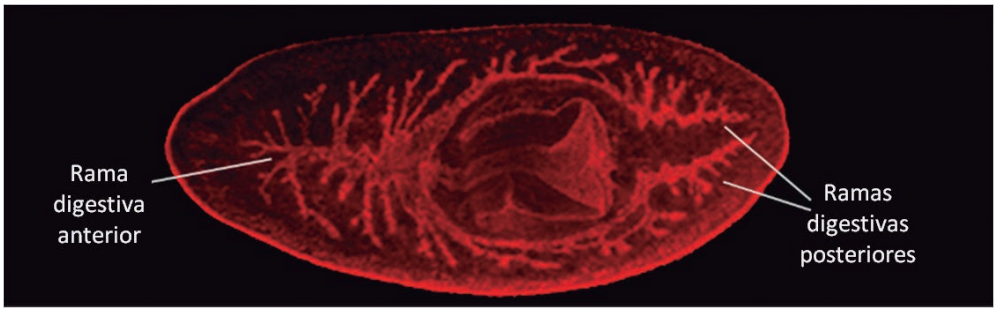


Figura 25. Inmunotinción del sistema digestivo utilizando el anticuerpo anti-βcatenina-2. [Imagen cedida por M. Almuedo-Castillo].

3.1.3 El sistema muscular y la epidermis

El sistema muscular (Figura 26) se compone de fibras musculares dispuestas en diferentes capas, cumpliendo también la función de sistema esquelético. Estas capas se disponen, desde el exterior al interior, en fibras musculares circulares, longitudinales y oblicuas. Además, se encuentran fibras longitudinales internas que atraviesan el parénquima. Diferentes órganos también presentan fibras musculares, como el digestivo, la faringe y el aparato copulador (Cebrià, 2016).

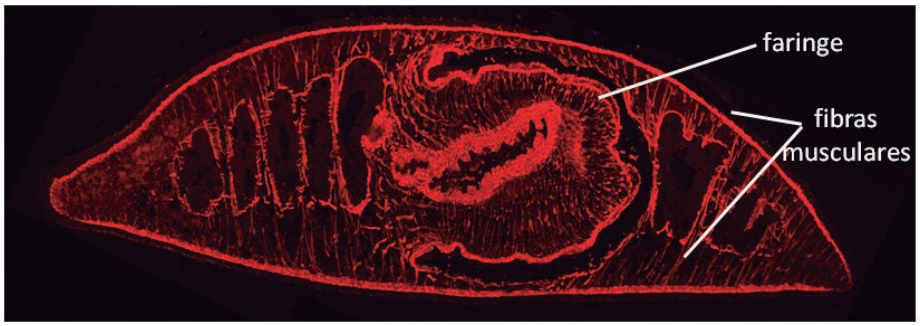


Figura 26. Inmunotinción del sistema muscular utilizando el anticuerpo 6G10 que marca la MHC-A. Sección transversal de la planaria.

Recientes estudios sugieren que la función del sistema muscular no es solo dotar de movimiento al animal, sino que también proporciona información posicional, actuando de "GPS" mediante la secreción de diferentes moléculas señalizadoras o morfógenos (Figura 27). Así pues, patronea al animal en los diferentes ejes (Witchley et al., 2013).

La epidermis se compone de una capa de células epiteliales monoestratificada y en su mayor parte ciliada. Entre estas células epiteliales encontramos células secretoras de moco, el cual forma una capa mucosa alrededor de la planaria. Esta capa tiene una función protectora (de defensa e inmunológica) y además facilita la locomoción del animal.

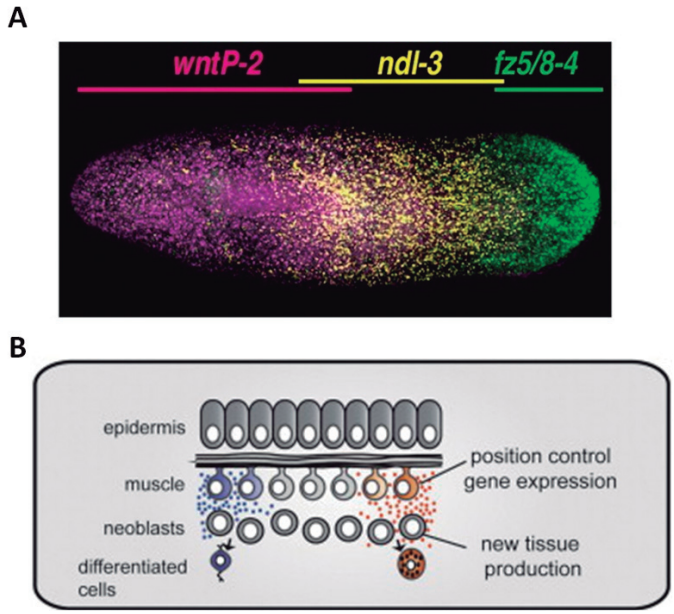


Figura 27. Modelo del control posicional mediante dos sistemas flexibles: neoblastos y musculatura. (A) Hibridación *in situ* fluorescente de *wntP-2*, *ndl-3* y *fz5/8-4*, expresados en células musculares en dominios concretos. Anterior es derecha. (B) Resumen del modelo. Los neoblastos pueden generar cualquier tipo celular y las células musculares les proporcionan las instrucciones posicionales para generar la región del cuerpo adecuada. [Adaptado de Witchley et al., 2013].

3.1.4 El parénquima: los neoblastos

El tejido que se encuentra entre la epidermis y los órganos internos se conoce como parénquima o mesénquima. Este tejido compacto incluye diferentes tipos de células, como las células secretoras y los neoblastos, los cuales pueden constituir entre un 20-30% de todas las células del parénquima (Baguñà et al., 1989). Los neoblastos son las células madre adultas pluripotentes que confieren a las planarias su gran plasticidad (Wagner et al., 2011). Se encuentran repartidos por todo el cuerpo excepto en la faringe y la punta de la cabeza (Figura 28 A). Son células de un tamaño entre 5-8 micras con una proporción alta núcleo/citoplasma (Figura 28 B). Además poseen pocos orgánulos celulares, cuerpos cromatoides perinucleares y cromatina laxa. Asimismo son las únicas células con capacidad proliferativa y, por lo tanto, son los responsables de los procesos de regeneración y homeostasis.

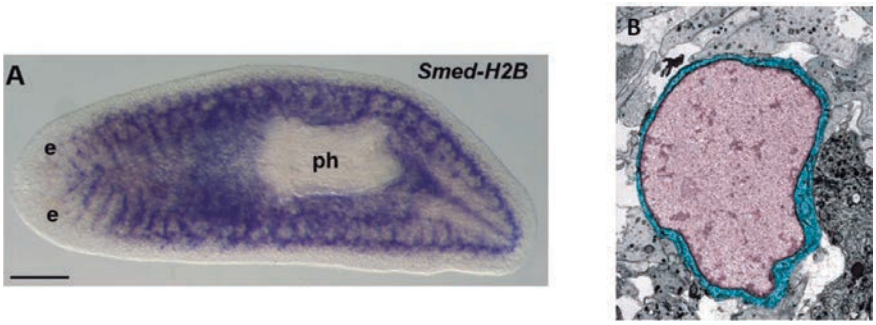


Figura 28. Los neoblastos. A) Hibridación *in situ* del gen *H2B* que marca todos los neoblastos. B) Imagen de microscopía electrónica que muestra el alto ratio núcleo/citoplasma. Núcleo resaltado en rosa, citoplasma resaltado en azul. [Adaptado de Solana et al., 2012 (A); y Reddien y Sanchez Alvarado, 2004 (B)].

En los últimos años se ha avanzado mucho en el conocimiento de los neobastos. Actualmente se propone que existen diferentes poblaciones de neoblastos (Molinaro and Pearson, 2016; van Wolfswinkel et al., 2014; Wagner et al., 2011):

- Los sigma-neoblastos: Esta población da lugar a las otras poblaciones de neoblastos. Se sugiere que una pequeña proporción de esta población son las verdaderas células madre pluripotentes, llamadas cNeoblastos o neoblastos clonogénicos.
- Los zeta-neoblastos: esta población se origina de los sigma, y da lugar al linaje de epidermis.
- Los gamma-neoblastos: origina el linaje digestivo.
- Los nu-neoblastos: dan lugar al linaje neural.

Los zeta/gamma/nu-neoblastos corresponden a células madre determinadas que todavía mantienen la capacidad de proliferar. Estas células darán lugar a las progenitoras postmitóticas y luego a las células terminalmente diferenciadas específicas de cada tejido (van Wolfswinkel et al., 2014). Los factores determinantes de la especificación de linajes específicos están siendo objeto de intensa investigación. Actualmente se conoce en bastante detalle los factores de transcripción que especifican diferentes linajes (Scimone et al., 2014).

3.1.5 El sistema reproductor

Las planarias del biotipo sexual, cuando alcanzan la madurez o un tamaño en concreto, desarrollan el sistema reproductor (Figura 29). Cabe recordar que son hermafroditas y por lo tanto poseen gónadas masculinas (testículos) y femeninas (ovarios). Además presentan una serie de órganos accesorios, como el aparato copulador o las glándulas vitelinas. Los testículos se encuentran distribuidos a lo largo del eje AP en dos líneas paralelas dorsales y son muy abundantes. Las gónadas femeninas se componen de un par de ovarios ventrales localizados justo detrás de cada ganglio cefálico. Las gónadas masculinas son las encargadas de la producción del esperma y las femeninas de los oocitos (Chong et al., 2011). Las gónadas están formadas por células madre diferentes que los neoblastos (Handberg-Thorsager and Saló, 2007; Sato et al., 2006; Wang et al., 2007), aunque se ha demostrado que pueden participar en la regeneración (Gremigni et al., 1980).

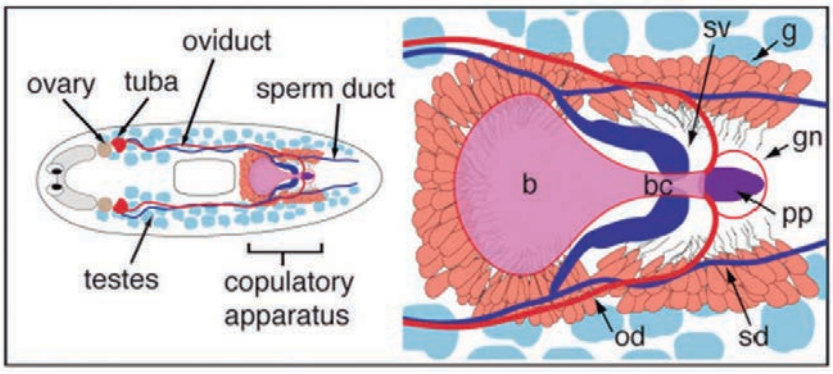


Figura 29. Esquema del sistema reproductor de la planaria. Bursa (b), canal de la bursa (bc), vesícula seminal (sv), espermiducto (sd), oviducto (od), glándulas (g), gonoporo (gn), papila peneana (pp). [Adaptado de Chong et al., 2011].

3.2 La regeneración

[La información relativa sobre la regeneración que se describe en los siguientes apartados proviene principalmente de los siguientes artículos científicos Baguñà, 2012; Baguñà et al., 1990; Reddien and Sanchez Alvarado, 2004; Saló, 2006]

Las planarias son especialmente conocidas por su gran capacidad regenerativa. Pocos animales tienen este potencial de regenerar un organismo entero partiendo de un fragmento muy pequeño. Citando la célebre frase de Dalyell ("Planarians can almost be called immortal under the edge of the knife"), las planarias se pueden considerar casi inmortales. La faringe y la punta de la cabeza (justo por delante de los ojos) son las únicas estructuras que no pueden dar lugar a un nuevo organismo, ya que estas zonas no poseen neoblastos.

La regeneración puede definirse como la habilidad de un organismo desarrollado de reemplazar estructuras del cuerpo amputadas o perdidas. Según la visión clásica propuesta por T. H. Morgan, la regeneración se clasifica en dos grandes mecanismos, morfalaxis y epimorfosis, dependiendo de la necesidad de actividad proliferativa o de desdiferenciación para la generación de nuevo tejido (Morgan, 1898b). Actualmente esta clasificación no se utiliza ya que los estudios moleculares demuestran que en la mayoría de organismos la regeneración es en realidad una mezcla de morfalaxis y epimorfosis.

3.2.1 El proceso de regeneración

Para explicar el proceso de regeneración de las planarias, utilizaremos como ejemplo la regeneración anterior después de la amputación de la cabeza (Figura 30), aunque puede generalizarse a cualquier parte del cuerpo. Después de amputar la cabeza, se produce una rápida contracción muscular que lleva a la confrontación de la epidermis dorsal con la ventral, cerrando la herida. Este proceso apenas dura unos treinta minutos. Después del cierre de la herida, los neoblastos empiezan a proliferar y migran hacia la herida o post-blastema (región adyacente al blastema) para formar una masa de tejido despigmentado o blastema, formado por células postmitóticas indiferenciadas. A medida que pasan los días, las células del blastema se diferencian dando lugar a las estructuras perdidas, como por ejemplo los ojos. Al cabo de unos 7 días se puede identificar claramente una nueva cabeza funcional.

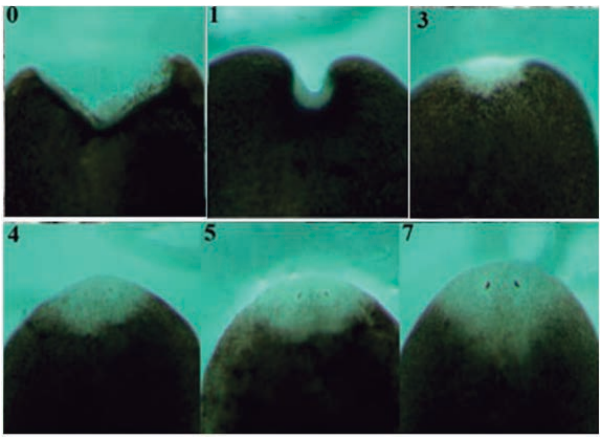


Figura 30. Etapas de la regeneración anterior en planaria. El numero de cada imagen representa el día post-amputación. [Adaptado de Saló, 2006].

En este proceso intervienen diversos eventos más complejos:

- Cierre de la herida: el cierre de la herida es básico para que el animal pueda regenerar. Este proceso se da gracias a la contracción muscular y la confrontación del epitelio dorsal y ventral. Cabe destacar que, aunque se han realizado diferentes estudios del cierre de la herida, aún no se ha encontrado ningún gen o molécula responsable de la activación de la contracción (Wenemoser et al., 2012).

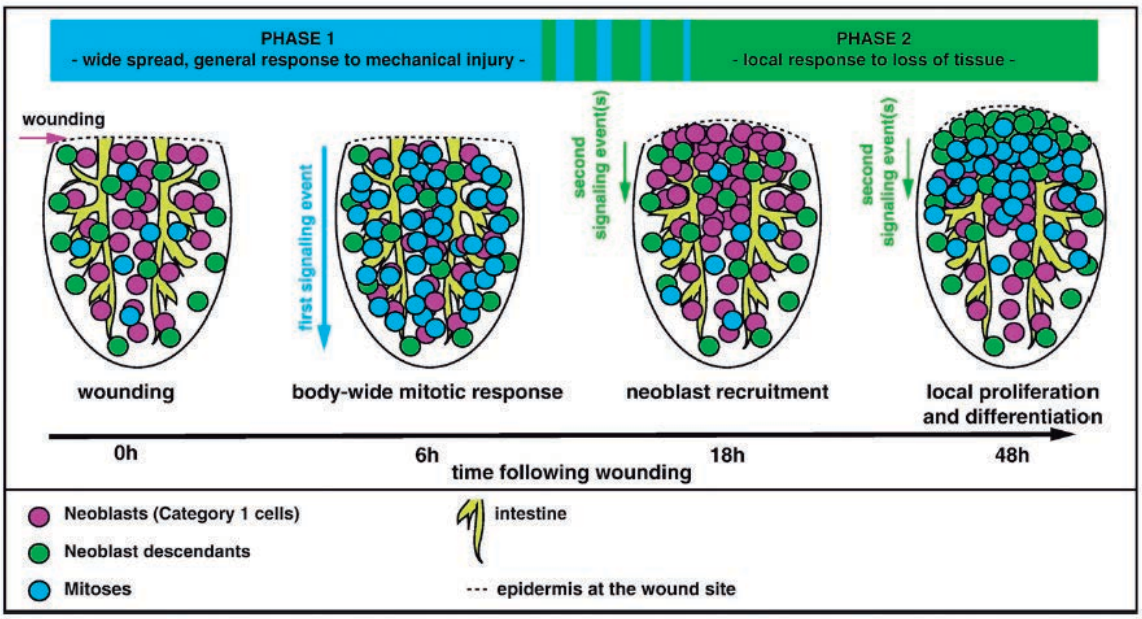


Figura 31. Modelo de la respuesta de los neoblastos a la herida. El esquema resume los cambios celulares durante el inicio de la regeneración en un fragmento cola. Ocurren dos fases distintas de respuesta de los neoblastos durante la regeneración inicial. La primera fase representa una respuesta genérica a la herida que se da por todo el cuerpo e induce el incremento de la proliferación de los neoblastos. La segunda fase ocurre solo cuando la pérdida de tejido es significativa e implica señales locales que inducen una proliferación local de los neoblastos y su posterior diferenciación en la región de la herida. [Adaptado de Wenemoser y Reddien, 2010]

- Proliferación y apoptosis: la regeneración de las planarias se basa en la producción de células nuevas a partir de la proliferación de los neoblastos. Así, tras una herida observamos un primer pico generalizado de proliferación a las 6 horas post-amputación y un segundo pico localizado en la zona del post-blastema a las 48 horas post-amputación (Saló and Baguñà, 1984; Wenemoser and Reddien, 2010). Mientras que el primer pico se activa después de cualquier lesión, por pequeña que sea, el segundo solo se activa cuando hay una perdida sustancial de tejido (Figura 31). Además, para que la respuesta regenerativa sea exitosa, también debe activarse la muerte celular por apoptosis. Después de una herida se produce un pico apoptótico a las 4 horas post-amputación localizado en la zona de la herida y un segundo pico a los 3 días, el cual está relacionado con la remodelación del organismo (Pellettieri et al., 2010). Esta remodelación está asociada con la restructuración de los tejidos preexistentes de acuerdo con su nuevo tamaño (Figura 32). Está descrito que existe una relación entre el control de la respuesta proliferativa y la apoptótica, por ejemplo por parte de vías de señalización tempranas como la de la JNK (Almuedo-Castillo et al., 2014). Sin embargo no se conoce si el primer pico apoptótico es necesario para la activación de la primera respuesta mitótica.

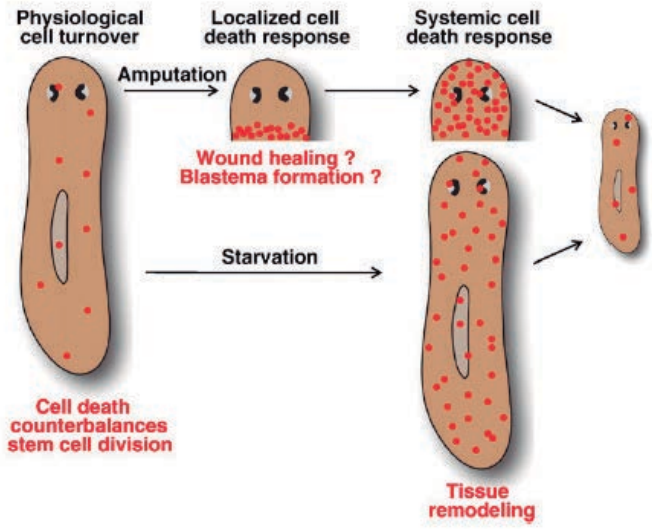


Figura 32. Modelo de la función apoptótica durante la remodelación de los tejidos. La amputación conduce a dos olas diferentes de apoptosis (círculos rojos) en fragmentos regenerantes. La ola inicial localizada de muerte celular se produce a las 4 horas post-amputación y promovería el cierre de la herida y/o la formación del blastema. La segunda ola sistémica estaría relacionada con la remodelación de los tejidos pre-existentes para acomodarlos y escalarlos al nuevo tamaño. Durante un periodo de ayunas también se induce la muerte celular sistémica que contribuye con la reducción del tamaño. [Adaptado de Pellettieri et al., 2010]

- Diferenciación de los tejidos: además de proliferar, los neoblastos deben diferenciarse correctamente para dar lugar a las estructuras perdidas. Se sugiere que los neoblastos se especializan en función de las diferentes señales que reciben. Estas señales inducen la expresión de factores de transcripción, los cuales determinan el linaje al que darán lugar (Reddien, 2013). Actualmente se postula que los neoblastos y su progenie reciben señales de la musculatura para determinar su posición y en qué diferenciarse (Witchley et al., 2013). Unas de las señales más estudiadas en este proceso son la vía Wnt/βcatenina y la vía BMP, responsables de restablecer los ejes AP y DV, respectivamente (Reddien, 2011).

3.3 La homeostasis

Las planarias son especialmente conocidas por su gran capacidad regenerativa, pero en los últimos años también se han convertido en un buen modelo para el estudio del control de la renovación de los tejidos durante la homeostasis y el crecimiento corporal. Como ya se ha comentado anteriormente, las planarias varían continuamente de tamaño, en función de la disponibilidad del alimento y las condiciones ambientales, como la temperatura (Figura 33). Además, después de regenerar una parte del cuerpo, los tejidos preexistentes deben remodelarse para adaptarse al nuevo tamaño del animal. Todas estas características convierten a la planaria en uno de los animales más plásticos que existen en el reino animal (Saló, 2006).

Podemos definir la homeostasis como el mantenimiento de los órganos y tejidos de un organismo de forma autoregulada. En las planarias, la homeostasis se mantiene indefinidamente en el tiempo y los neoblastos son los responsables de este fenómeno. Aunque la regeneración y la homeostasis están muy ligadas entre sí, no siguen exactamente los mismos procesos. Por ejemplo, el balance entre proliferación y muerte celular es diferente. Si el animal dispone de alimento, la proliferación es más alta mientras que la muerte celular se da de forma basal. En cambio, si el animal está en ayunas, la proliferación tiene lugar de forma basal mientras que la muerte celular aumenta para proporcionar energía y reescalar los tejidos debido a su decrecimiento. De esta forma,

controlando el balance proliferación-muerte celular, el animal puede crecer o decrecer (Gonzalez-Estevez et al., 2012). Recientes estudios han demostrado que tanto la vía de la Insulina como la vía mTOR están directamente relacionadas con este fenómeno (González-Estévez et al., 2012; Miller and Newmark, 2012).

Durante la homeostasis, la planaria no solo debe mantener los tejidos, también debe mantener los ejes de simetría. Se ha descrito que la vía Wnt/βcatenina y la vía BMP, además de controlar la polaridad AP y DV durante la regeneración, también la mantienen durante la homeostasis (Gurley et al., 2008; Iglesias et al., 2008; Molina et al., 2007; Orii and Watanabe, 2007; Petersen and Reddien, 2008; Reddien et al., 2007).

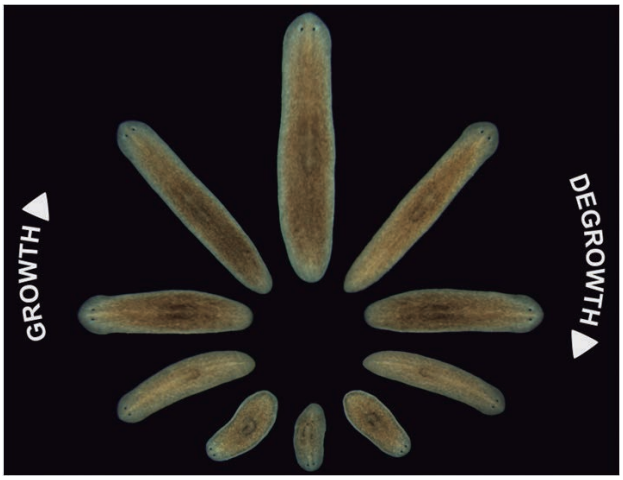


Figura 33. Crecimiento y decrecimiento de las planarias. En función de la disponibilidad de alimento y la temperatura, las planarias pueden crecer y decrecer continuamente. [Imagen cedida por M. Almuedo-Castillo].

3.4 La reproducción

Las planarias según su biotipo pueden reproducirse de forma sexual o asexual. La reproducción asexual consiste en la escisión de una parte del organismo, normalmente la cola, formando dos fragmentos. Ambos fragmentos regeneran las partes ausentes, en un caso la cola y en el otro la cabeza y el tronco. Por otro lado, la reproducción sexual conlleva la introducción del aparato copulador de un individuo en otro individuo y viceversa. Esto produce un estímulo que da lugar a la formación de un huevo o cocoon (Vreys and Michiels, 1998). Sin embargo, la formación del cocoon puede darse de dos maneras:

- Por fecundación cruzada, el esperma del individuo A fecunda el oocito del individuo B.
- Por partenogénesis, sin la participación del esperma del individuo A, el oocito del individuo B genera un embrión (D'Souza et al., 2004).

El cocoon contiene entre uno y siete embriones encapsulados junto con las células vitelinas, y es depositado en el medio exterior. Pasados unos 14 días, del cocoon saldrán los juveniles, con la apariencia del adulto pero más pequeños y sin el sistema reproductor.

Los biotipos sexuales, por norma general, tenderán a reproducirse de forma sexual para generar más variabilidad genética. Esto no excluye que puedan reproducirse de forma asexual.

Las planarias utilizadas en esta tesis, *S. mediterranea* y *S. polychroa*, son biotipo asexual y sexual respectivamente.

3.5 La embriogénesis

S. mediterranea y *S. polychroa* pertenecen al grupo Neophora, que se caracteriza por generar huevos ectolecitos (vitelo periférico separado del óvulo) (Benazzi-Lentati, 1970). Además, aunque están dentro del clado de los espirales, presentan un desarrollo derivado, diferente al de sus ancestros espirales (Martín-Durán et al., 2012).

Las planarias durante la cópula depositan el esperma en el otro individuo. Este esperma puede ser utilizado inmediatamente para fecundar un oocito, almacenarse y utilizarse más adelante para el mismo propósito, o ser degradado y utilizado como nutriente. Una vez el oocito es fecundado, baja por el oviducto pasando por las glándulas vitelinas que incorporarán las células vitelinas o vitolecitos. Finalmente el embrión y los vitelocitos son encapsulados por una cubierta de naturaleza no quitinosa formando el cocoon o cápsula embrionaria (Cardona et al., 2005).

Diferentes autores han descrito y dividido la embriogénesis de planarias en diversos estadios (Figura 34) (Cardona et al., 2005; Le Moigne, 1966; Martín-Durán et al., 2010):

- Estadio 1: dentro del cocoon se encuentran varios zigotos rodeados por células vitelinas. El primer evento es la segmentación del zigoto, que da lugar a los blastómeros, las células embrionarias. Estos blastómeros permanecen aislados unos de otros, a diferencia de todos los tipos de segmentación descritos. Al cabo de unas 2-3 rondas de mitosis, algunos vitelocitos se fusionan formando un sincitio alrededor de cada embrión. Una vez formado el sincitio, los blastómeros continúan dividiéndose pero aún permanecen aislados entre ellos.

- Estadio 2: durante este estadio se forma la larva, en la cual se distingue una epidermis embrionaria ciliada, una faringe embrionaria musculada y una cavidad embrionaria conectada a la faringe. Entre la epidermis y la cavidad embrionaria se encuentran blastómeros indiferenciados y núcleos de células vitelinas provenientes del sincitio, que han sido rodeados por la epidermis embrionaria. Todos los órganos que aparecen en este estadio son transitorios y no se encuentran en el adulto.

- Estadio 3: la larva comienza a ingerir las células vitelinas externas que no han participado en la formación de los diferentes sincitios. A medida que la larva come, la cavidad embrionaria se llena de vitelocitos generando presión hacia la periferia, de forma que los blastómeros y los núcleos de los vitelocitos se agregan formando la banda germinal. Durante este estadio los blastómeros continúan dividiéndose y la larva aumenta en tamaño.

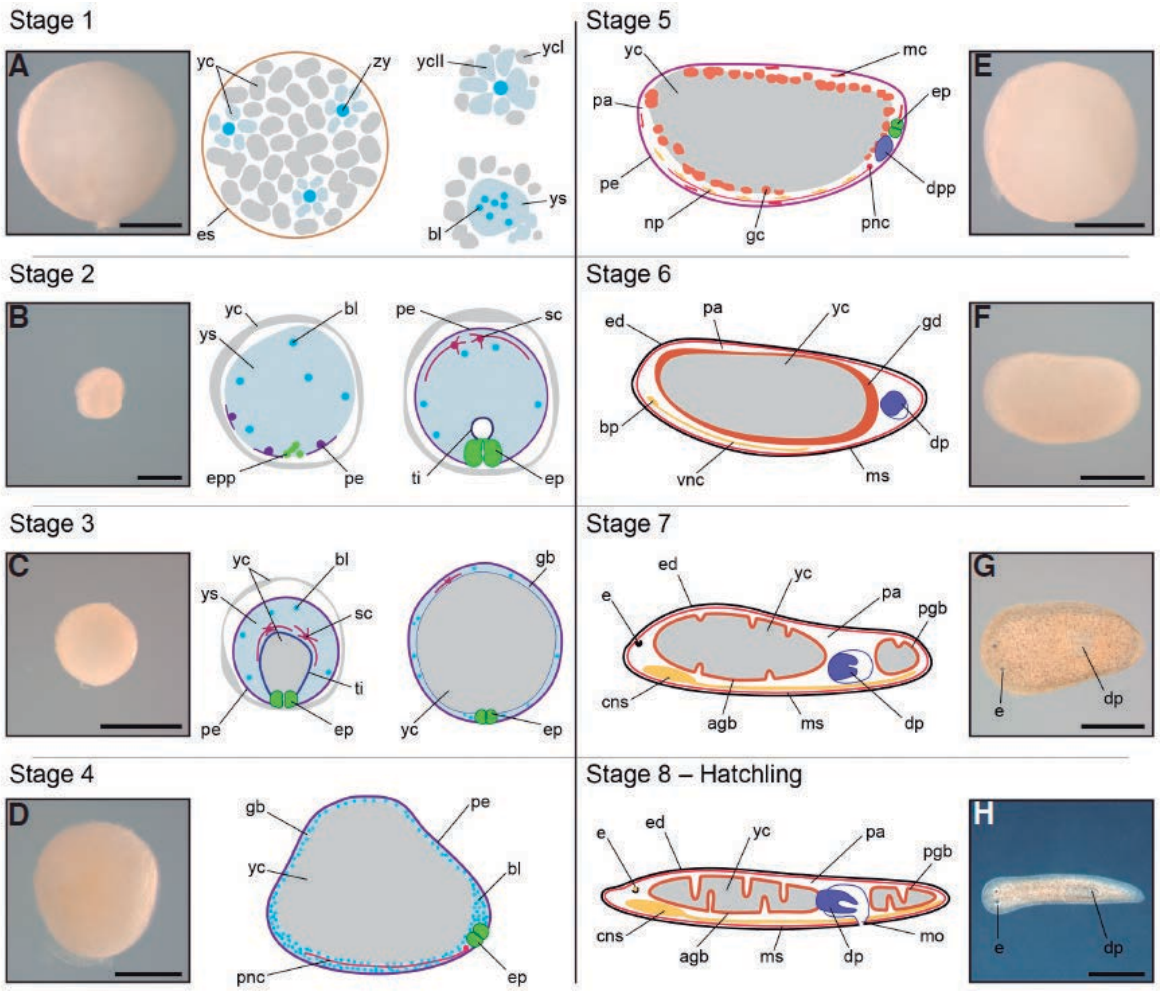


Figura 34. Desarrollo embrionario de la planaria *S. polychroa*. (A-H) Embriones diseccionados de estadio 1, 2, 3, 4, 5, 6 ,7 y juvenil respectivamente. (A) Masa de células vitelinas, en cuyo interior se forma el sincitio y las primeras divisiones de los blastómeros. (B) Larva prematura con sus estructuras transitorias características (epidermis primaria, faringe embrionaria e intestino transitorio). (C) Debido a la ingesta del vitelo, la larva crece en tamaño y el sincitio inicial queda restringido a la periferia de la larva formando la banda germinal. (D) Los blastómeros de la banda germinal proliferan y se empiezan a formar los cordones nerviosos pioneros. (E) Especificación de las identidades axiales definitivas y diferenciación de las células adultas, formación del primordio de la faringe definitiva. (F,G) Durante los estadios 6-7 los órganos definitivos se desarrollan hasta adquirir la morfología del juvenil (H). Células vitelinas (yc), zigoto (zy), envoltorio del huevo (es), blastómeros (bl), sincitio vitelino (ys), primordio de faringe embrionaria (epp), epidermis embrionaria (pe), faringe embrionaria (ep), intestino temporal (ti), banda germinal (gb), parénquima (pa), precursor neuronal (np), célula gastrodérmica (gc), primordio de cordón nervioso (pnc), primordio de faringe definitiva (dpp), célula muscular (mc), epidermis (ed), faringe definitiva (dp), musculatura (ms) cordones nerviosos ventrales (vnc), primordio de cerebro (bp), ojo (e), sistema nervioso central (cns), boca (mo), rama digestiva anterior (agb), rama digestiva posterior (pgb) [Adaptado de Martín-Durán et al., 2012].

- Estadio 4: durante este estadio la larva alcanza su mayor tamaño y concluye su ingesta de vitelocitos. En este estadio da comienza la metamorfosis de la larva.

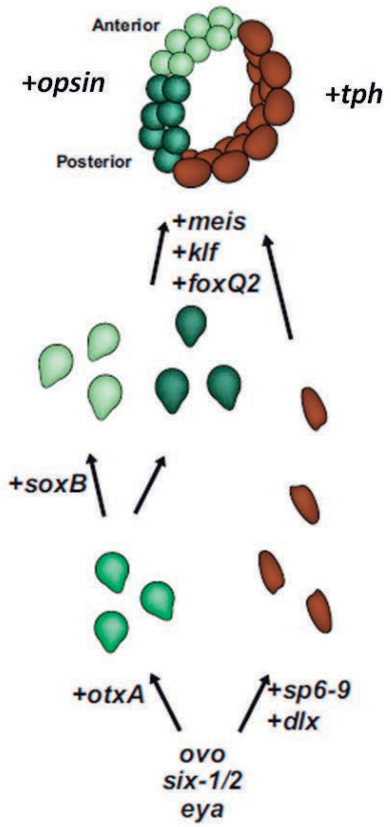
- Estadio 5: la larva deja de ser esférica para formar una especie de "lacasito" (término acuñado por el Dr. Rafa Romero). La faringe embrionaria se desplaza hacia un extremo del "lacasito", el cual se considera el extremo posterior. Por tanto, se puede diferenciar una polaridad AP (en términos morfológicos). Durante este estadio embrionario se produce una diferenciación masiva. Los blastómeros cambian de forma y dan lugar a los diferentes precursores de los tejidos adultos (epidermis,

musculatura, nervioso, etc.). Además, aparece el primordio de la faringe definitiva en una posición cercana a la faringe embrionaria, que empieza a degenerar. En la región anterior tiene lugar una acumulación de células neuronales que origina los ganglios cefálicos.

- Estadio 6: durante este estadio el embrión adquiere una forma fusiforme debido a la diferenciación de las fibras musculares. Se pueden distinguir los ganglios cefálicos, los cordones nerviosos ventrales y la faringe definitiva.
- Estadio 7: aparecen los ojos y se continúan desarrollando los órganos definitivos, como el digestivo.
- Estadio 8: el embrión o juvenil finaliza la organogénesis y está preparado para salir del cocoon con todos los rasgos del adulto, a excepción del sistema reproductor.

3.6 La genética del sistema visual

En los últimos años, el sistema visual ha sido básico para entender el proceso de especialización y diferenciación de los neoblastos (Saló et al., 2002).



Los neoblastos son la fuente de nuevas células tanto durante la homeostasis como la regeneración. Existen subpoblaciones de neoblastos que definen un linaje celular en concreto. Uno de esos linajes es el que origina el sistema visual (Figura 35). Estos neoblastos especializados se caracterizan por expresar el factor de transcripción *ovo* (Lapan and Reddien, 2012), además de diferentes marcadores generales de neoblastos como son *h2b* o *piwi*. El factor de transcripción *ovo* se expresa en todas las células de ojos, sean neoblastos especializados, progenie más diferenciada o células totalmente diferenciadas, (fotoreceptores y células pigmentarias), y no se expresa en ningún tipo celular más. A medida que los neoblastos especializados se van diferenciando, pierden los marcadores de pluripotencia (*h2b* o *piwi*), mantienen *ovo* y empiezan a expresar marcadores de línea pigmentaria (*sp6-9*), o bien de fotoreceptores (*otxA*), según sea su destino. Esta expresión de marcadores de línea pigmentaria y fotoreceptores se mantiene hasta el estado

Figura 35. Modelo de la genética del ojo. Las células dibujadas en verde representa el linaje de fotoreceptor y las células dibujadas en marrón representa el linaje pigmentario. El precursor de ojo expresa *ovo*, *six-1/2* y *eya*. Las células que expresan *otxA* pasan a ser progenitoras de fotoreceptor y a medida que se diferencian expresan otros factores de transcripción (*soxB, meis*, etc.) hasta expresar marcadores de célula diferenciada como *opsin*. Las células que expresan *sp6-9* y *dlx* pasan a ser progenitoras de célula pigmentaria hasta expresar marcadores de célula diferenciada como *tph*. [Adaptado de Lapan y Reddien, 2012].

totalmente diferenciado.

Cuando las células forman parte de la estructura del ojo, cada linaje expresa genes con funciones específicas de cada tipo celular, como puede ser *opsin* en fotoreceptores o *tph* en pigmentarias (Lapan and Reddien, 2011; Lapan and Reddien, 2012; Saló et al., 2002). El proceso de diferenciación celular se da en el tiempo y también en el espacio. Los neoblastos se especializan a una cierta distancia de la propia estructura del ojo; sus descendientes, a medida que se diferencian, se encuentran cada vez más cerca de la estructura del ojo, hasta que las células diferenciadas se agrupan para formar el ojo en sí. Cabe resaltar la formación del ojo durante la embriogenesis de planaria exhibe una gran similitud con el proceso de regeneración del mismo (Martín Duran et al., 2012).

3.7 La planaria como modelo de estudio del establecimiento del eje AP

Desde finales del siglo XIX, las planarias se han utilizado para estudiar la regeneración. Uno de los procesos que desde entonces ha despertado más atención es cómo se generan los ejes AP y DV después de una amputación: ¿por qué un trozo central de una planaria sabe donde ha de regenerar la cabeza y la cola?

T.H. Morgan utilizó las planarias para estudiar la regeneración. Observó que tras cortar una planaria en fragmentos muy pequeños en algunas ocasiones daban lugar a regeneraciones aberrantes, formando en determinados casos dos cabezas en ambos extremos (Morgan, 1898a). Morgan ya postuló que debía haber algún tipo de molécula o información posicional que podría estar implicada en el establecimiento de la polaridad (Morgan, 1904a; Morgan, 1904b; Morgan, 1905). Sin embargo, en aquel momento no se disponía del conocimiento y las técnicas que existen hoy en día. Por ese motivo se abandonaron las planarias para este objetivo. Actualmente, gracias a las técnicas moleculares y genéticas desarrolladas en planarias, estas se han convertido en un gran modelo para estudiar cómo se mantiene, se establece y re-establece el eje AP.

3.7.1 La vía Wnt en las planarias

La vía Wnt es posiblemente la vía de señalización más estudiada en planarias.

Como tantos otros organismos, las planarias presentan componentes de la vía canónica y no canónica. Se han caracterizado diferentes elementos (Tabla 1), tanto de la vía canónica como la no canónica.

Mediante el estudio de los componentes de la vía Wnt se ha demostrado que la vía canónica establece y mantiene la identidad posterior en planarias (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Petersen and Reddien, 2009b). Además, se ha demostrado que la vía no canónica PCP también está presente en planarias y organiza los cilios de la epidermis. Por otra parte, se ha demostrado que la vía no canónica está implicada en el guiado de los axones (Almuedo-Castillo et al., 2011).

Componentes de la vía	Nº elementos	Referencias
Secreción de Wnts		
Porcupine	2	(Gurley et al., 2008)
Evi/Wntless	1	(Adell et al., 2009)
Inhibidores de Wnts		
sFRP	3	(Gurley et al., 2008)
Notum	1	(Petersen and Reddien, 2011)
WIF	1	(Riddiford and Olson, 2011)
Wnts	9	(Gurley et al., 2008; Petersen and Reddien, 2008; Adell et al., 2009; Petersen and Reddien, 2009; Gurley et al., 2010; Riddiford and Olson, 2011)
Frizzled	10	(Gurley et al., 2008; Riddiford and Olson, 2011; Currie et al., 2016)
Ptk7	1	(Lander and Petersen, 2016)
Dishevelled	2	(Gurley et al., 2008; Almuedo-Castillo et al., 2011)
Complejo de destrucción de Bcat		
Axina	2	(Iglesias et al., 2011)
APC	1	(Gurley et al., 2008; Petersen and Reddien, 2008; Iglesias et al., 2011)
GSK3	3	(Adell et al., 2008)
Bcatenina	3	(Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Chai et al., 2010)

Tabla 1. Componentes descritos de la vía Wnt.

Vía Wnt dependiente de la βcatenina

La función mas estudiada de la vía Wnt dependiente de βcatenina en planarias es la del establecimiento del eje AP. Diferentes grupos de investigación demostraron que la inhibición de la *βcatenina*, en concreto *βcatenina-1*, mediante ARN de interferencia (ARNi) produce la aparición de una cabeza en lugar de una cola en la región posterior. Esta transformación, fenotipo llamado "dos cabezas", se obtiene después de inhibir la *βcatenina-1* durante la homeostasis o después de inducir la regeneración (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Iglesias y colaboradores demostraron que el fenotipo producido después de inhibir *βcatenina-1* es dependiente de la dosis, tanto en cantidad como en tiempo de inhibición. Por tanto, después de inhibir

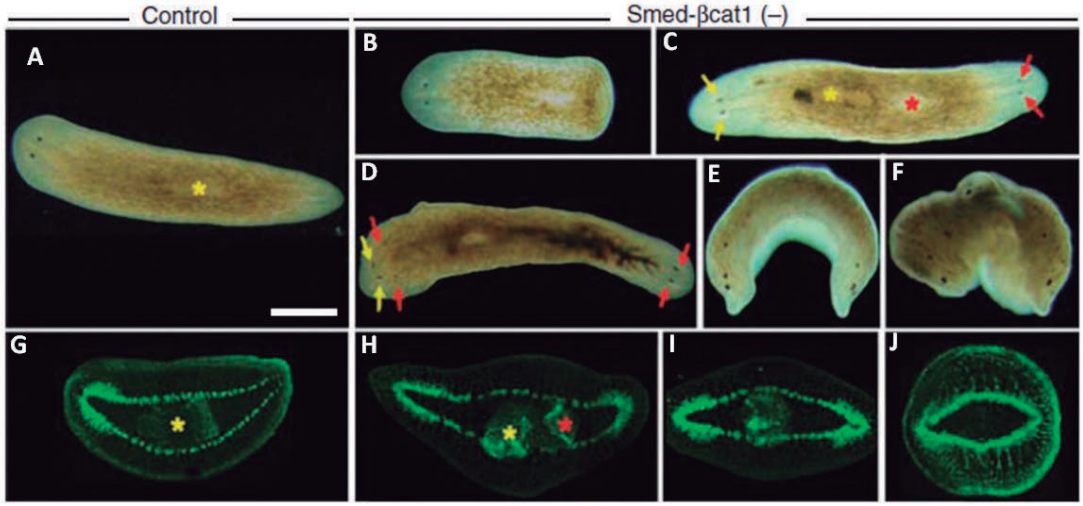


Figura 36. Gradación de fenotipos generados tras inhibir *βcatenina-1*. (A-F) Imágenes *in vivo*, (G-J) inmunotinción del sistema nervioso utilizando el anticuerpo 3C11 que marca la sinapsina. Se observa como a medida que se incrementa la dosis y el tiempo de la inhibición de la *βcatenina-1*, la anteriorización de la región posterior es más severa (B-D,H,I) hasta convertirse en una planaria radial hipercefalizada (E,F,J). Las flechas amarillas señalan ojos normales, las flechas rojas señalan ojos ectópicos, el asterisco amarillo señala faringe original, el asterisco rojo señala faringe ectópica. [Adaptado de Adell et al., 2010].

βcatenina-1 se obtienen planarias "sin cola", con "dos cabezas", con "dos cabezas y ojos ectópicos" y finalmente el fenotipo más severo de todos, una planaria "hipercefalizada" con morfología radial (Figura 36) (Adell et al., 2010; Iglesias et al., 2008).

Por otra parte, estos mismos autores describieron que las planarias poseen dos βcateninas. La inhibición de *βcatenina-1* produce los defectos de polaridad, mientras que la inhibición de *βcatenina-2* no produce ningún defecto (Iglesias et al., 2008).

Chai y colaboradores demostraron que en realidad esta duplicación de la βcatenina en planarias ha dado lugar a una segregación funcional completa. La βcatenina-1 se ha especializado en transcripción y la βcatenina-2 en adhesión celular. Esto se demostró mediante el estudio *in silico* de sus dominios proteicos y análisis funcionales *in vitro*. Además, el análisis de la localización subcelular de la βcatenina-2 (mediante un anticuerpo especifico) demostró que solo se encuentra en la membrana celular (Chai et al., 2010).

El fenotipo "dos cabezas" se obtiene tras inhibir a la *βcatenina-1* directamente o a través de inhibir a sus activadores, los Wnts. En concreto, la inhibición de *wnt1* fenocopia los fenotipos "sin cola" y "dos cabezas" cuando se induce la regeneración (Adell et al., 2009; Petersen and Reddien, 2009b). Cabe destacar que la inhibición de *evi/wntless*, proteína necesaria para la secreción de los Wnt, también produce el fenotipo "dos cabezas". Sin embargo, en este caso también se observan otros defectos clasificados como no canónicos (posición del sistema nervioso). Esta mezcla de defectos tras inhibir *evi/wntless* es debida a que se inhibe la secreción de todos los Wnts, tanto canónicos (Wnt1) como no canónicos (Wnt5) (Adell et al., 2009). Este mismo fenotipo (planarias con dos cabezas y defectos en el sistema nervioso) también se observa tras inhibir a *dishevelled* (Almuedo-Castillo et al., 2011).

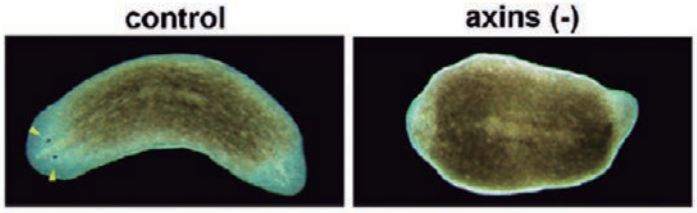


Figura 37. Fenotipo obtenido tras inhibir las *axinas*. La planaria axina-negativa presenta una cola en ambos extremos. Las flechas amarillas indican presencia de ojos. [Adaptado de Iglesias et al., 2011].

Mientras que la inhibición de la actividad de βcatenina-1 produce la aparición de una cabeza en la región posterior, la sobreactivación de βcatenina-1 da lugar a un fenotipo opuesto: la aparición de una cola en la región anterior. La sobreactivación de la vía se puede conseguir inhibiendo elementos del complejo de destrucción de la βcatenina, como es *APC* o *axina* (Figura 37). También se puede sobreactivar la βcatenina-1 inhibiendo reguladores negativos de los Wnts, como *notum*. La inhibición de estos reguladores negativos tras inducir la regeneración en planarias produce la aparición de una cola en la región anterior (Gurley et al., 2008; Iglesias et al., 2011; Petersen and Reddien, 2011).Todos estos datos demuestran que la vía Wnt dependiente de βcatenina especifica la identidad posterior en planarias, en concreto a través de βcatenina-1.

El estudio de la localización del ARN mensajero de los diferentes componentes de la vía Wnt llevó a proponer la hipótesis de que un gradiente de actividad βcatenina-1 desde

la cabeza hasta la cola de la planaria especifica el eje AP. Este gradiente de actividad de βcatenina-1 se da a nivel proteico y los Wnts expresados en posterior son los responsables de establecerlo y mantenerlo. En anterior se encuentran los inhibidores de los Wnts, como son *notum* o *sfrp-1*. Estas dos proteínas inhiben la acción de los Wnts y por tanto la actividad de βcatenina-1 en anterior (Adell et al., 2010). Sin embargo, la existencia de este gradiente no ha sido demostrada

Los Wnts posteriores

Además del *wnt1*, también se han caracterizado parcialmente otros *wnts* expresados en posterior que pertenecen a la familia del *wnt11*. Estos *wnts* posteriores (*wnt1, wnt11-1, wnt11-2* y *wnt11-5*) se expresan en las células musculares en forma de gradiente posterior (Figura 38) (Gurley et al., 2010; Witchley et al., 2013). Diferentes trabajos sugieren que tanto el Wnt11-2 como el Wnt11-5 pueden activar a la βcatenina-1. La inhibición de *wnt11-2* provoca el fenotipo "sin cola" tras inducir la regeneración (Figura 39), fenotipo igual al obtenido tras inhibir *βcatenina-1* a bajas dosis (Adell et al., 2009; Gurley et al., 2010). Por este motivo, Adell y colaboradores consideran que la función de Wnt11-2 es activar a la βcatenina-1 (Adell et al., 2009). En cambio Gurley y colaboradores sugieren que Wnt11-2 tiene un papel en el establecimiento de la línea media posterior y que su función es elongar la región posterior (Gurley et al., 2010).

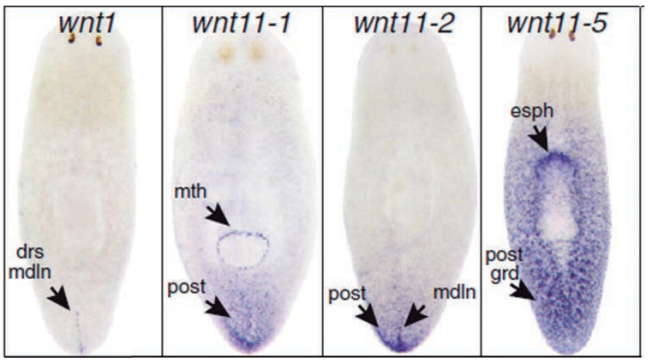


Figura 38. Patrón de expresión de los *wnts* posteriores. *wnt1* se expresa en la línea media posterior dorsal. *wnt11-1* se expresa en la boca y en forma de gradiente posterior en la cola. *wnt11-2* se expresa en forma de gradiente posterior en la cola y de forma concentrada en la línea media posterior. *wnt11-5* se expresa en forma de gradiente desde la región prefaríngea a la cola y de forma concentrada en el esófago de la planaria. [Adaptado de Gurley et al., 2010]

Por otra parte, la inhibición de *wnt1*, además de producir fenotipo "dos cabezas", también produce con mayor frecuencia el fenotipo "sin cola". En cambio, esta menor frecuencia del fenotipo "dos cabezas" incrementa hasta casi el 100% si se inhibe *wnt1* conjuntamente con *wnt11-5*, sugiriendo que Wnt11-5 también puede activar a la βcatenina-1 (Petersen and Reddien, 2009b). Sin embargo, no se ha descrito ningún fenotipo producido tras inhibir solo *wnt11-5* y, por tanto, no se conoce una función específica. En cuanto a *wnt11-1*, no se había descrito ninguna función hasta la fecha.

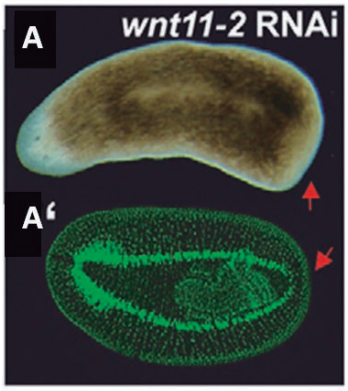


Figura 39. Fenotipo "sin cola" obtenido tras inhibir *wnt11-2*. A) imagen *in vivo*. A') inmunotinción del sistema nervioso utilizando el anticuerpo 3C11 que marca la sinapsina. [Adaptado de Adell et al., 2009].

Vía Wnt independiente de βcatenina

Una de las funciones de la vía Wnt independiente de βcatenina es el correcto posicionamiento del sistema nervioso. En las planarias, la inhibición de *wnt5* produce el desplazamiento lateral del sistema nervioso, la no conexión entre los dos ganglios cerebrales y la interrupción del quiasma óptico. Este fenotipo también se obtiene tras inhibir *evi/wntless* y *dishevelled*, como ya se ha mencionado anteriormente.

Por otra parte, se ha descrito la función de *wnt11-6*, el cual se expresa en la parte posterior del cerebro. La inhibición de *wnt11-6* produce la elongación del cerebro, sugiriendo que su función está relacionada con la formación del patrón cerebral (Adell et al., 2009; Kobayashi et al., 2007).

Asimismo, se ha descrito la conservación de la función de la vía PCP en planarias. Almuedo-Castillo y colaboradores demostraron que la vía PCP controla el correcto posicionamiento de los cilios en la epidermis (Almuedo-Castillo et al., 2011).

3.7.2 Otras vías implicadas en el establecimiento del eje AP

En planarias la vía Wnt dependiente de βcatenina no es la única que coordina y especifica el eje AP. La vía Hedgehog también tiene un papel fundamental en el establecimiento del eje. Dos estudios (Rink et al., 2009; Yazawa et al., 2009) realizados en diferentes especies de planaria demostraron que la activación de la vía Hedgehog (mediante el silenciamiento del receptor *patched*) produce el mismo fenotipo que la sobreactivación

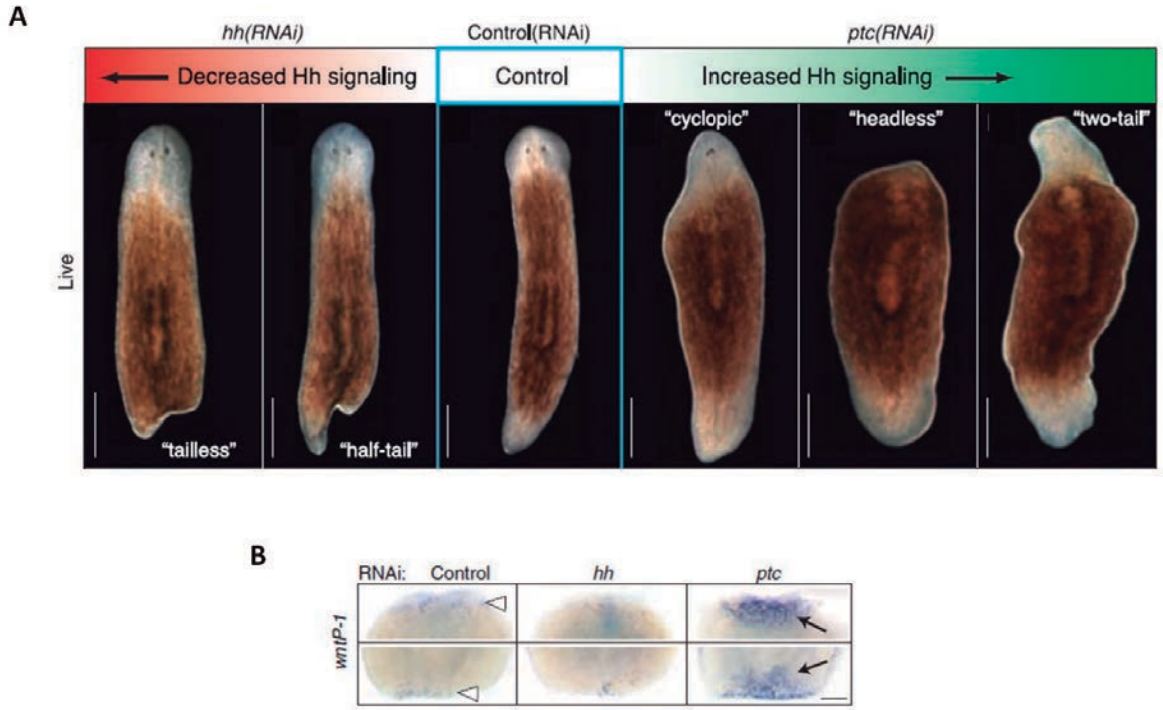


Figura 40. Defectos observados tras modificar la vía Hedgehog. A) Imágenes *in vivo* de los fenotipos observados tras inhibir la vía Hedgehog (rojo) y tras sobreactivarla (verde). B) Hibridación *in situ* del gen *wntP-1* tras inhibir *hh* y *ptc*. Las flechas blancas indican la señal en condiciones normales y las flechas negras indican el incremento de la expresión. [Adaptado de Rink et al., 2009].

de la vía Wnt durante la regeneración, es decir, planarias con "dos colas" (Figura 40 A). Por otra parte, la inhibición del ligando *hedgehog* produce el efecto contrario, es decir, planarias con "dos cabezas". Cabe resaltar que este fenotipo solo se obtiene en planarias de la especie *Dugesia japonica*. En la especie *S. mediterranea* la inhibición de *hedgehog* produce defectos en la cola pero nunca la formación de una cabeza. En conclusión, ambos trabajos demostraron que la vía Hedgehog regula a la vía Wnt dependiente de βcatenina a través del control de la expresión del ligando *wnt1* (Figura 40 B). Por tanto, los fenotipos obtenidos son debidos a la alteración de la expresión del ligando *wnt1*.

Por otra parte, también se ha descrito que las uniones de tipo Gap afectan a la polaridad del eje AP. Oviedo y colaboradores describieron que el bloqueo de las uniones de tipo Gap, si se amputa la cabeza y la cola, produce un fenotipo "dos cabezas". Sin embargo, si solo se amputa la cola, no produce ningún fenotipo. Por último, observaron que si se trunca el cordón nervioso y se amputa la cola, se reproduce el fenotipo "dos cabezas". Estos resultados llevan a proponer a estos autores que el cerebro envía señales por los cordones nerviosos a través de las uniones de tipo Gap hacia posterior, y estas señales inhiben la formación de una cabeza en posterior. Por tanto, el sistema nervioso también pueden estar implicado en el establecimiento del eje AP (Oviedo et al., 2010).

OBJETIVOS

El principal objetivo de esta tesis doctoral es profundizar en el rol de la vía Wnt durante el desarrollo embrionario, la regeneración y la homeostasis de las planarias, con la finalidad de entender mejor el mecanismo molecular que permite la especificación de los ejes corporales y de los diferentes tipos celulares durante el desarrollo de las planarias. La información que se desprenda del estudio en planarias también mejorará nuestra comprensión del desarrollo en los metazoos en general. Para este fin los objetivos concretos planteados son:

1. Caracterización funcional de los Wnts posteriores durante la regeneración y la homeostasis de las planarias.

2. Estudio de la localización subcelular de la βcatenina-1 en las planarias para testar la veracidad de la hipótesis de que la especificación del eje anteroposterior es determinado por un gradiente de actividad de βcatenina-1.

3. Caracterización funcional de la βcatenina-3 y 4, nuevas βcateninas de planarias, durante la regeneración y la homeostasis de las planarias.

4. Descripción de la dinámica de expresión de la proteína βcatenina-1 durante el desarrollo embrionario de las planarias.

RESULTADOS

Informe de la directora de tesis sobre la participación del doctorando y el índice de impacto de las publicaciones

La memoria de la tesis doctoral presentada por Miquel Sureda Gómez, titulada "Caracterización del mecanismo molecular por el cual la vía Wnt/βcatenina especifica el eje anteroposterior y controla la organogénesis en las planarias", de la que soy directora, incluye cinco artículos. Cuatro de ellos están incluidos en el apartado de resultados, de los cuales dos han sido publicados en revisas científicas internacionales, uno ha sido enviado (en segunda revisión) y el ultimo será enviado tras añadir algunos experimentos y ultimas reflexiones. El otro artículo, una revisión, está incluido en el apartado de anexos y ha sido igualmente publicado en una revista científica internacional. Todas estas revistas constan en el PubMed, la base de datos más importante de ciencias biomédicas. También están incluidas en la ISI Web of Science y en todos los casos se trata de publicaciones que han pasado por el filtro de evaluadores anónimos designados por los editores. A continuación se detallan la participación del doctorando en cada publicación y los índices de impacto y la posición en el listado ordenado de especialidades correspondientes (datos del ISI Web of Science, edición 2015).

Artículo 1:

Posterior Wnts Have Distinct Roles in Specification and Patterning of the Planarian Posterior Region.

Miquel Sureda-Gómez, Eudald Pascual-Carreras y Teresa Adell. **International Journal of Molecular Sciences** (2015) 16:26543-26554.

Factor de impacto: 3,257

Posición en el área: 110/289 (Q2, área Biochemistry and Molecular Biology)

El trabajo experimental presentado en este artículo ha sido realizado por el doctorando Miquel Sureda Gómez con la ayuda del doctorando Eudald Pascual Carreras bajo la supervisión de la Doctora Teresa Adell Creixell. Así mismo, Miquel Sureda Gómez ha contribuido activamente en el diseño de los experimentos y en la discusión de los resultados. La escritura del manuscrito, producción y diseño de las imágenes ha sido realizada por el doctorando con la ayuda del doctorando Eudald Pascual Carreras bajo la supervisión de la Doctora Teresa Adell Creixell.

Artículo 2:

Localization of planarian βCATENIN-1 reveals multiple roles during anterior-posterior regeneration and organogenesis. **Miquel Sureda-Gómez**, José M. Martín-Durán y Teresa Adell. **Development** (2016) 143:4149-4160.

Factor de impacto: 6,059

Posición en el área: 4/41 (Q1, área Developmental Biology)

El trabajo experimental presentado en este artículo ha sido realizado por el doctorando Miquel Sureda Gómez bajo la supervisión de la Doctora Teresa Adell Creixell. Miquel Sureda Gómez, en colaboración con el Doctor José Mª Martín Durán, realizaron la puesta a punto del protocolo de immunohistoquímica del anticuerpo contra la Bcatenina-1, bajo la supervisión de la Doctora Teresa Adell Creixell. Así mismo, Miquel Sureda Gómez ha contribuido activamente en el diseño de los experimentos y en la discusión de los resultados. La escritura del manuscrito, producción y diseño de las imágenes ha sido realizado por el doctorando bajo la supervisión del Doctor José Mª Martín Durán y la Doctora Teresa Adell Creixell.

Artículo 3:

A C-terminally Truncated Form of β-catenin Acts as a Novel Regulator of Wnt/β-catenin Signaling in Planarians. Hanxia Su, **Miquel Sureda-Gómez**, Neus Rabaneda-Lombarte, Maria Gelabert, Jianlei Xie, Wei Wu y Teresa Adell. **PLOS Genetics**, en segunda revisión.

Factor de impacto: 6,661

Posición en el área: 15/166 (Q1, área Genetics and Heredity)

El trabajo experimental presentado en este artículo ha sido realizado por el doctorando Miquel Sureda Gómez conjuntamente con la doctoranda Hanxia Su, con la ayuda de la estudiante de grado Neus Rabaneda Lombarte y bajo la supervisión de la Doctora Teresa Adell Creixell. En colaboración con la estudiante de grado Maria Gelabert realizaron la puesta a punto del protocolo de immunohistoquímica del anticuerpo contra la Bcateni-

na-4, bajo la supervisión de la Doctora Teresa Adell Creixell. Así mismo, Miquel Sureda Gómez ha contribuido activamente en el diseño de los experimentos y en la discusión de los resultados. La escritura del manuscrito, producción y diseño de las imágenes ha sido realizada por el doctorando conjuntamente con la doctorando Hanxia Su y bajo la supervisión de la Doctora Teresa Adell Creixell.

Artículo 4:

βcatenin-1 dynamics during the embryonic development of the planaria Schmidtea polychroa.

Miquel Sureda-Gómez, José M. Martín-Durán y Teresa Adell. Manuscrito en preparación.

El trabajo experimental presentado en este artículo ha sido realizado por el doctorando Miquel Sureda Gómez bajo la supervisión de la Doctora Teresa Adell Creixell. Miquel Sureda Gómez, en colaboración con el Doctor José Mª Martín Durán, realizaron la puesta a punto del protocolo de immunohistoquímica del anticuerpo contra la Bcatenina-1, bajo la supervisión de la Doctora Teresa Adell Creixell. Así mismo, Miquel Sureda Gómez ha contribuido activamente en el diseño de los experimentos y en la discusión de los resultados. La escritura del manuscrito, producción y diseño de las imágenes ha sido realizada por el doctorando bajo la supervisión de la Doctora Teresa Adell Creixell.

Revision 1 (Anexo 1):

Wnt signaling in planarians: new answers to old questions. María Almuedo-Castillo , **Miquel Sureda-Gómez** y Teresa Adell. **International Journal of Developmental Biology** (2012): 56: 53-65.

Factor de impacto: 1,753

Posición en el área: 34/41 (Q4, área Developmental Biology)

El doctorando Miquel Sureda Gómez ha contribuido en la escritura de la revisión junto con las Doctoras María Almuedo Castillo y Teresa Adell Creixell. La producción y diseño de las imágenes las realizó la Doctora María Almuedo Castillo con la ayuda del Doctor José Mª Martín Durán y bajo la supervisión de la Doctora Teresa Adell.

Firmado,

La directora,

Dra. Teresa Adell

Artículo 1:

Posterior Wnts Have Distinct Roles in Specification and Patterning of the Planarian Posterior Region

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International Journal of Molecular Sciences, 16:26543-26554;

Índice de impacto (2015): 3,257

Resumen en castellano:

Los Wnts posteriores tienen distintas funciones en la especificación y en la formación del patrón de la región posterior de la planaria.

La vía Wnt dependiente deβcatenines un mecanismo de comunicación intercelular esencial en la especificación del destino celular, el patrón de los tejidos y la identidad regional. La vía Wnt/βcatenina dependiente es la encargada de especificar el eje anteroposterior en planarias, tanto durante la regeneración de nuevos tejidos como la homeostasis. Por otro lado, cuatro ligandos *wnts* (*wnts* posteriores) se encuentran expresados de manera solapada en la región central y posterior de la planaria. En este trabajo hemos analizado la función específica de cada uno de estos *wnts* posteriores y su posible cooperación en la especificación y la formación del patrón de la región central y posterior. Nuestros resultados muestran que cada Wnt posterior ejerce una función distinta durante la re-especificación y mantenimiento de la región central y posterior de la planaria, y que de la integración de las diferentes señales de los wnts (dependiente e independiente de βcatenina) subyace la formación del patrón del eje anteroposterior desde la región central hasta la punta de la cola. Basándonos en nuestros datos y los que provienen de la literatura, proponemos un modelo de como se forma el patrón del eje anteroposterior de planarias.

International Journal of Molecular Sciences

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Article

Posterior Wnts Have Distinct Roles in Specification and Patterning of the Planarian Posterior Region

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Abstract: The wnt signaling pathway is an intercellular communication mechanism essential in cell-fate specification, tissue patterning and regional-identity specification. A βcatenin-dependent signal specifies the AP (Anteroposterior) axis of planarians, both during regeneration of new tissues and during normal homeostasis. Accordingly, four *wnts* (posterior *wnts*) are expressed in a nested manner in central and posterior regions of planarians. We have analyzed the specific role of each posterior *wnt* and the possible cooperation between them in specifying and patterning planarian central and posterior regions. We show that each posterior *wnt* exerts a distinct role during re-specification and maintenance of the central and posterior planarian regions, and that the integration of the different wnt signals (βcatenin dependent and independent) underlies the patterning of the AP axis from the central region to the tip of the tail. Based on these findings and data from the literature, we propose a model for patterning the planarian AP axis.

Keywords: patterning; identity specification; wnt signaling; planarians

1. Introduction

The wnt signaling pathway is an intercellular communication mechanism with essential roles in cell-fate specification, tissue patterning and specification of regional identity [1]. Wnts, the secreted elements of the pathway, interact with the membrane receptors Frizzleds and their co-receptors (LRP4/5, Ror, Ryk) to transduce different signals that branch mainly in three pathways: the canonical or βcatenin-dependent wnt signaling, and two non-canonical or βcatenin-independent signals, which regulate either JNK (c-Jun N-terminal kinase) or PKC (Protein kinase C) pathways [1–3]. The βcatenin-dependent pathway exerts its function by regulating the nuclear translocation of βcatenin, and is mainly involved in cell-fate specification. One of the most conserved roles of βcatenin-dependent Wnt signaling is the specification of the AP (Anteroposterior) axis, where βcatenin is required to confer posterior features in most developmental models studied [4]. βcatenin-independent pathways are mainly involved in the control of cell shape and movements [5,6].

Planarians are an ideal model for the study of cell-fate specification and patterning, since they are extremely plastic. They are bilateral animals with a complex cephalized nervous system and a three-branched gut which converges into a pharynx, which takes in food and expulses debris through a ventral mouth [7]. Planarians can regenerate any amputated part, even the head, in a few days, and they continuously remodel their tissues while they grow and shrink according to food availability and temperature. Those capabilities are due to the presence of a population of totipotent stem cells all around their body, the neoblasts, which are able to differentiate to any cell type [7–9]. Because of its astonishing regenerative abilities, planarians have been established as a unique model to understand stem cell biology and the molecular mechanisms underlying patterning and

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regional identity specification. Specifically, the function of the Wnt signaling pathway has been extensively studied in planarians [10–19]. Due to its plasticity, even in the adult stage, the phenotypes generated when silencing Wnt pathway elements had no precedent in the field of developmental biology and were extremely informative. RNAi experiments demonstrate that *Smed-βcatenin1* is essential to pattern the AP axis in planarians, since its inhibition generates anteriorized phenotypes ranging from "tailless" planarians to "two-headed" planarians and, most strikingly, "radial-like hypercephalized" planarians [12,20]. Moreover, the study of several elements of the pathway confirms this function, since inhibition of *APC* and *axin*, elements of the βcatenin destruction complex, lead to posteriorization of planarians [11,18]. Interestingly, the *Smed-βcatenin1*-dependent Wnt signal is required to specify AP identities both during planarian regeneration and during homeostasis [12,17,18].

Consistent with the role of the βcatenin-dependent Wnt signal in AP axial specification, 4 *wnts* are expressed in the posterior part of planarians in a nested manner, which we name in this study posterior *wnts* (*Smed-wnt1*, *Smed-wnt11-1*, *Smed-wnt11-2* and *Smed-wnt11-5*) [15,17–19]. Since planarians such as *S. mediterranea* typically measure at least 1–2 mm in length, the field is too large to be patterned by a single morphogen. It has therefore been proposed that cooperation between posterior *wnts* could be required to pattern the AP axis [20]. Out of the four posterior *wnts*, however, only *Smed-wnt1* and *Smed-wnt11-2* have been studied functionally. During regeneration of the tail, *Smed-wnt1* inhibition leads to "tailless" or "two-headed" planarians, and *Smed-wnt11-2* inhibition leads to "tailless" planarians [14,15,19]. Although those two *wnts* seem to be regulators of βcatenin activity, because its silencing produces an anteriorized phenotype, the strong anteriorization of planarians produced after *Smed-βcatenin1* silencing has never been phenocopied by the inhibition of any *wnt*. The purpose of the present study is to analyze the specific role of each posterior *wnt* and the possible cooperation among them both during regeneration and maintenance of the AP axis. Our data demonstrates that each posterior *wnt* exerts a distinct function during posterior regeneration, and that the inhibition of all of them generates a stronger anteriorization than the inhibition of any of them alone. During homeostasis, simultaneous silencing of the four posterior *wnts* also generates a stronger phenotype than silencing any *wnt* alone, although a shift of posterior to anterior identity is never achieved. We conclude that the integration of the different Wnt signals (βcatenin dependent and independent) underlies the patterning of the AP axis from the central region to the tip of the tail.

2. Results

2.1. Individual Posterior Wnts Exert Specific Roles during Posterior Regeneration

To study the role of each posterior *wnt* during posterior regeneration, we first analyzed their expression pattern by *in situ* hybridization. In agreement with previous reports, the four posterior *wnts* are found to be expressed in a graded manner along the AP axis in intact planarians (Figure S1A) [19]. *Smed-wnt1* expression is restricted to few cells in the posterior midline; *Smed-wnt11-1* and *Smed-wnt11-2* are expressed from the mouth to the tip of the tail, and *Smed-wnt11-1* also in the mouth itself; and *Smed-wnt11-5* is expressed from the pre-pharyngeal region to the tip of the tail. Interestingly, all of them are expressed as a gradient, higher in the most posterior tip. Moreover, posterior *wnts* are also expressed in a temporal manner during posterior regeneration, being *Smed-wnt1* the first one, expressed few hours after cutting (Figure S1B) [14,19], followed by *Smed-wnt11-1* and *Smed-wnt11-2*, which are detected 2 days after cutting (Figure S1B) [19]. *Smed-wnt11-5* is expressed at all regeneration stages, since its expression is not lost after cutting the tail but just re-patterned (Figure S1B) [19]. Those expression patterns suggest that each posterior *wnt* could exert a specific role during posterior specification and patterning, and that the cooperation between them could enable a correct and complete posterior pattern.

To test the specific role of each posterior *wnt*, we analyzed the morphology and pattern of the tail regenerated by planarians in which each posterior *wnt* alone was silenced. Phenotypes

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were analyzed by morphological observation and by immnohistochemistry with anti-synapsin and anti-βcatenin2 antibodies, to visualize the nervous and the digestive system, respectively (Figure 1 and Figure S2). As expected, inhibition of *Smed-wnt1* led to "tailless" and "two-headed" planarians (Figure 1A and Figure S1). Immunohistochemical analysis showed that "two-headed" planarians always differentiate a second pharynx in the opposite direction to the original one, according to the new axis generated in the posterior tip (Figure 1A(D′)). "Tailless" planarians showed a rounded closure of ventral nerve cords (VNCs) and an undefined posterior tip (Figure 1A(B′,C′)) [15]. Among "tailless" planarians two different phenotypes could be distinguished: animals in which only one pharynx was observed (sometimes in an opposite orientation) (Figure 1A(B′)) and animals in which two pharynges in opposite orientation could be observed (Figure 1A(C′)). Silencing of *Smed-wnt11-1* lead to the regeneration of shorter tails, in which the distance from the pharynx to the posterior tip was clearly shorter (Figure 1 and Figure S2). Immunohistochemical analysis showed that those animals close properly the VNCs in the posterior tip, and no signal of anteriorization can be observed (Figure 1A(E′)). Again, two different phenotypes could be distinguished when analyzing the central region, since in some animals a second pharynx appeared in parallel and very close to the pre-existing one (Figure 1A(F′)). Interestingly, two-pharynged *Smed-wnt11-1* RNAi animals never showed two mouths (Figure 1A(F′′)). Silencing of *Smed-wnt11-2* always lead to the regeneration of "tailless" planarians, as had been already reported (Figure 1 and Figure S1) [15,19]. Immunohistochemical analysis demonstrated that although *Smed-wnt11-2* RNAi animals only show the normal pre-existing pharynx, a second mouth appears in half of the animals (Figure 1A(G′,G′′)). A second pharynx associated to the second mouth has not been observed, although in few cases a pharynx primordium could be guessed (Figure 1A(H′)). *Smed-wnt11-5* RNAi animals apparently regenerated a perfect tail (Figure S2). Immunohistochemical analysis corroborates that VNCs close normally in the posterior tip. However, in most of the cases a second pharynx, oriented either in the same or in opposite direction with respect to the original one, can be observed (Figure 1A(I′,J′)). A second mouth always differentiates associated to the second pharynx (Figure 1A(I′′)). Thus, in *Smed-wnt11-5* RNAi animals, posterior identity appears normal but the central region appears duplicated. The quantification of the different phenotypes observed after silencing each posterior *wnt* alone allows the visualization of the different degrees of anteriorization generated (Figure 1B).

We then analyzed posterior identity specification of planarians in which posterior *wnts* were silenced. Posterior *wnts* and *fz4* were used as markers [18]. Results show that after *Smed-wnt1* RNAi the rest of posterior *wnts* and *fz4* disappear or decrease significantly, demonstrating the loss of posterior identity in "tailless" and "two-headed" phenotypes (Figure 1C). In contrast, after *Smed-wnt11-1* and *Smed-wnt11-5* RNAi, all posterior markers were expressed in the same pattern and levels as in controls, in agreement with the normal posterior closure of the VNCs in the posterior tip (Figure 1C). Thus, *Smed-wnt11-1* and *Smed-wnt11-5* RNAi animals have normal posterior identity. *Smed-wnt11-2* RNAi animals displayed a significant decrease in the expression of posterior markers, according to the "tailless" phenotype observed. Expression of *Smed-wnt1* appeared not only diminished but totally disorganized (Figure 1C) [19]. Interestingly, expression of *Smed-wnt11-1* and *Smed-wnt11-2* was found to be dependent on *Smed-wnt1*, although it remains unclear whether this is a direct regulation or a consequence of the loss of posterior identity.

Taken together, these results suggest that *Smed-wnt1* and *Smed-wnt11-2* specify posterior identity, although only *Smed-wnt1* RNAi animals exhibit a shift in polarity. Moreover, *Smed-wnt11-2* exerts a role in patterning or specifying central identity, since its inhibition duplicates the mouth. *Smed-wnt11-1* and *Smed-wnt11-5* are not required to specify the identity of the posterior tip. However, they have a role in patterning or specifying the central region, since ectopic pharynges differentiate when they are silenced. *Smed-wnt11-1* would be also required to properly elongate the tail.

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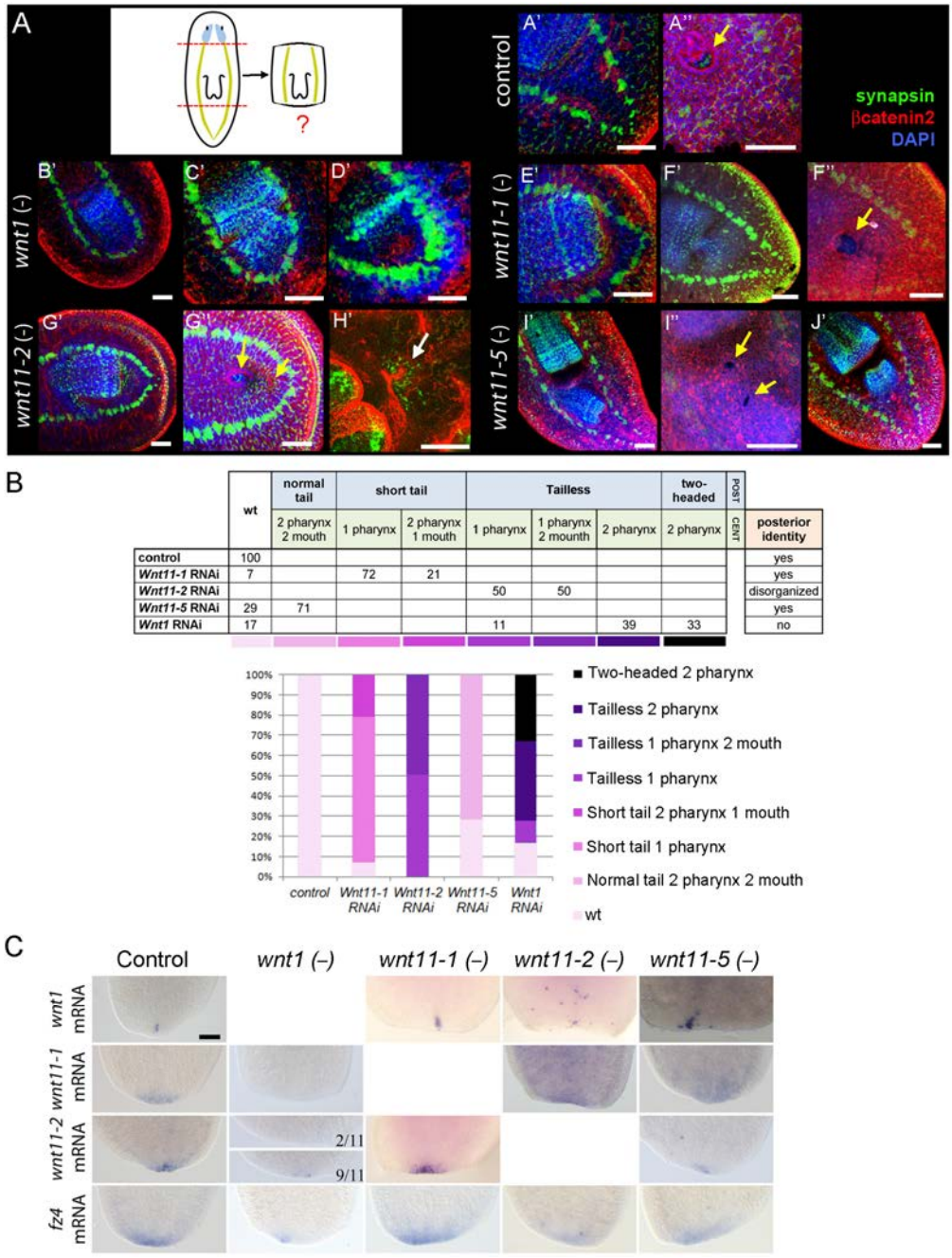

	wt	normal tail	short tail		Tailless			two-headed	POST	posterior identity
		2 pharynx 2 mouth	1 pharynx	2 pharynx 1 mouth	1 pharynx	1 pharynx 2 mounth	2 pharynx	2 pharynx	CENT	
control	100									yes
Wnt11-1 RNAi	7		72	21						yes
Wnt11-2 RNAi					50	50				disorganized
Wnt11-5 RNAi	29	71								yes
Wnt1 RNAi	17				11		39	33		no

Figure 1. Each posterior *wnt* exerts a distinct function during planarian posterior regeneration. (**A**) Immunohistochemical analysis of planarian tail after silencing of *Smed-wnt1* (**B′**–**D′**), *Smed-wnt11-1* (**E**–**F′′**), *Smed-wnt11-2* (**G′**–**H′**) and *Smed-wnt11-5* (**I′**–**J′**). Anti-synapsin labels the nervous system (green), anti-βcatenin1 labels adherent junctions (red), and nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole)(blue). **A′′**, **F′′**, **G′′** and **I′′** show a magnification of the plane corresponding to the mouth opening of **A′**, **F′**, **G′** and **I′**, respectively (mouth openings are indicated with yellow arrows). A primordium of a second pharynx in *Smed-wnt11-2* RNAi animals is shown in **H′** (white arrow). Animals were fixed at 20 days of regeneration. All images correspond to confocal z-projections; (**B**) Quantification of the different phenotypes observed after silencing each posterior *wnt*. (The number of animals analyzed for each condition was at least $n = 14$.). wt; wild type; (**C**) *In situ* hybridization analysis of the expression of posterior markers in 3-day regenerating posterior *wnt* RNAi. (The number of animals analyzed for each condition was at least $n = 11$.). Anterior is left/up, posterior is right/down in (**A**); anterior is up, posterior is down in **C**. Scale bar: 100 μm (**A**,**C**).

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2.2. Smed-wnt11-2 and Smed-wnt11-5 but not Smed-wnt11-1 Cooperate with Smed-wnt1 in Specifying Posterior Identity

To study whether posterior *wnts* play a cooperative role in posterior specification and patterning, we silenced *Smed-wnt1* (the only *wnt* that leads to shift of posterior to anterior identity upon silencing) simultaneously with *Smed-wnt11-1*, *Smed-wnt11-2* or *Smed-wnt11-5*. The resulting phenotypes were analyzed by immunohistochemistry with anti-synapsin and anti-βcatenin2 antibodies to visualize the nervous and digestive systems (Figure 2A). The phenotypes obtained after double inhibition were quantified and compared with those obtained after single inhibition of each posterior *wnt* (Figure 2A), allowing visualization of the degree of cooperation between the different posterior *wnts* in central and posterior specification. In these experiments, the penetrance of the phenotypes of single *wnt* RNAi was milder than in the experiments shown above, since half the amount of dsRNA was injected for each gene in order to maintain the total amount of dsRNA injected per animal (see Section 4.2). Quantification of the different phenotypes shows that simultaneous silencing of *Smed-wnt1* together with *Smed-wnt11-2* or *Smed-wnt11-5* increased the number of "two-headed" planarians from 20% in *Smed-wnt1* RNAi planarians to 70% in the doubles [14]. In contrast, simultaneous silencing of *Smed-wnt1* together with *Smed-wnt11-1* decreased the frequency of "two-headed" planarians from 20% to 8%. Interestingly, two new phenotypes not observed in the single inhibition experiment appeared in these experiments. Firstly, we observed "tailless" planarians with two pharynges in parallel, which is the addition of the suppression of the posterior identity after *Smed-wnt1* silencing together with the appearance of an ectopic pharynx after *Smed-wnt11-1*. In addition, "tailless" planarians were observed with two pharynges in tandem and in the same orientation, which is the addition of the suppression of the posterior identity after *Smed-wnt1* silencing and the duplication of the central identity produced by *Smed-wnt11-5* silencing (Figure 2A(A′,B′)). According to the phenotypes observed, analysis of the posterior marker *fz4* in the double RNAi planarians revealed a loss or reduction in *Smed-wnt1/Smed-wnt11-2* and *Smed-wnt1/Smed-wnt11-5* RNAi planarians (Figure 2B). *Smed-wnt1/Smed-wnt11-1* RNAi animals also displayed a mild reduction of *fz4* expression, possibly due to the inhibition of *Smed-wnt1*. Taken together, these results demonstrate that *Smed-wnt11-2* and *Smed-wnt11-5*, but not *Smed-wnt11-1*, cooperate with *Smed-wnt1* in specifying posterior identity. The contribution of *Smed-wnt11-2* in posterior specification could be predicted according to its requirement in single RNAi experiments. However, the contribution of *Smed-wnt11-5* in posterior specification should be in cooperation with *Smed-wnt1*, since its inhibition alone never induces posterior defects. The possible cooperation between *Smed-wnt11-1*, *Smed-wnt11-2* and *Smed-wnt11-5* in the specification and patterning of the central region requires further attention.

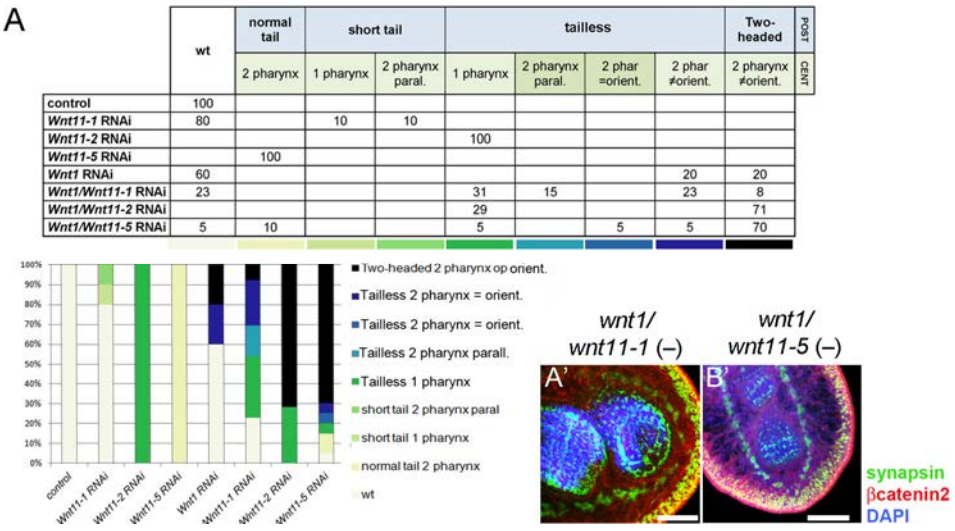

Figure 2. *Cont.*

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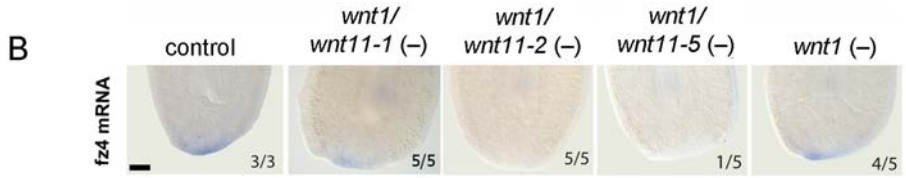

Figure 2. Cooperation of posterior *wnts* to specify posterior identity during regeneration. (**A**) Quantification of the different phenotypes observed after silencing each posterior *wnt* alone and *Smed-wnt1* in combination with the other posterior *wnts*. Two new phenotypes appeared after silencing *Smed-wnt1/Smed-wnt11-1* (**A′**) and *Smed-wnt1/Smed-wnt11-5* (**B′**), both of which show a "tailless" morphology next to the differentiation of a second pharynx alongside the original one. Animals were fixed at 20 days of regeneration. (The number of animals analyzed for single RNAi was n = 4–10 and for double n = 7–20.). **A′**,**B′** images correspond to confocal z-projections; and (**B**) *In situ* hybridization analysis of the expression of posterior markers in 3-day regenerating animals in which *Smed-wnt1* was silenced together with the other posterior *wnts*. (The number of animals analyzed for each condition was n = 3–5.). Anterior is left/up, posterior is right/down in **A′**,**B′**; anterior is up, posterior is down in (**B**). Scale bar: 100 μm (**A**,**B**).

2.3. Silencing of All Posterior Wnts Together Is Insufficient to Transform Posterior Identity into Anterior during Homeostasis

βcatenin-dependent Wnt signaling is also required for the maintenance of posterior identity and pattern during planarian homeostasis, since *Smed-βcatenin1* inhibition in intact planarians produces the appearance of ectopic eyes and brain in the posterior tip [12,17,18]. To analyze the possible cooperation between posterior *wnts* in the maintenance and pattern of the AP axis during homeostasis, we silenced them simultaneously and analyzed the resulting phenotypes after 6 weeks of inhibition. As a previous step, we silenced each *wnt* alone and showed that posterior eyes were not induced in any case (Figure S3). However, *Smed-wnt11-1* and *Smed-wnt11-2* RNAi planarians did show "tailless" phenotypes. RNAi of the four posterior *wnts* simultaneously produced an evident reduction of the tail, generating a strong "tailless" phenotype. In those animals, the pattern of the central region was also affected, and 3 types of central phenotypes could be distinguished: animals with two pharynges in opposite orientation, animals with a disorganized pharynx, and animals without a pharynx, due to its expulsion (Figure 3A,B). Despite the strong phenotype observed in posterior *wnt* RNAi planarians, the differentiation of ectopic anterior structures never occurred. The analysis of anterior and posterior identity markers corroborates the "tailless" phenotype, since RNAi planarians completely lost the expression of the posterior marker *fz4*, and the anterior markers *sFRP* [18] and *notum* [16] never appear in the posterior region (Figure 3C). Moreover, *sFRP* staining also revealed disorganization of the pharynx (Figure 3C).

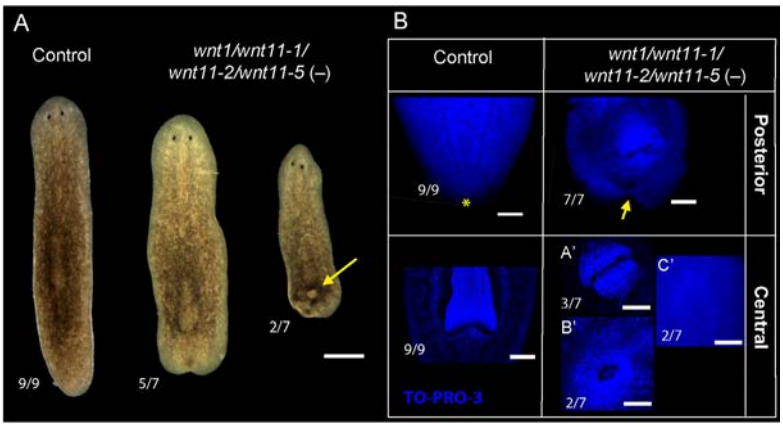

Figure 3. *Cont.*

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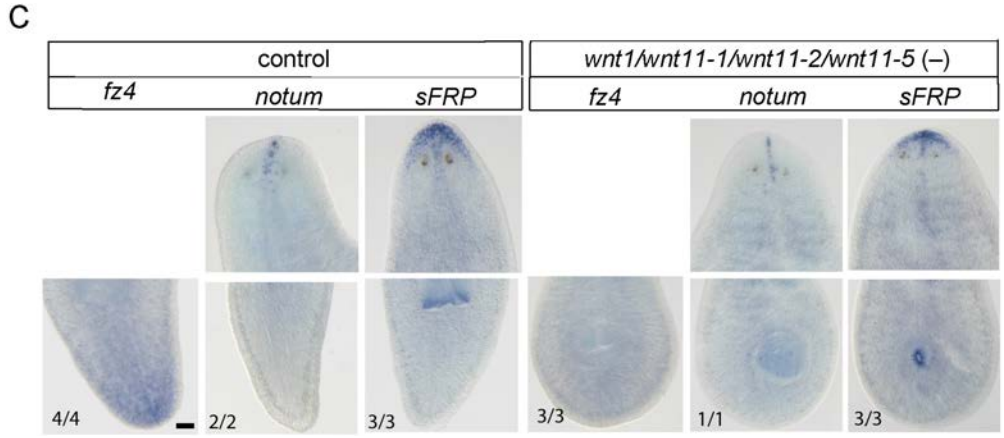

Figure 3. Silencing of all posterior *wnts* during homeostasis generates a strong "tailless" phenotype, without neither posterior nor anterior identity (**A**) After 6 rounds of *Smed-wnt1*/*Smed-wnt11-1*/*Smed-wnt11-2*/*Smed-wnt11-5* inhibition, planarians show a "tailless" phenotype in which the central region is also affected, since the pharynx cannot be maintained (yellow arrow points to a hole generated after the expulsion of the pharynx). (The number of animals analyzed for each condition was n = 7–9); (**B**) TO-PRO-3 staining of the nucleus shows the "tailless" shape of RNAi planarians (yellow arrow) compared to controls (yellow asterisk), and the disorganization of the central region (**A′**, two pharynges; **B′**, disorganized pharynx; **C′**, no pharynx, after expulsion). All images correspond to confocal z-projections; and (**C**) "Tailless" *Smed-wnt1*/*wnt11-1*/*wnt11-2*/*wnt11-5* RNAi animals do not show expression of either posterior (*Fz4*) or anterior (*sFRP*, *notum*) markers in the posterior region. Anterior markers are normally expressed in the anterior region. Anterior is up, posterior is down in **all images**. Scale bar: 500 μm (**A**), 100 μm (**B**, **A′**, **B′** and **C′**) and 100 μm (**C**).

Taken together, these results show that disruption of the central and posterior regions in intact planarians is much stronger when silencing all posterior *wnts* simultaneously than when they are silenced individually, providing evidence of cooperation in the patterning of these regions. However, in contrast to the results reported for *Smed-βcatenin1* silencing [12], a shift of posterior identity to anterior was not observed under homeostatic conditions.

3. Discussion

Depending on the dose and time of inhibition, *Smed-βcatenin1* RNAi induces a gradual anteriorization of planarians, from "tailless" to "radial-like hypercephalized" animals. Consequently, it has been proposed that the graded activation of Smed-βcatenin1 from posterior to anterior is responsible for specifying the whole AP axis in planarians [12,20]. However, the *wnts* responsible for the nuclear localization of Smed-βcatenin1 in such a broad domain remained mainly elusive. Until now, only the involvement of *Smed-wnt1* in Smed-βcatenin1 nuclearization had been suggested, since it is the only *wnt* for which inhibition induces the appearance of a posterior head during posterior regeneration [14,15]. However, the strong anteriorization observed after *Smed-βcatenin1* silencing has never been observed following inhibition of any *wnt*. In this study, we analyzed the function of the four *wnts* which are expressed in the posterior part of planarians (posterior *wnts*) and explored the possibility that they cooperate to pattern planarian AP axis (Figure 4). Our results confirm that *Smed-wnt1* is the only *wnt* for which inhibition leads to a shift in posterior polarity during regeneration, when posterior identity must be re-specified. Moreover, we reproduce the "tailless" phenotypes after inhibition of *Smed-wnt11-2* [15,19], which also must exert a role in posterior specification, according to the decreased and disorganized pattern of posterior markers. In contrast, our results demonstrate that *Smed-wnt11-1* and *Smed-wnt11-5* are not required for posterior specification, since the tip of the tail in those RNAi animals regenerates normally and posterior markers are normally expressed. Interestingly, our data point to a role for *Smed-wnt11-5* in the

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specification or patterning central identity, since *Smed-wnt11-5* RNAi animals regenerate a second pharynx and mouth posteriorly to the pre-existing one. The shorter tail of *Smed-wnt11-1* RNAi planarians could indicate a role for this *wnt* in the extension of the tail. Moreover, our data suggests that *Smed-wnt11-1* could exert a direct role in the formation of the mouth, since it is expressed in this organ, and *Smed-wnt11-1* RNAi planarians never duplicate the mouth despite the presence of two pharynges.

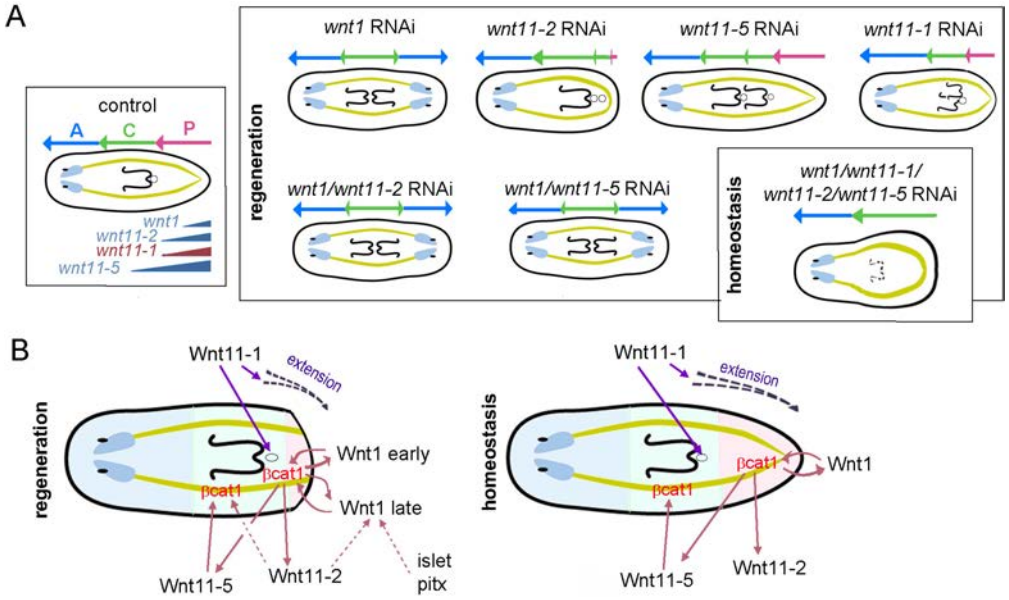

Figure 4. Summary and working model. (**A**) Scheme of the phenotypes generated after silencing the different posterior *wnts* (blue A, anterior; green C, central; pink P, posterior). The strongest phenotype is represented; (**B**) Proposed model of the function of posterior *wnts* in central and posterior specification and patterning (in the planarian: blue is anterior; green is central and pink is posterior).

Based on the results obtained in this study, we hypothesize that *Smed-wnt1*, *Smed-wnt11-2* and *Smed-wnt11-5* could act in a *βcatenin*-dependent manner, nuclearizing Smed-βcatenin1 in different domains along the AP axis (Figure 4B). Whereas *Smed-wnt1* and *Smed-wnt11-5* could be direct regulators of the βcatenin destruction complex in the posterior and central region, respectively, *Smed-wnt11-2* could be modulating Smed-βcatenin1 activity indirectly, at least in the posterior region. This possibility is supported by the observation that *Smed-wnt1* does not disappear but shows a disorganized pattern in *Smed-wnt11-2* RNAi planarians (Figure 4B). At this point, it should be noted that two different stages of *Smed-wnt1* expression occur during regeneration: an early *Smed-wnt1* expression, which occurs during wounding and is stem-cell independent, and a late *Smed-wnt1* expression, localized in the most posterior tip (the area which would correspond to the posterior organizer), that is stem-cell dependent [19]. We hypothesize that posterior identity is established by early *Smed-wnt1* expression, which triggers the sustained activation of *Smed-β*catenin1 in posterior regions through the subsequent activation of the late *Smed-wnt1* expression (Figure 4B). *Smed-wnt11-2*, for which inhibition leads to "tailless" planarians, would be required for the proper expression pattern of the late *Smed-wnt1*. The concentration of *Smed-wnt1* in the posterior tip would be essential for the establishment of the organizing region, which is responsible for growth and pattern rather than for identity specification. Additional factors, such as *Smed-pitx* or *Smed-islet*, could cooperate with *Smed-wnt11-2*, since their inhibition leads to suppression of late *Smed-wnt1* expression and regeneration of "tailless" planarians [21,22]. It has been proposed that the "tailless" phenotype could also be the result of *Smed-wnt11-2* acting in the establishment of the posterior midline [19]. In our view, the abolishment of the posterior midline goes together

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with the disruption of the posterior organizer. *Smed-wnt11-1* RNAi animals regenerate a shorter tail showing a proper terminal identity. Moreover, their occasionally duplicated pharynx never locates in tandem, like in *Smed-wnt11-5* or *Smed-wnt1* RNAi planarians. For that reason, we hypothesize that *Smed-wnt11-1* would not function in a *βcatenin*-dependent manner but it would be involved in the non-canonical/βcatenin-independent Wnt signaling, a well known mechanism to regulate migration and cell movement, which are the main morphogenetic processes required for tissue extension and epithelial rearrangements [6]. The possible non-canonical function of *Smed-wnt11-1* and *Smed-wnt11-2* compared to the βcatenin-dependent function of *Smed-wnt11-5* is further supported by their evolutionary origin, since phylogenetic analysis shows that *Smed-wnt11-5* does not branch with Wnt11 but with the Wnt4 family [23]. Moreover, a *wnt4* has been suggested to act in a βcatenin-dependent manner in the platyhelminth *Schistosoma* [24]. Altogether, our results suggest that posterior *wnts* act in cooperation to provide a precise spatiotemporal control of the AP axis, from the pre-pharyngeal region to the tip of the tail.

The cooperation and integration of βcatenin-dependent and -independent Wnt signaling has been demonstrated to be essential also in the patterning of the AP neuroectoderm axis in sea urchin [25]. In cnidarians, it has been suggested that the patterning of the oral-aboral axis could be established by the cooperation between different Wnts, a "Wnt code", which would exert the function of the Hox code in bilatelians [26]. If the cooperation of posterior *wnts* is also required for maintenance of the AP pattern during homeostasis in planarians, then we expect that inhibition of the whole posterior *wnt* complement would lead to the abolishment of the identities from the pre-pharynx to the tail. Our results show that inhibition of posterior *wnts* during homeostasis one by one never induces the appearance of ectopic anterior structures but only generates mild "tailless" phenotypes. In contrast, inhibition of all posterior *wnts* together leads to a strong "tailless" phenotype, in which posterior markers disappear and also the central region is affected, since the pharynx cannot be maintained, which in fact is a feature of *Smed-βcatenin1* RNAi animals. This result confirms the hypothesis that posterior *wnts* cooperate to pattern the AP axis, including central and posterior regions. However, inhibition of the whole posterior *wnt* complement never induces the appearance of ectopic anterior structures, as occurs after *Smed-βcatenin1* silencing. One reason could be that silencing all posterior *wnts* simultaneously affects not only the bcatenin-dependent but also the bcatenin-independent Wnt signaling, which could prevent cell tip specification. Further RNAi analysis with different combinations of posterior *wnts* should be performed. A second reason could be that RNAi inhibition of the secreted elements of the pathway is less efficient than inhibition of the intracellular element, particularly considering that we are silencing four genes simultaneously. However, it must be noted that silencing of *Smed-wnt1* alone produces a strong anteriorization of planarians during regeneration but has no apparent phenotype during homeostasis. This observation could indicate that the signals which trigger posterior identity are different in the context of regeneration, when the posterior organizer must be re-specified, compared with the context of homeostasis, when the posterior organizer must be only maintained. A robust signaling network could underlie the maintenance of the posterior organizer (high levels of Smed-βcatenin1). Only the inhibition of *Smed-βcatenin1* itself or downstream elements, like *Smed-teashirt* [27], or the removal of the organizer after a posterior amputation, enables its re-specification towards a different fate.

4. Experimental Section

4.1. Planarian Culture

Planarians used in the presented experiments correspond to the clonal strain of *S. mediterranea* known as BCN-10 biotype. They were maintained as previously described [28]. Planarians used in these experiments were 4–6 mm length and were starved for 1 week before used for experiments.

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4.2. RNAi Analysis

Double-stranded RNAs (dsRNAs) used in these experiments were synthesized by *in vitro* transcription (Roche) as previously described [29]. dsRNA microinjections were performed in the digestive system of planarians following the standard protocol of a 3 × 32 nL/injection of double-stranded (ds) RNA for three consecutive days before being amputated [29]. In regeneration experiments, 2 consecutive rounds of dsRNA injections were performed (1 round corresponds to 1 week, in which animals are injected on the first 3 days and amputated on the fourth). Animals were amputated transversally in 3 parts (heads, trunks and tails). In homeostasis experiments, 1 round of injection corresponds to 1 week in which dsRNA is injected on the first 3 days. Control animals were injected with dsRNA for the green fluorescent protein (GFP) sequence. In simultaneous gene-silencing experiments, the total amount of dsRNA injected for each gene and also the total amount of dsRNA injected in each animal was maintained constant by injecting the amount of GFP required.

4.3. Whole-Mount in Situ Hybridization

The RNA probes used in the present experiments were synthesized *in vitro* using Sp6 or T7 polymerase (Roche, Sant Cugat del Vallès, CAT, Spain) and DIG-modified ribonucleotides (Roche). Afterwards they were purified by ethanol precipitation and 7.5 M ammonium acetate addition. For *in situ* hybridization, animals were killed with HCl 2%, and fixed in Carnoy. An *in situ* Pro hybridization robot (Abimed/Intavis, Tübinguen, BW, Germany) was used for the *in situ* protocol, as previously described [30,31]. The temperature used for hybridizations was 56 °C, and were carried out for 16 h. A Leica MZ16F microscope (Leica Microsystems, Mannhiem, BW, Germany) was used to observe the samples. Images were captured with a ProgRes C3 camera from Jenoptik (Jena, TH, Germany).

4.4. Immunostaining

Immunostaining was carried out as described in previous studies [32]. The antibodies used in these experiments were: anti-synapsin (anti-SYNORF1,1:50, Developmental Studies Hybridoma Bank, Iowa City, IA, USA), anti-Smed-β-catenin2 (1:1000) [33] and anti-α-tubulin (AA4, 1:20, Developmental Studies Hybridoma Bank). Alexa 488-conjugated goat anti-mouse (1:400, Molecular Probes, Waltham, MA, USA) and Alexa 568-conjugated goat anti-rabbit (1:1000, Molecular Probes) were used as a secondary antibodies. Nuclei were stained with DAPI (1:5000) or TO-PRO®-3 (1:3000, Thermo Fisher Scientific, Waltham, MA, USA). A Leica TCS-SP2 (Leica Lasertechnik, Heidelberg, BW, Germany) adapted for an inverted microscope (Leitz DMIRB, Leica Lasertechnik, Heidelberg, BW, Germany) and a Leica TCS SPE (Leica Microsystems, Mannhiem, BW, Germany) were used to obtain confocal images. Representative confocal stacks for each experimental condition are shown.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/16/11/25970/s1.

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Author Contributions: Teresa Adell and Miquel Sureda-Gómez conceived and designed the experiments; Miquel Sureda-Gómez and Eudald Pascual-Carreras performed the experiments; Teresa Adell, Miquel Sureda-Gómez and Eudald Pascual-Carreras analyzed the data; Teresa Adell and Miquel Sureda-Gómez wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary Information

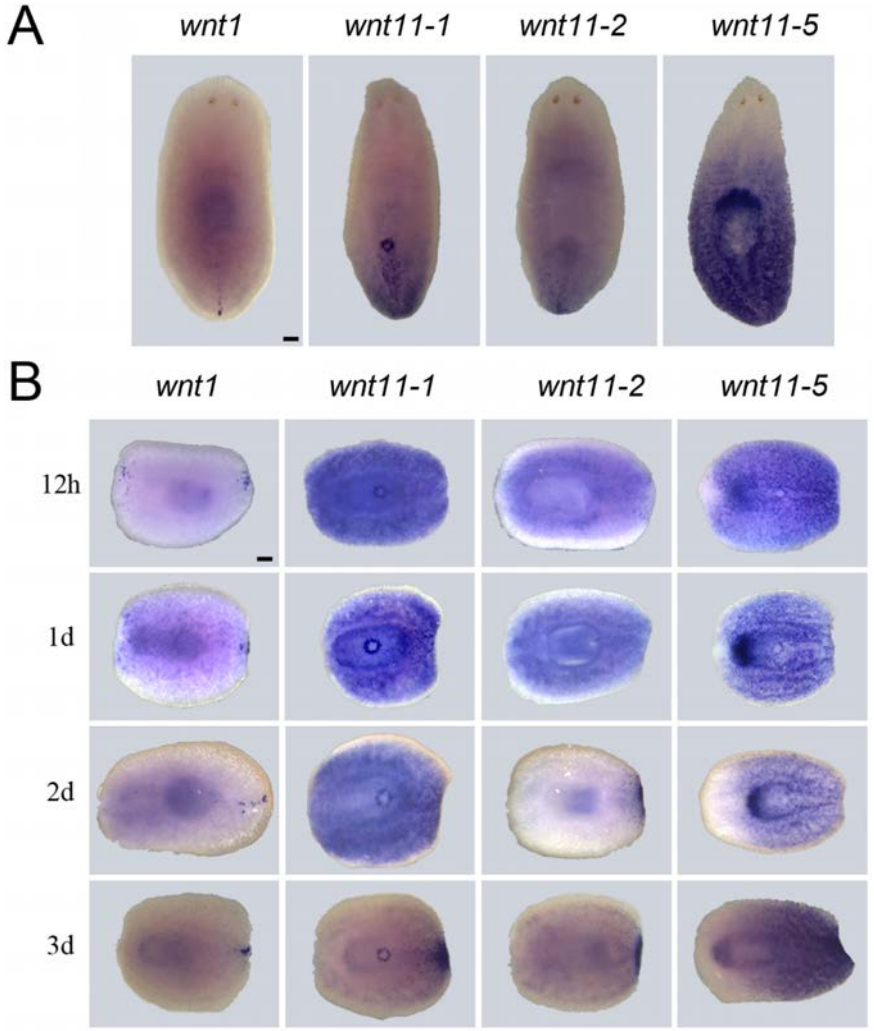

Figure S1. Expression pattern of posterior *wnts* in intact and regenerating animals. (**A**) *In situ* hybridization of posterior *wnts* in intact animals. *Smed-wnt1* is expressed as a stripe of cells in the posterior dorsal midline; *Smed-wnt11-1* is expressed in the mouth and as a posterior gradient from the mouth to the tail; *Smed-wnt11-2* is expressed as a posterior gradient, concentrated in the posterior midline; and *Smed-wnt11-5* is expressed in the esophagus and as a gradient from the prepharynx to the tail; and (**B**) *In situ* hybridization of posterior *wnts* in regenerating trunks at 12 h, 1 day, 2 days and 3 days post-amputation. Anterior blastemas are shown on the left and posterior blastemas on the right. The first wnt to be expressed in the regenerating region is *Smed-wnt1*, at 12 h. At 1 day, *Smed-wnt1* decreases its expression in anterior blastemas and concentrates in the posterior. At 2 days, it recovers the expression pattern observed in intact animals (posterior dorsal midline) and disappears in anterior blastemas. *Smed-wnt11-1* expression is only maintained in the mouth during early regeneration stages, and appears in the regenerating region at day 2, at the same time that *Smed-wnt11-2*. *Smed-wnt11-5* keeps the expression observed in intact animals. At 2 days of regeneration, it starts to re-scale from anterior to posterior to recover the gradient seen in intact animals. At 3 days of regeneration, the expression of all posterior *wnts* resembles the one observed in intact planarians. (The number of animals analyzed for each condition was at least $n = 5$.) Scale bar: 100 μm (**A**,**B**).

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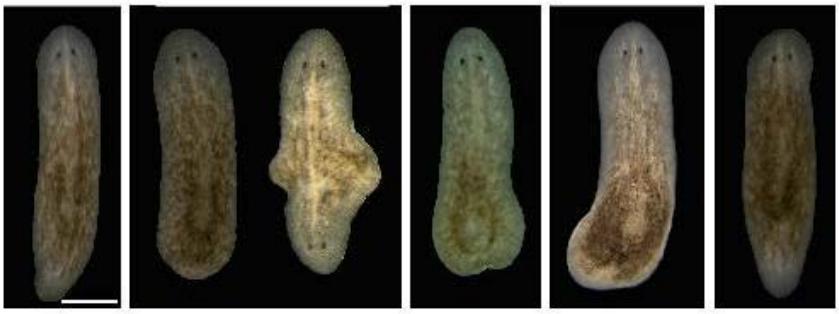

Figure S2. Phenotypes of posterior *wnts* RNAi during regeneration. Stereomicroscope views of regenerating trunk pieces showing the different phenotypes. From left to right: control animals have a wild-type appearance; *Smed-wnt1* RNAi planarians exhibit 2 different phenotypes, "tailless" (50% of animals) and "two-headed" (33% of animals); *Smed-wnt11-1* RNAi generates "short tail" planarians (93% of animals); *Smed-wnt11-2* RNAi generate "tailless" planarians (100% of animals); and *Smed-wnt11-5* RNAi planarians have a wild type tail. Images correspond to 20 days regenerating animals. (The number of animals analyzed for each condition was at least $n = 14$.) Scale bar: 500 μm.

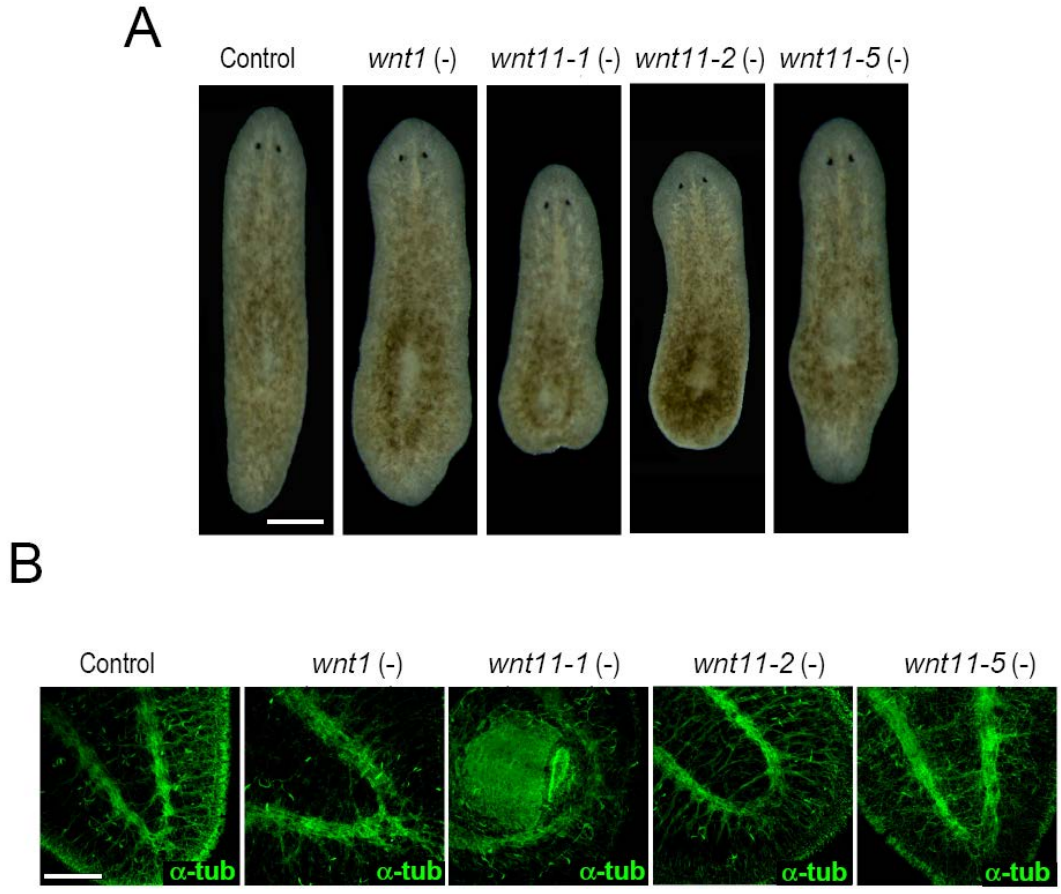

Figure S3. Phenotypes of posterior *wnt* RNAi during homeostasis. (**A**) Stereomicroscope view of the different phenotypes following RNAi in intact planarians. From left to right: controls resemble wild type; *Smed-wnt1* RNAi planarians also resemble wild type; *Smed-wnt11-1* RNAi generates "short tail" planarians; *Smed-wnt11-2* RNAi generates "tailless" planarians (100% of animals); and *Smed-wnt11-5* RNAi planarians have a tail that resembles wild type. (The number of animals analyzed for each condition was at least $n = 10$.); and (**B**) α-Tubulin immunostaining showing the morphology of the ventral nerve cords in the posterior tip of planarians after silencing posterior *wnts*. All images correspond to 20 days regenerating animals. Scale bar: 500 μm (**A**) and 100 μm (**B**).

Artículo 2:

Localization of planarian βCATENIN-1 reveals multiple roles during anterior-posterior regeneration and organogenesis.

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Resumen en castellano:

La localización de la proteína βCATENINA-1 de planarias revela múltiples roles durante la regeneración anteroposterior y la organogénesis.

La vía Wnt dependiente de βcatenina ejerce múltiples roles dependientes del contexto en tejidos embrionarios y adultos. En planarias se acepta que la βcatenina-1 especifica identidad posterior a través de la generación de un gradiente anteroposterior. Sin embargo, no se ha demostrado la existencia de tal gradiente. En este articulo, mediante el uso de un anticuerpo policlonal específico, demostramos que existe un gradiente anteroposterior nuclear de βCATENINA-1 desde la región prefaríngea hasta la cola de la planaria *Schmidtea polychroa*. Altos niveles en la región posterior disminuyen de forma constante hacia la región prefaríngea para luego incrementar de nuevo en la región de la cabeza. Durante la regeneración, la βCATENINA-1 se nuclearíza tanto en blastemas anteriores como posteriores, pero la nuclearización posterior solo está influenciada por el ligando canónico WNT1. Además, la βcatenina-1 es necesaria para la correcta morfogénesis en anterior, de forma consistente con los altos niveles observados en esta región. Finalmente demostramos que la βCATENINA-1 se encuentra de forma abundante durante el desarrollo y la diferenciación de los órganos y de forma particular se requiere para la especificación de la línea germinal. En conjunto, nuestros resultados proporcionan la primera evidencia directa de la existencia de un gradiente anteroposterior nuclear de βCATENINA-1 en planarias adultas, además de presentar nuevos roles de βcatenina-1 dependientes del contexto durante la regeneración y la organogénesis.

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STEM CELLS AND REGENERATION

RESEARCH ARTICLE

Localization of planarian β-CATENIN-1 reveals multiple roles during anterior-posterior regeneration and organogenesis

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ABSTRACT

The β-catenin-dependent Wnt pathway exerts multiple context-dependent roles in embryonic and adult tissues. In planarians, *β-catenin-1* is thought to specify posterior identities through the generation of an anteroposterior gradient. However, the existence of such a gradient has not been directly demonstrated. Here, we use a specific polyclonal antibody to demonstrate that nuclear β-CATENIN-1 exists as an anteroposterior gradient from the pre-pharyngeal region to the tail of the planarian *Schmidtea polychroa*. High levels in the posterior region steadily decrease towards the pre-pharyngeal region but then increase again in the head region. During regeneration, β-CATENIN-1 is nuclearized in both anterior and posterior blastemas, but the canonical WNT1 ligand only influences posterior nuclearization. Additionally, *β-catenin-1* is required for proper anterior morphogenesis, consistent with the high levels of nuclear β-CATENIN-1 observed in this region. We further demonstrate that β-CATENIN-1 is abundant in developing and differentiated organs, and is particularly required for the specification of the germline. Altogether, our findings provide the first direct evidence of an anteroposterior nuclear β-CATENIN-1 gradient in adult planarians and uncover novel, context-dependent roles for *β-catenin-1* during anterior regeneration and organogenesis.

KEY WORDS: β-catenin, Gradient, Wnt, Axial patterning, Organogenesis, Planarians

INTRODUCTION

The canonical (β-catenin-dependent) Wnt pathway is an evolutionarily conserved signaling pathway in animals which controls multiple crucial events during development, adult homeostasis and body regeneration (Clevers et al., 2014; Huelsken and Birchmeier, 2001; Martin and Kimelman, 2009; van Amerongen and Nusse, 2009; Yokoyama et al., 2007). During canonical signaling, secreted Wnt ligands bind to their Frizzled receptors to induce cytoplasmic accumulation and nuclear translocation of β-catenin (Clevers and Nusse, 2012; Mikels and Nusse, 2006), which is the key intracellular component of the transduction cascade. In the nucleus, β-catenin binds to the TCF/LEF transcription factors, triggering changes in chromatin conformation and gene transcription (Komiya and Habas, 2008; Valenta et al., 2012). Thus, β-catenin acts as a master regulatory gene, responsible for the control of multiple processes. During early animal embryogenesis, β-catenin specifies the site of endoderm internalization and promotes posterior fates in most studied systems (Byrum and Wikramanayake, 2013; Darras et al., 2011; Haegel et al., 1995; Henry et al., 2008; Lee et al., 2006; Logan et al., 1999; Srivastava et al., 2014; Wikramanayake et al., 2003). In later stages of development, the canonical Wnt pathway influences the formation of multiple cell types, tissues and organ systems (Aulehla et al., 2008; Grigoryan et al., 2008; Hari et al., 2002; Holland et al., 2005; Kiecker and Niehrs, 2001; Lewis et al., 2004; Petersen and Reddien, 2009a; Schneider and Bowerman, 2007; Tan et al., 2006; Watanabe et al., 2014), and most prominently, the anteroposterior (AP) patterning of the vertebrate central nervous system (Ciani and Salinas, 2005). In adult organisms β-catenin is essential for tissue homeostasis and its deregulation leads to degenerative diseases and cancers (Clevers and Nusse, 2012). Thus, β-catenin plays multiple, context-dependent roles in development, homeostasis and regeneration.

Planarian flatworms are a powerful model to analyze the multiple roles of β-catenin-dependent Wnt signaling. Their striking regenerative capacity coupled with the continuous remodeling of their tissues to adapt their body size to food availability (Saló, 2006) allows analysis of signaling pathways in the intact organism in multiple situations. The planarians *Schmidtea mediterranea* and *Schmidtea polychroa* have two β-catenin paralogs, namely *β-catenin-1* and *β-catenin-2* (Gurley et al., 2008; Iglesias et al., 2008; Martín-Durán and Romero, 2011; Martín-Durán et al., 2010). While *β-catenin-2* is involved in cell-cell adhesions (Chai et al., 2010), inhibition of planarian *β-catenin-1* results in a striking 'radial-like' hypercephalyzed phenotype, revealing a central role for canonical Wnt signaling in the specification of posterior identities during adult regeneration and homeostasis (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). In contrast, inhibition of negative regulators of the canonical Wnt pathway, such as the components of the β-catenin destruction complex, APC and axin, leads to the duplication of posterior territories (i.e. posteriorized phenotype) (Gurley et al., 2008; Iglesias et al., 2011). To explain these phenotypes, it is widely postulated that there is a gradient of increasing β-catenin activity from anterior to posterior that specifies and maintains AP axial identity in planarians (Adell et al., 2010). This model, which departs from classic regeneration experiments (Child, 1911, 1941; Morgan, 1904, 1905), is further supported by the range of phenotypes generated after *β-catenin-1* inhibition, in which anterior and posterior structures are gradually affected according to the dose and time of inhibition (Adell et al., 2010; Iglesias et al., 2008). These phenotypes vary from loss of the most posterior structures ('tail-less' phenotype) to 'radial-like' hypercephalization with no posterior or central identity (Adell et al., 2010; Iglesias et al., 2008). Moreover, it is known that several Wnt ligands are expressed posteriorly, whereas inhibitors (e.g. *notum* and *sFRP*) localize to the anterior pole, and their inhibition leads to anteriorized or posteriorized phenotypes, respectively (Adell et al.,

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2009; Gurley et al., 2010; Lander and Petersen, 2016; Petersen and Reddien, 2009b, 2011; Scimone et al., 2016; Sureda-Gómez et al., 2015). Interestingly, recent reports have suggested that *β-catenin-1* exerts additional roles in eye and brain patterning (Hill and Petersen, 2015; Owen et al., 2015), which is consistent with the ubiquitous mRNA expression of *β-catenin-1* (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). To date, however, no studies have described the localization of β-CATENIN-1 protein either as an AP gradient or in specific developing tissues.

Since β-catenin is regulated at the protein level (Clevers, 2006), analysis of its different roles in intact and regenerating planarians requires tools to visualize the localization of β-catenin protein. In this study, we analyze the role of β-catenin using a polyclonal antibody generated against the N-terminal region of planarian β-CATENIN-1. Immunohistochemistry demonstrates the existence of a gradient of nuclear β-CATENIN-1 along the AP axis, with the lowest levels in the pre-pharyngeal region and the highest levels at the tip of the tail. β-CATENIN-1 levels are also high in the head region. We demonstrate that the WNT1 ligand is specifically required for the nuclear localization of β-CATENIN-1 in posterior regions. β-CATENIN-1 is also abundant in the anterior pole, where it controls proper brain and head regeneration. Finally, immunodetection of β-CATENIN-1 also reveals high levels of nuclear protein in specific organs during adult homeostasis and regeneration, as well as during embryogenesis. In this context, we demonstrate an essential function of β-CATENIN-1 in female and male germline development. Altogether, our findings validate the main assumptions of the current model for AP specification in planarian flatworms, and uncover new roles for the canonical Wnt pathway in embryonic and adult organogenesis.

RESULTS

β-CATENIN-1 displays a nuclear gradient along the AP axis, from the pre-pharyngeal region to the tail

To analyze the localization of β-CATENIN-1 in planarians, we generated a polyclonal antibody against the N-terminal region of the *Schmidtea mediterranea* β-CATENIN-1 protein (anti-βCAT-1) (Fig. S1A). Western blot analysis on protein extracts of adult specimens of *S. mediterranea* showed a specific band of the expected size (107 kDa) (Fig. S1B). Importantly, anti-βCAT-1 also recognizes the β-CATENIN-1 protein from *Schmidtea polychroa* (Fig. S1B), which is the sister species of *S. mediterranea* (Alvarez-Presas et al., 2008). The region used for raising the antibody exhibits 98% amino acid identity between both species (Fig. S1A). We found that the antibody produced a more reliable signal for immunohistochemistry in *S. polychroa*. Since *S. polychroa* is a sexual species, which enables the study of the reproductive system and the embryogenesis, we therefore focused all subsequent analyses on this species.

To confirm that anti-βCAT-1 specifically recognizes β-CATENIN-1, we performed western blot analysis on protein extracts from *β-catenin-1(RNAi)* and *APC(RNAi)* animals after 5 weeks of treatment. In *S. polychroa*, these animals did not yet show any AP morphological transformation (Fig. S2A). As expected, inhibition of *β-catenin-1* transcription resulted in a reduction of β-CATENIN-1 levels, and inhibition of *APC*, which downregulates β-catenin (Clevers, 2006), produced an increase in β-CATENIN-1 levels (Fig. S2B). qPCR analysis of *β-catenin-1* mRNA levels in those samples showed that *β-catenin-1* was downregulated after *β-catenin-1* RNAi but was not upregulated after *APC* RNAi (Fig. S2C). This observation further supports the specificity of the antibody, since APC induces the destruction of β-CATENIN-1 protein but does not act at the transcriptional level (Clevers, 2006). We further validated the specificity of the anti-βCAT-1 antibody by quantifying its immunoreactivity in anterior and posterior regions in control, *β-catenin-1(RNAi)* and *APC(RNAi)* intact animals after 2 weeks of treatment. Consistent with the previous western blot results, immunoreactivity decreases in *β-catenin-1(RNAi)* animals and increases in *APC(RNAi)* planarians compared with controls (Fig. S3).

To examine the subcellular localization of β-CATENIN-1, we performed immunohistochemistry on paraffin sections of uninjured animals. β-CATENIN-1 was mostly located in the nuclei in both anterior and posterior regions (Fig. 1A). Although we cannot rule out residual localization in the cytoplasm of scattered cells, β-CATENIN-1 was never observed in the cell membrane, consistent with the functional specialization of the planarian β-catenin genes (Chai et al., 2010). We next analyzed distribution of β-CATENIN-1 along the AP axis of uninjured planarians in sagittal sections (Fig. 1B; Fig. S4A). Immunoreactivity for β-CATENIN-1 was abundant in the mesenchyme, mostly posteriorly, and in the brain and pharynx (Fig. 1B; Fig. S4A). A heat map of the localization of β-CATENIN-1 demonstrated that the highest levels were found in the brain, the distal part of the pharynx and the posterior end of the animal (Fig. 1B; Fig. S4A). We next quantified the signal of nuclear β-CATENIN-1 along the AP axis to detect differences in any region of the body (Fig. 1C; Fig. S4C). Although β-CATENIN-1 was almost always located in the nucleus, we applied a nuclear mask to specifically quantify the nuclear signal (Fig. S4B). For this analysis, we considered sagittal sections corresponding only to the region around the midline, and we excluded the brain and pharyngeal signal, which we assumed was related to organogenesis rather than to axial positional information (Fig. 1C). We quantified nuclear β-CATENIN-1 levels in eight consecutive regions along the AP axis of the animal, which demonstrated that the highest levels of nuclear β-CATENIN-1 occurred in the most posterior region of the animal, whereas the lowest levels were observed in the pre-pharyngeal area (Fig. 1C). Remarkably, we detected higher levels of nuclear β-CATENIN-1 in the head than in the pre-pharyngeal region. Importantly, the gradient distribution of β-CATENIN-1 along the AP axis was statistically significant (Fig. 1C; Fig. S4C; Table S1). Although we cannot specify the cell types that contribute to the gradient, we observed nuclear localization of β-CATENIN-1 in sub-epidermal muscular cells, which are the source of positional information cues (Witchley et al., 2013) and in proliferating cells, which correspond to neoblast and early progeny stem cells (Reddien, 2013) (Fig. S4D). Therefore, our findings demonstrate that the nuclear localization of β-CATENIN-1 exhibits a gradient along the AP axis, with the highest levels posteriorly and the lowest levels in the pre-pharynx. High levels of nuclear localization are also observed in the head.

wnt1 controls β-CATENIN-1 localization in posterior blastemas

Because *β-catenin-1* mRNA is expressed in both anterior and posterior blastemas from the first hours after amputation (Fig. S5A) (Iglesias et al., 2008), we analyzed β-CATENIN-1 localization in anterior and posterior blastemas at different time points during regeneration on sagittal sections (Fig. 2A; Fig. S5B). At 6 h post amputation (hpa), β-CATENIN-1 localized to the nucleus of cells located in the tip of anterior and posterior wounds (Fig. S5B). With the formation of the blastema at 12 hpa and 1 dpa, we observed accumulation of β-CATENIN-1 in both anterior and posterior blastemas compared with the adjacent pre-existing tissue (Fig. 2A; Fig. S5B,C). This situation was apparently maintained 3 and 5 days

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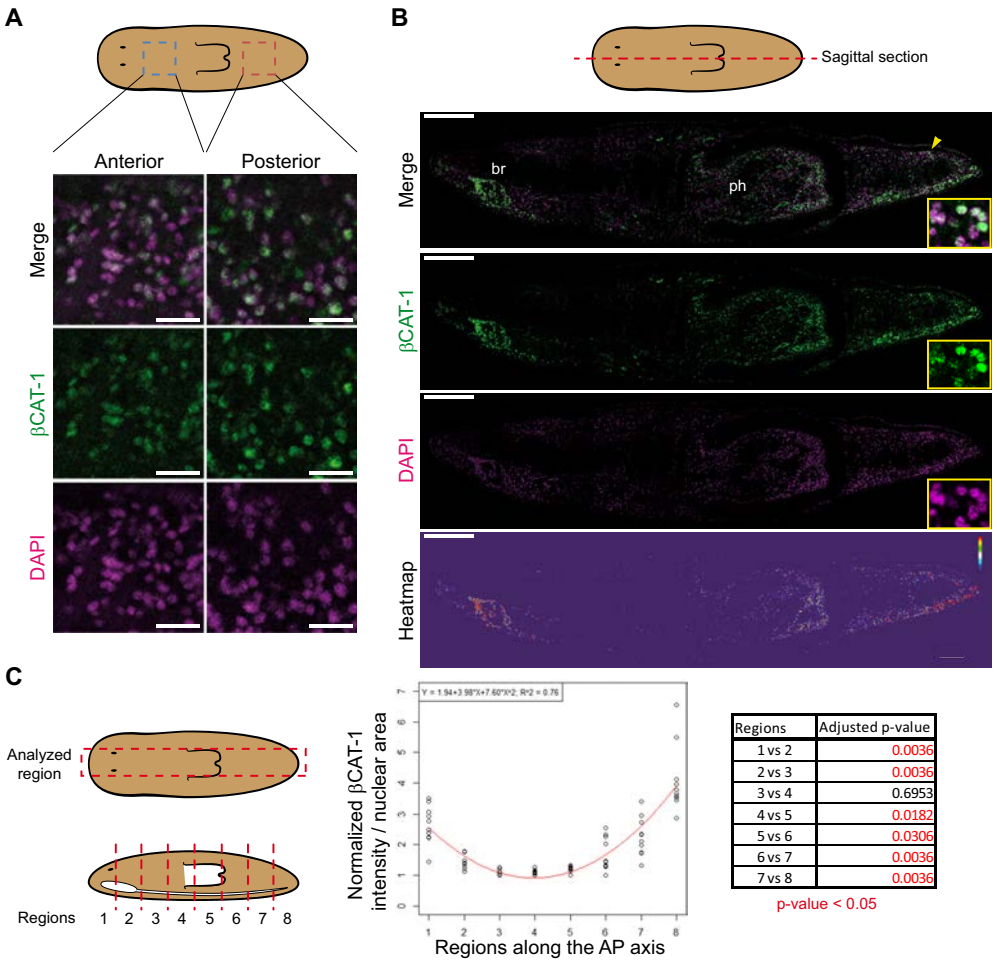

Fig. 1. β-CATENIN-1 is expressed as a nuclear gradient from the prepharyngeal region to the tail. (A) Immunostaining on paraffin sections against β-CATENIN-1 (green) and nuclear staining with DAPI (magenta) in uninjured planarians. β-CATENIN-1 is localized in the nucleus both in anterior and posterior regions. (B) Immunostaining on a sagittal section corresponding to the region around the midline against β-CATENIN-1 (green) and nuclear staining with DAPI (magenta) in uninjured animals. The images shown correspond to a mosaic of sub-images. The rainbow heat map of β-CATENIN-1 (bottom) illustrates the intensity of the signal (red, maximum). Dorsal, top. Arrowhead indicates areas shown at higher magnification in insets. (C) Quantification of nuclear β-CATENIN-1 intensity on sagittal sections corresponding to the region around the midline normalized with the nuclear area of each section (red lines indicate the different regions analyzed along the AP axis), excluding the brain and pharynx (white areas) and relative to the lower value (n=10 sections corresponding to four different animals). Values are represented using a polynomial regression and were analyzed using the Wilcoxon signed-rank test. Table shows the *P*-values obtained comparing the different regions. *P*-values in red are significant. br, brain; ph, pharynx. Scale bars: 25 µm (A), 200 µm (B). In all images, anterior is left.

post amputation (dpa), although β-CATENIN-1 became more abundant in the ventral side of the anterior blastema as the brain formed (Fig. 2A; Fig. S5B). Therefore, nuclear localization of β-CATENIN-1 during regeneration does not depend on the identity of the blastema.

The observation that *wnt1* is expressed in a few cells of the posterior dorsal midline (Fig. S5A) and that *wnt1* silencing phenocopies *β-catenin-1(RNAi)* (Fig. S6A) suggests that *wnt1* specifies posterior identity via activation of *β-catenin-1* (Adell et al., 2009; Petersen and Reddien, 2009b). However, *wnt1* is expressed in anterior and posterior regenerating regions at early stages of regeneration, from 12 hpa to 1 dpa (Fig. S5A) (Gurley et al., 2010; Petersen and Reddien, 2009b). We therefore analyzed whether *wnt1* is responsible for β-CATENIN-1 localization in anterior and posterior blastemas during the earliest stages of regeneration. Western blots of protein extracts from 1 dpa anterior and posterior wounds showed that β-CATENIN-1 levels only decayed in posterior blastemas in *wnt1(RNAi)* animals (Fig. 2B). We confirmed this specific decrease by immunohistochemistry on anterior and posterior 1 day regenerating blastemas (Fig. 2C; Fig. S6B). Altogether, our results indicate that *wnt1* positively controls β-CATENIN-1 nuclear levels in posterior blastemas at 1 dpa, but it does not have a significant role in maintaining β-CATENIN-1 levels in anterior regenerating regions.

β-CATENIN-1 is required for proper anterior regeneration

The high levels of nuclear β-CATENIN-1 in anterior blastemas suggest a role for *β-catenin-1* in anterior regeneration and

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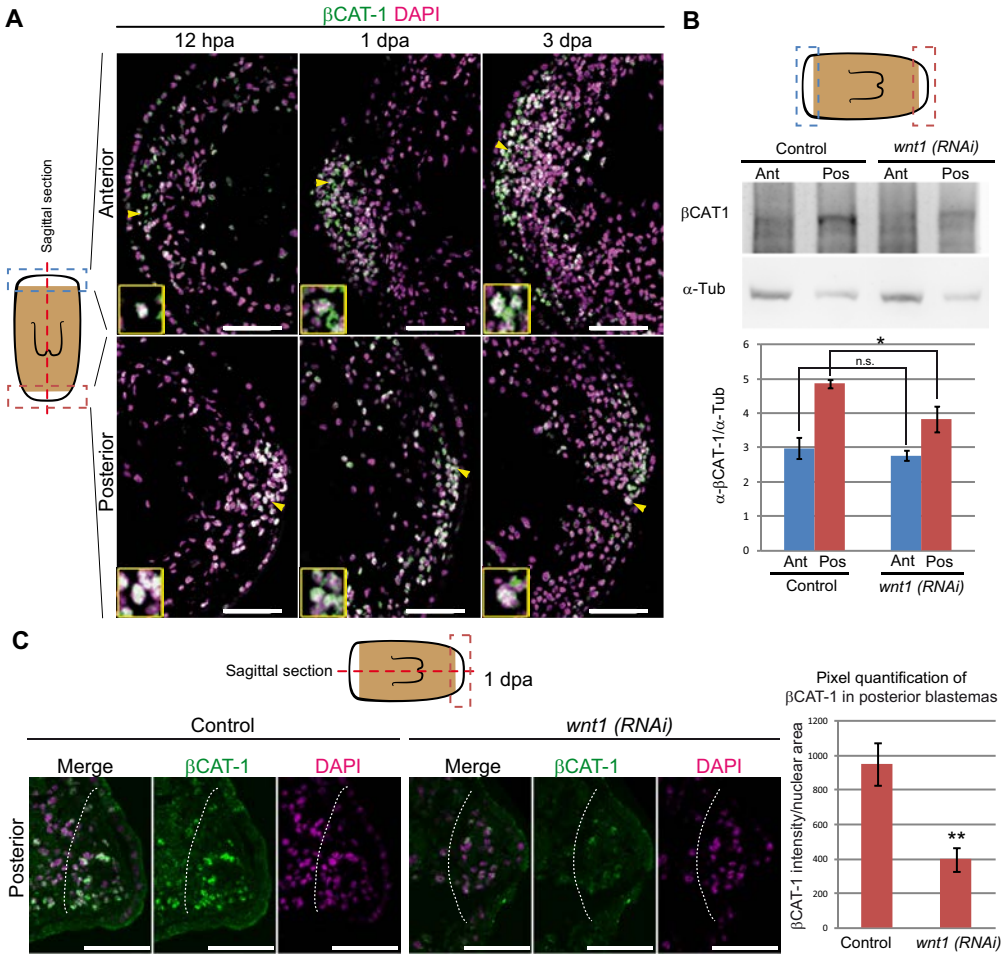

Fig. 2. β-CATENIN-1 is localized in the nucleus of anterior and posterior blastemas but only posterior β-CATENIN-1 depends on WNT1. (A) Immunoreactive pattern of the βCAT-1 antibody (green) on sagittal sections and nuclear staining with DAPI (magenta) in 12 h, 1 day and 3 day bipolar regenerating trunks. A nuclear mask was applied to show nuclear signal (original images are shown in Fig. S5B). Dorsal, top; anterior, left. Arrowheads indicate areas shown at higher magnification in insets. (B) Western blot with anti-βCAT-1 antibody of protein extracts from anterior and posterior regenerating regions of 1 day regenerating control and *wnt1(RNAi)* animals. α-Tubulin antibody was used as a loading control. Bar chart shows the quantification of β-CATENIN-1 signal with respect to α-tubulin, normalized with respect to the control anterior region value (n=5). β-CATENIN-1 signal decreases after *wnt1(RNAi)* in posterior but not in anterior regions. Error bars indicate s.d., *P<0.05; n.s., not significant. (C) Immunostaining on sagittal sections with anti-βCAT-1 antibody (green) and DAPI (magenta) of control and *wnt1(RNAi)* posterior regenerating regions 1 day after amputation. Bar chart shows the quantification of β-CATENIN-1 signal in the blastema region (delimited by the white dotted line). Error bars indicate s.d., **P<0.005. *wnt1(RNAi)* animals show a decrease in posterior nuclear β-CATENIN-1 signal compared with controls (n=6). Dorsal, top; anterior, left. Scale bars: 50 µm (A,C).

homeostasis. Analysis of β-CATENIN-1 in the anterior region revealed nuclear localization of the protein in neural cells of the cephalic ganglia and in the head margin (α-tubulin^{+}), as well as in non-neural cells (α-tubulin^{-}) located in the most anterior dorsal midline (Fig. 3A). Interestingly, this region corresponds to the expression domain of the Wnt inhibitor *notum*, which induces the regeneration of anterior tails when silenced (Petersen and Reddien, 2011). A similar distribution was observed in 3 dpa anterior blastemas, where β-CATENIN-1 localized to dorsal scattered cells at the dorsal midline in addition to neural cells of the brain primordia (Fig. 3B). In order to study the role of β-CATENIN-1 in anterior regeneration, we analyzed the morphology and patterning of anterior blastemas in *β-catenin-1(RNAi)* animals. We first observed that the eyes formed closer to the pre-existing tissue in *β-catenin-1(RNAi)* planarians compared with controls (Fig. S7A). Quantitative analysis of the expression of a dopaminergic (*th-1*$^{+}$) and an octopaminergic (*tbh*$^{+}$) neuronal marker in anterior blastemas revealed a significant decrease in both cell populations after 3 and 8 dpa (Fig. 3C). Similarly, the number of mechanosensory cells (*cintillo*$^{+}$) in the head margin was also significantly reduced after *β-catenin-1(RNAi)* (Fig. S7B) (Hill and Petersen, 2015; Owen et al., 2015). Quantification of the brain area by DAPI staining and analysis of the distance from the anterior tip of the brain to the anterior tip of the head revealed that the brain ganglia in *β-catenin-1 (RNAi)* animals were smaller and formed closer to the head margins than in control planarians (Fig. 3C). In addition, the midline furrow

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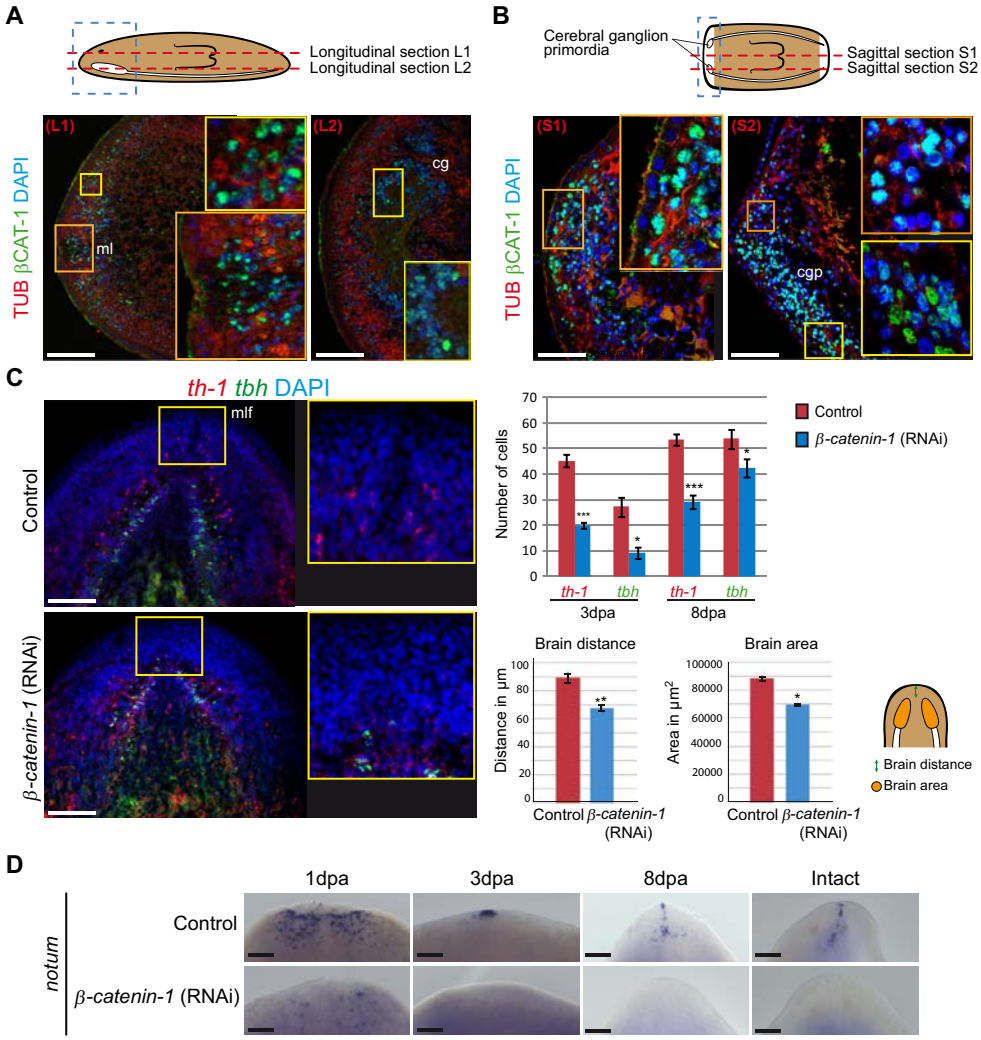

Fig. 3. *β-catenin-1* is required for proper anterior regeneration. (A) Immunostaining on longitudinal sections of uninjured planaria with anti-βCAT-1 (green) and anti-α-tubulin (red). Nuclei are labeled with DAPI (blue). Yellow and orange squares indicate regions corresponding to neural and non-neural cells, respectively, which are shown in higher magnification. Anterior, left. (B) Immunostaining on sagittal sections with anti-βCAT-1 (green) and anti-α-tubulin (red) of 3 dpa anterior regions. Nuclei are labeled with DAPI (blue). Yellow and orange squares indicate regions corresponding to ventral and dorsal areas, respectively, which are shown in higher magnification. Anterior, left. (C) Dopaminergic (*th-1*, red) and serotoninergic (*tbh*, green) neurons visualized by whole-mount *in situ* hybridization in control and *β-catenin-1(RNAi)* animals fixed 8 dpa. DAPI (blue) labels the nucleus. Yellow squares indicate regions corresponding to the midline ventral furrow (mlf) shown in higher magnification. Quantification of *th-1*$^+$ and *tbh*$^+$ cells showing a decrease of both neural populations in *β-catenin-1(RNAi)* planarians. Quantification of the distance from the tip of the cephalic ganglia to the anterior tip of the animal and of the total brain area, both visualized by DAPI nuclear staining, shows a decrease of both measures in *β-catenin-1(RNAi)* planarians. Error bars indicate s.d., **P*<0.05, ***P*<0.005, ****P*<0.0005 (two-tailed *t*-test); *n*=5. Anterior, top. (D) Whole-mount *in situ* hybridization showing *notum* expression in control and *β-catenin-1(RNAi)* planarians at 1, 3 and 8 days of regeneration and in uninjured animals. *notum* expression disappears in *β-catenin-1(RNAi)* animals. Anterior, top. cg, cephalic ganglia; ml, midline; cgp, cephalic ganglia primordium; mlf, midline furrow. Scale bars: 100 µm (A,C,D), 50 µm (B).

between the brain ganglia was not correctly patterned in *β-catenin-1 (RNAi)* planarians (Fig. 3C), suggesting a problem in restoration of the anterior tip. We then analyzed whether these morphological defects affected the domain of expression of *notum* in regenerating and intact *β-catenin-1(RNAi)* animals. As early as 1 dpa, the expression of *notum* decreased after *β-catenin-1(RNAi)* and subsequently disappeared in regenerating and uninjured 14-day-old dsRNA-injected animals (Fig. 3D) (Hill and Petersen, 2015; Petersen and Reddien, 2011; Scimone et al., 2014). Both the tip- and brain-related domains of *notum* expression were affected by *β-catenin-1(RNAi)* treatment. Altogether, these results indicate that β-CATENIN-1 is required for the proper patterning and morphogenesis of the brain and head margin structures. Further work will be required to determine which specific anterior defects are caused by the function of β-CATENIN-1 as an element of the anterior organizing region (e.g. as a *notum* regulator) and which are

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due to its probable role in central nervous system (CNS) patterning and morphogenesis.

β-CATENIN-1 is stabilized in the nucleus during adult and embryonic organogenesis

In addition to the strong β-CATENIN-1 immunoreactivity in the brain observed with our anti-βCAT-1 antibody, we also observed signal in other mature organs (Fig. 4A; Fig. S8A). In the nervous system, β-CATENIN-1 localized preferentially to the external part of the brain ganglia and the brain branches, and in scattered cells in the most ventral part. Additionally, we observed strong signal in the ventral nerve cords and apparently in the eye (Fig. 4A; Fig. S8A). β-CATENIN-1 was also localized in the nucleus of the digestive system and the pharynx, where it was found to be more abundant in

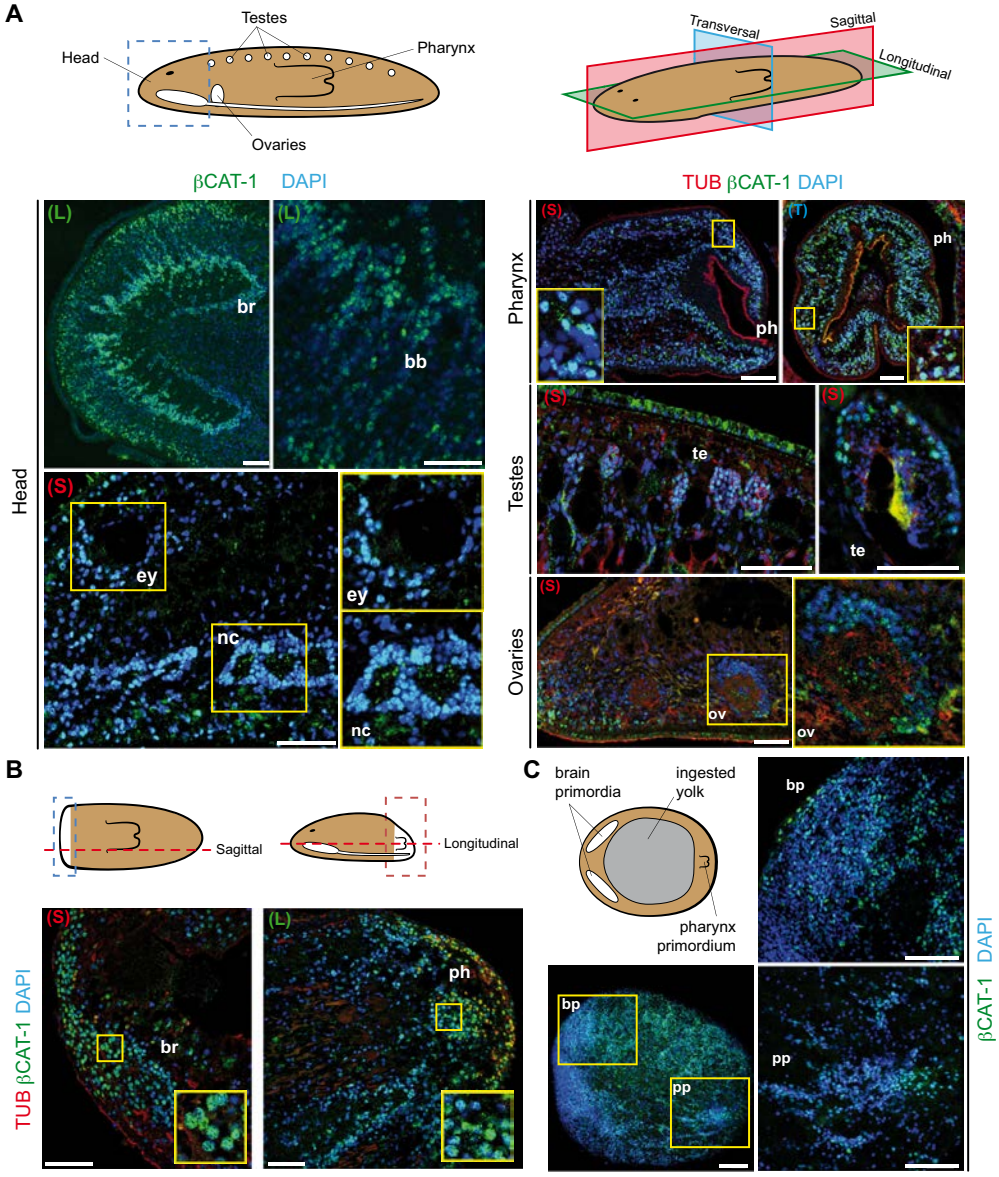

Fig. 4. Nuclear β-CATENIN-1 is upregulated in specific organs during maintenance and *de novo* formation. (A) Immunostaining on sections with anti-βCAT-1 (green) and anti-α-tubulin (red) of uninjured animals. Nuclei are labeled with DAPI (blue). Yellow squares indicate regions shown at higher magnification. Analyzed regions are indicated in the scheme at the top left and planes are indicated in the top right scheme. (B) Immunostaining on sections (planes are indicated in scheme at top) with anti-βCAT-1 (green) and anti-α-tubulin (red) of regenerating animals. Nuclei are labeled with DAPI (blue). Yellow squares indicate images shown at higher magnification. (C) Immunostaining on whole-mount of a 6-day-old embryo (stage 5) with anti-βCAT-1 (green) and anti-α-tubulin (red). Nuclei are labeled with DAPI (blue). Yellow squares indicate images shown at higher magnification. br, brain; bb, brain branches; nc, nerve cord; ph, pharynx; te, testes; ov, ovaries; bp, brain primordium; pp, pharynx primordium. Scale bars: 50 µm (A,B), 200 µm (C).

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the most distal part (Fig. 4A; Fig. S8A), consistent with the *β-catenin-1* expression pattern (Fig. S5A) (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Remarkably, we detected high levels of nuclear β-CATENIN-1 in the male gonads (the testis), particularly in the germ cells and in the nucleus of the cells around the ovaries, where female germ cells are found (Hoshi et al., 2003) (Fig. 4A). We also observed signal for β-CATENIN-1 in the copulatory apparatus (Fig. S8A). We next analyzed the distribution of β-CATENIN-1 in these organs during regeneration, between 3 and 5 dpa. We observed an increase in the levels of β-CATENIN-1 in the nuclei of the primordial cells of each organ, compared with the surrounding cells (Fig. 4B; Fig. S8B). To further investigate the distribution of β-CATENIN-1 during organogenesis, we characterized its localization in stage 5 embryos, which corresponds to the onset of *de novo* cell differentiation and early organogenesis during planarian embryonic development (Martín-Durán and Romero, 2011; Monjo and Romero, 2015). We detected a widespread distribution of β-CATENIN-1 throughout the entire embryo, and in particular in the presumptive anlagen of the definitive pharynx and brain, which at this stage appear as an anterior bilobed cluster of cells that are already positive for neural markers (Martín-Durán and Romero, 2011; Monjo and Romero, 2015) (Fig. 4C; Fig. S8C). Therefore, in addition to its nuclear localization in the main organs of planarians during homeostasis, β-CATENIN-1 is also dynamically regulated during regeneration and embryogenesis, consistent with a role for *β-catenin-1* during planarian organogenesis.

β-CATENIN-1 is required for germline specification

To further elucidate the role of *β-catenin-1* in the development and regeneration of the mature organ systems of planarians, we decided to focus on the possible role of *β-catenin-1* in germline specification and gonad maturation, since β-CATENIN-1 was strongly detected in male and female gonads (Fig. 4A). *β-catenin-1* dsRNA was injected in 1- to 3-day-old hatchlings, prior to expression of *nanos* (*nos*), a germline marker required for proper germline specification (Fig. S9A) (Wang et al., 2007). After 4 weeks of RNAi treatment, control animals had normally developing gonads, with *nos* expression in small clusters along two dorsolateral lines, corresponding to the testis and in two larger ventral clusters behind the brain, corresponding to the ovaries (Fig. 5A). In contrast, *β-catenin-1(RNAi)* animals did not show any *nos* expression (Fig. 5A). The same result was obtained using *germinal histone H4* (*gh4*) as a marker of male germline specification (Wang et al., 2007) (Fig. S9B). Our results thus show that *β-catenin-1* is required for germ cell specification and gonad development, uncovering a role for this multi-functional protein in controlling planarian organogenesis, in addition to its well-established axial role.

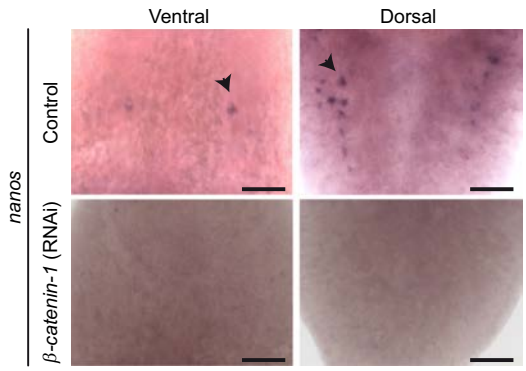

Fig. 5. β-CATENIN-1 is required for germline specification. Analysis of expression of *nanos* by whole-mount *in situ* hybridization in control and *β-catenin-1 (RNAi)* 4-week-old hatchling planarians (*n*=16). *β-catenin-1(RNAi)* (12/16) animals lack expression of *nanos* in ovaries and testes compared with controls (16/16). Black arrows indicate testes and ovaries. Anterior, up. Scale bars: 200 µm.

DISCUSSION

Planarians are an ideal system in which to understand the complex context-dependent roles of β-catenin, since it is possible to study multiple cellular and genetic processes in the whole organism during embryonic development, regeneration and adult homeostasis. To date, however, a major challenge for planarian biology has been to analyze protein expression with specific antibodies. In this study, we successfully generated a specific anti-β-CATENIN-1 antibody that allowed analysis of the functional behavior of β-CATENIN-1 in the whole planarian and in different developmental stages.

Our study provides the first direct confirmation of the hypothesized AP gradient of nuclear β-CATENIN-1. Interestingly, however, we found that there are two regions with high levels of nuclear β-CATENIN-1, one in the tail and one in the head, with the lowest levels of nuclear localization of β-CATENIN-1 found in the pre-pharyngeal region. During regeneration, β-CATENIN-1 is actively nuclearized in posterior wounds in a *wnt1*-dependent manner. Similar nuclear accumulation was observed in anterior blastemas and in the organ primordia, highlighting an additional role of *β-catenin-1* in head patterning and organogenesis, and in particular for germline development.

Importantly, β-CATENIN-1 signal was mostly found in the nucleus and never in the membrane. This result contrasts with the results reported from other systems, mainly in adult stages, where β-catenin is only occasionally detected in the nucleus, and at much lower levels in comparison to its membrane localization (Anderson et al., 2002). This is explained by the functional specialization of planarian β-catenin paralogs, where *β-catenin-1* is dedicated to nuclear signaling and *β-catenin-2* to cell-cell adhesion (Chai et al., 2010). Moreover, the significant levels of β-CATENIN-1 found in the nucleus are striking, and indicate the degree of Wnt signaling activation required in this unique species in the adult stage. Since nuclear localization of β-catenin is considered to be a hallmark of Wnt activation, the fact that planarians have evolved separate nuclear- and membrane-localized β-catenin proteins facilitate the analysis of specific context-dependent functions for nuclear β-catenin. In addition, the high expression levels of nuclear β-catenin in planarians improve visualization of the protein at different levels. Thus, analysis of nuclear β-catenin function in planarians may shed light on potential roles that were previously overlooked in other systems.

A gradient of β-CATENIN-1 along the AP axis

The hypothesis that a dynamic morphogenetic gradient of axial potential underlies the re-establishment of the AP polarity during planarian regeneration is rooted in classic cutting experiments performed more than a century ago (Child, 1911, 1941; Lewis et al., 1977; Meinhardt, 1978; Morgan, 1904, 1905). In recent years, functional characterization of the key elements of the canonical Wnt pathway suggested that the morphogenetic role of the *wnt* ligands secreted by a posterior organizing center could generate a gradient of nuclear β-CATENIN-1 responsible for the specification and maintenance of the planarian AP axis (Adell et al., 2009, 2010; Almuedo-Castillo et al., 2012; Lander and Petersen, 2016; Petersen

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and Reddien, 2009b; Scimone et al., 2016; Sureda-Gómez et al., 2015). The nested expression of several Wnt ligands (*wnt1*, *wnt11-1*, *wnt11-2* and *wnt11-5*) in the pharyngeal and tail region of the planarian body, and the expression of Wnt inhibitors (*notum* and *sFRP*) in the anterior tip of the head gave support to the gradient hypothesis (Adell et al., 2010; Gurley et al., 2010; Lander and Petersen, 2016; Petersen and Reddien, 2011; Scimone et al., 2016; Sureda-Gómez et al., 2015). Additionally, inhibition of these posterior Wnt ligands leads to 'tail-less' or 'two-headed' planarians, as does the inhibition of *β-catenin-1* itself (Adell et al., 2009; Gurley et al., 2010; Petersen and Reddien, 2009b; Sureda-Gómez et al., 2015). However, silencing of *notum* and *APC* results in the opposite phenotype, characterized by the regeneration of 'two-tailed' planarians (Gurley et al., 2008; Iglesias et al., 2011; Petersen and Reddien, 2011). The fact that the expansion of anterior fates after *β-catenin-1(RNAi)* depends on the time and dose of inhibition (Adell et al., 2010; Iglesias et al., 2008) and that the cutting position along the AP axis influences the severity of the phenotype after *APC (RNAi)* (Iglesias et al., 2011) are also in agreement with a gradient hypothesis. Although β-CATENIN-1 is widely present in specific organs of adult planarians, our findings robustly demonstrate the existence of a nuclear gradient of β-CATENIN-1 (Fig. 1; Fig. S4; Fig. 6A). The highest levels of nuclear β-CATENIN-1 occur in posterior territories, but strikingly, the lowest levels are not found in the anterior pole, as proposed in the gradient hypothesis, but rather in the pre-pharyngeal region. We found differentiated (e.g. muscle) and proliferating cell types endowing nuclear β-CATENIN-1, recognizing that the specific cell types that contribute to this gradient deserve further studies. Although the observation of the nuclear β-CATENIN-1 gradient confirms the main assumption of the gradient hypothesis, it also suggests that the patterning of the head region involves more complex epistatic interactions between different signaling molecules (see below). In this context, the role of the anterior Wnt inhibitors (*notum* and *sFRPs*) might not merely result in complete inhibition of *β*-CATENIN-1, but rather in its fine regulation.

During bi-polar regeneration, *β-catenin-1* is expressed in both blastemas (Iglesias et al., 2008). The secreted elements *wnt1* and *notum* are first expressed in all wounds, and later become restricted to the posterior and anterior blastemas, respectively (Petersen and Reddien, 2009b, 2011; Wenemoser et al., 2012). Our results demonstrate that there is abundant nuclear β-CATENIN-1 in both anterior and posterior blastemas at any stage (Fig. 2A). However, 1 day after amputation, when the axial identities of the blastemas are established, nuclear localization of β-CATENIN-1 depends on *wnt1* activity only in posterior blastemas (Fig. 2B,C; Fig. 6B). This observation supports the hypothesis that the *wnt1* ligand, secreted by the newly formed posterior organizer, is the morphogen that sustains nuclear translocation of β-CATENIN-1 at the posterior pole during regeneration (Adell et al., 2009; Petersen and Reddien, 2009b). Later on, the nested expression of the other posterior Wnt ligands would control the re-establishment and maintenance of the gradient (Hayashi et al., 2011; Lander and Petersen, 2016; März et al., 2013; Scimone et al., 2016; Sureda-Gómez et al., 2015) (Fig. 6A). Therefore, the signaling network that specifies the AP axial identity behaves differently according to the regenerating context. However, our findings indicate that β-CATENIN-1 could be a component of both the anterior and the posterior organizers, and that it is the fine-tuning of β-CATENIN-1 that is essential for the organizing function in each pole. This is further supported by the observation that *β-catenin-1* inhibition restores the ability to regenerate a head in anterior wounds in planarian species that cannot

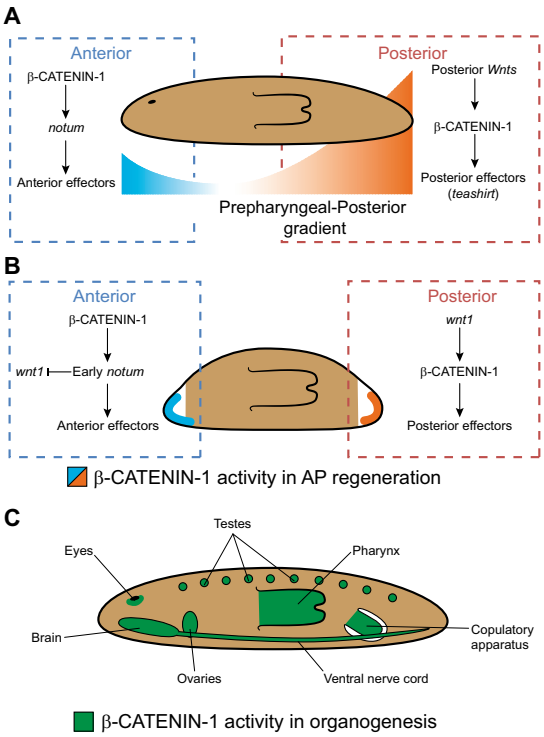

Fig. 6. Summary of β-CATENIN-1 function in planarians. (A) β-CATENIN-1 displays a gradient from the prepharyngeal region to the tail. Nuclear localization of β-CATENIN-1, which is responsible for activation of effectors of posterior identity, like *teashirt* (Reuter et al., 2015), would depend on the action of the posterior Wnts (WNT1, WNT11-1, WNT11-2, WNT11-5). In the anterior region, β-CATENIN-1 is required for expression of *notum* among other effectors of anterior morphogenesis. (B) During bi-polar regeneration, β-CATENIN-1 is localized in both anterior and posterior blastemas. Posterior β-CATENIN-1, which is responsible for conferring posterior identity, would be specifically activated by *wnt1*. Anterior β-CATENIN-1 is also required for *notum* expression during regeneration. Early *notum* could be the signal which inhibits the action of WNT1 in anterior-facing wounds. It remains unknown which signal triggers anterior β-CATENIN-1 activation during regeneration and homeostasis. (C) β-CATENIN-1 is localized in the nucleus of several planarian organs. It controls embryonic and adult organogenesis and is specifically required for brain patterning and for normal development of the spermatogonia and oogonia.

regenerate anteriorly (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). Similarly, β-CATENIN-1 stabilization also restores the ability to regenerate anterior wounds in these animals, even though this experiment causes the expected polarity reversal and the planarians develop a two-tailed phenotype (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013). Nevertheless, further work will be necessary to determine which specific upstream regulators generate this context-specific behavior and enable the differential roles of nuclear β-CATENIN-1 in anterior and posterior blastemas.

β-CATENIN-1 controls anterior patterning

In addition to its role in posterior development, the canonical Wnt pathway appears to be implicated in the regeneration of the planarian head and brain (Fraguas et al., 2014; Hill and Petersen,

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2015; Iglesias et al., 2011; Owen et al., 2015). In this respect, β-CATENIN-1 localizes abundantly to the head region, both in the cephalic ganglia and in the mesenchymal cells (Figs 1, 3 and 4). A closer examination revealed that these mesenchymal cells with active β-CATENIN-1 include neural and non-neural cell types. The latter are mostly located in the dorsal-anterior tip of the animal, where *notum* is expressed (Petersen and Reddien, 2011). As in intact animals, β-CATENIN-1 is active both ventrally, where the brain primordium forms, and dorsally, in anterior regenerating blastemas. In accordance with this complex pattern of activity, our findings show that *β-catenin-1* is required to regenerate a normal sized brain, with the correct number of neural cell populations, as well as a normal head sensory margin (Hill and Petersen, 2015; Owen et al., 2015). Importantly, the midline ventral furrow and *notum* expression in the anterior midline also disappears in *β-catenin-1 (RNAi)* animals, which suggest a problem in anterior tip patterning and the establishment of the anterior organizing region. Considering that not only the tip-related expression of *notum* but also the brain-related expression completely disappears after *β-catenin-1* RNAi, *notum* may be directly regulated by *β-catenin-1*, generating a regulatory loop to control *β-catenin-1* levels in the anterior region of planarians (Hill and Petersen, 2015; Petersen and Reddien, 2011; Scimone et al., 2014). Our findings thus support three major roles of *β-catenin-1* in anterior regeneration: (1) the specification and maintenance of the anterior signaling center, defined by the tip-related expression of *notum* (Petersen and Reddien, 2011) and other genes (Chen et al., 2013; Gaviño et al., 2013; Roberts-Galbraith and Newmark, 2013; Scimone et al., 2014; Vásquez-Doorman and Petersen, 2014; Vogg et al., 2014) (Fig. 6A,B); (2) the regeneration of the brain, through the regulation of the brain-related expression of *notum* (Hill and Petersen, 2015); and (3) the specification of different neuronal cell types, as described in most animal models (Ciani and Salinas, 2005; Hari et al., 2002; Lewis et al., 2004; Machon et al., 2003; Watanabe et al., 2014; Yu and Malenka, 2003; Zechner et al., 2003).

Further experiments will be required in order to determine the exact nature of the signaling network that enables the specific roles of β-CATENIN-1 in anterior patterning and the mechanisms that activate the different pools of β-CATENIN-1 in the head region. Of the two Wnt genes expressed in the head (*wnt2* and *wnt11-6*), a function has only been reported for *wnt11-6* (Adell et al., 2009; Kobayashi et al., 2007). Hill and Petersen (2015) demonstrate that *wnt11-6* regulates the brain-related expression of *notum* through β-CATENIN-1 and that a *wnt11-6*/*notum* system would control the size of the brain. However, *wnt11-6* does not control the expression of *notum* in the anterior pole. Consequently, the mechanism through which β-CATENIN-1 is activated in this specific area remains unclear. Since no Wnt has been found to be expressed in this region, it is possible that β-CATENIN-1 is activated in a Wnt-independent manner, for instance, through WNK (with no Lysine) kinases (Serysheva et al., 2013) or via growth factors such as platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) (Couffinhal et al., 2006).

β-CATENIN-1 controls embryonic and adult organogenesis

Besides its role in axial patterning, the involvement of β-catenin in organogenesis has been broadly reported in many developmental systems, such as mouse, zebrafish and *Drosophila* (Clevers, 2006; Huelsken and Birchmeier, 2001; Lewis et al., 2004; Orsulic and Peifer, 1996; Tan et al., 2006). Our knowledge of this additional role of the canonical Wnt pathway during planarian regeneration is still poor, primarily because of the strong axial phenotypes obtained after *β-catenin-1(RNAi)* treatment that can mask other more subtle defects. Our findings demonstrate that there is nuclear β-CATENIN-1 in the brain, ventral nerve cords, pharynx, eyes, copulatory apparatus, and the male and female gonads (summarized in Fig. 6C). Moreover, we observed strong nuclear signal during the regeneration of the organ primordia and during embryogenesis. Although the role of the canonical Wnt pathway in all these organs (e.g. in the pharynx and the eyes) is still unclear, *β-catenin-1* appears to be required for brain patterning (see above) (Hill and Petersen, 2015), as well as for the proper specification and development of the spermatogonia and oogonia (Fig. 5). This latter observation contrasts with the fact that *β-catenin-1(RNAi)* treatment does not produce any apparent neoblast-related phenotype. The germline and the neoblasts (adult stem cells) are the only two cell types with the ability to self-maintain in adult planarians. Therefore, our results uncover different requirements for *β-catenin-1* in the two dividing cell populations of planarians, indicating that β-catenin does not confer a universal stemness property to cells (Clevers and Nusse, 2012). In our model, the canonical Wnt pathway might be conferring particular cell identities, rather than maintaining stemness.

Altogether, our findings demonstrate that the canonical Wnt pathway, and in particular β-CATENIN-1, is a multifaceted signaling network during planarian regeneration and homeostasis. It defines the posterior identity through an activity gradient, participates in the morphogenesis of anterior structures by controlling anterior gene expression and promotes normal organogenesis, for instance, of the brain and reproductive organs. The analysis and visualization of β-CATENIN-1 protein distribution fills a key practical and theoretical gap in the study of planarian biology and more generally in the understanding of the multiple context-dependent roles of β-catenin. Our study thus delivers an important tool that will improve future investigations on the molecular basis of planarian regeneration and homeostasis, as well as on the multiple roles of β-catenin-dependent Wnt signaling.

MATERIALS AND METHODS

Planarian culture

An asexual clonal strain population of *S. mediterranea* BCN-10 biotype was maintained as previously described (Fernandéz-Taboada et al., 2010). A sexual population of *S. polychroa* from Sant Celoni (Barcelona, Spain) was maintained in the lab as described elsewhere (Martín-Durán et al., 2008). Animals were starved for 1 week before conducting any experiment. Egg capsules were collected and fixed as described previously (Cardona et al., 2005a).

Generation of the anti-βCAT-1 polyclonal antibody

The cDNA region of the first 201 N-terminal amino acids of *Smed*-β-CATENIN-1 was cloned into the pET-His tagged vector (Novagen) and 200 µg of the recombinant protein was used as immunogen to produce polyclonal IgGs in three rabbits (Innoprot, Spain). Three more immunizations were performed using 100 µg of the recombinant protein. The post-immunization serum was precipitated in ammonium sulfate and stored at 4°C. Before use in immunohistochemistry, IgGs were purified using a Protein A Antibody Purification Kit (Sigma-Aldrich).

Western blot assays

Intact planarians and regenerating blastemas were dissociated in lysis buffer (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% SDS). Protein extracts were denatured for 15 min at 95°C in lysis buffer, run on 8% SDS-PAGE gels and transferred to western blot membranes. After incubation with anti-βCAT-1 (1:30,000) and anti-α-tubulin (Sigma, T9026; 1:10,000) antibodies, signal was developed using the Clarity Western ECL Substrate (Bio-Rad) or the Amersham ECL Prime Western Blotting Detection Reagent and chemiluminescence was detected using a C-DiGit Chemiluminescent

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Author contributions
Conceptualization: M.S.-G., J.M.M.-D., T.A.; Formal analysis and investigation: M.S.-G., J.M.M.-D.; Writing - original draft preparation and review and editing: M.S.-G., J.M.M-D., T.A.; Funding acquisition: T.A.; Supervision: T.A.

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Supplementary information
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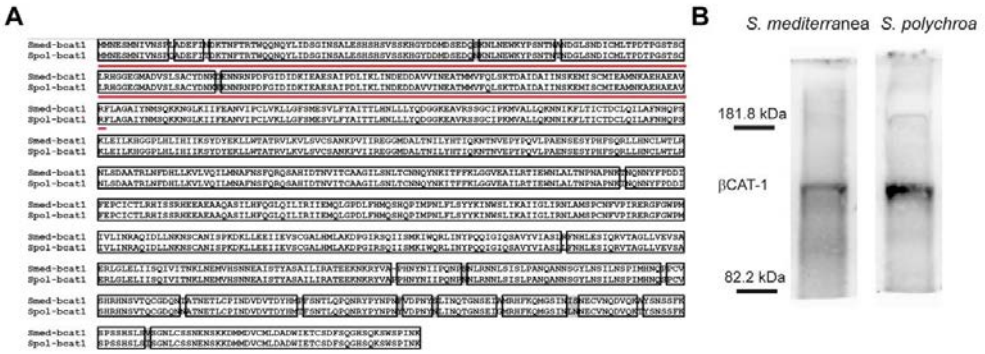

Supplementary figure 1. Inmunoreactivity of the anti-βCAT-1 antibody. (A) *S. mediterranea* and *S. polychroa* β-CATENIN-1 protein sequence alignment. Boxed sequences indicate 100% similarity. Red line indicates the region used to produce the polyclonal anti-βCAT-1 antibody. (B) Western blot of protein extracts from uninjured planarians corresponding to *S. mediterranea* and *S. polychroa*, immunoblotted with the anti-βCAT-1 antibody (n=3). A band of the expected size (107 kDa) was detected in extracts of both species.

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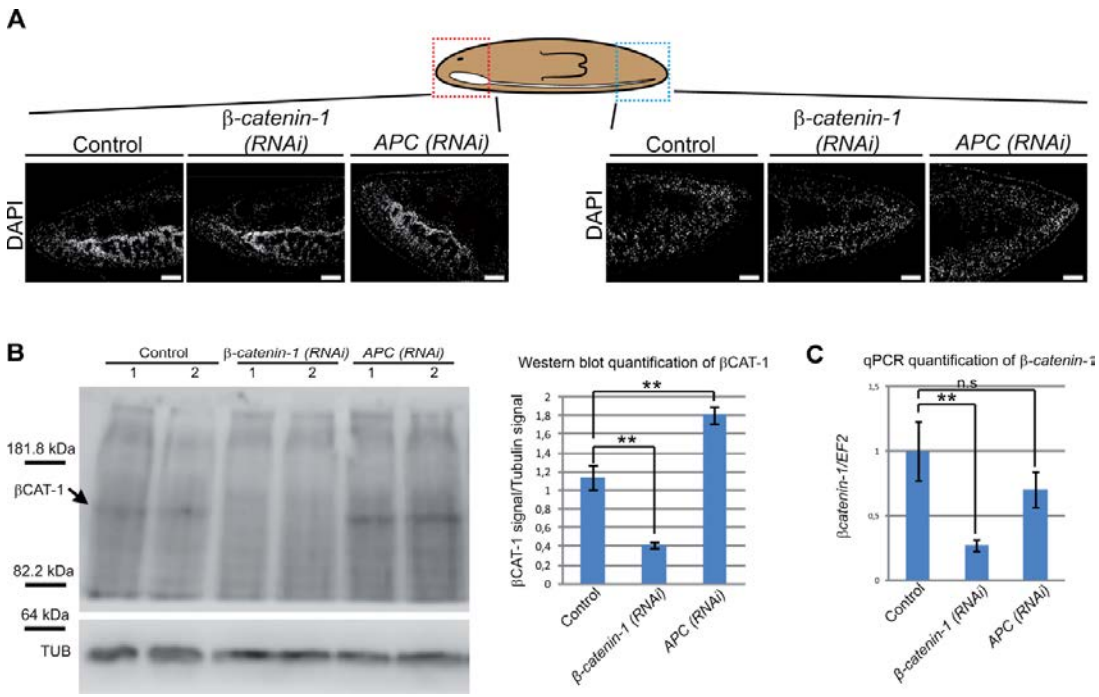

Supplementary figure 2. Specificity of the anti-βCAT-1 antibody by Western Blot

(A) DAPI immunostaining (white) of sagittal sections corresponding to the anterior and posterior region of *β-catenin-1 (RNAi), APC (RNAi)* and control uninjured planarians after 5 weeks of RNAi treatment. (B) Western blot of control, *β-catenin-1 (RNAi)* and *APC (RNAi)* planarian protein extracts immunoblotted with the anti-βCAT-1 antibody. Extracts correspond to uninjured planarians after 5 weeks of RNAi treatment. Bar chart shows βCATENIN-1 signal quantification relative to control sample 1 and normalized to α-Tubulin levels. Two replicates per condition (n=3). (C) Real-time PCR quantification of *β-catenin-1* mRNA levels in samples analyzed in C, relative to control sample and normalized by *Elongation Factor 2* (EF2). Dorsal, top; Anterior, left. Scale bar – 50 µm.

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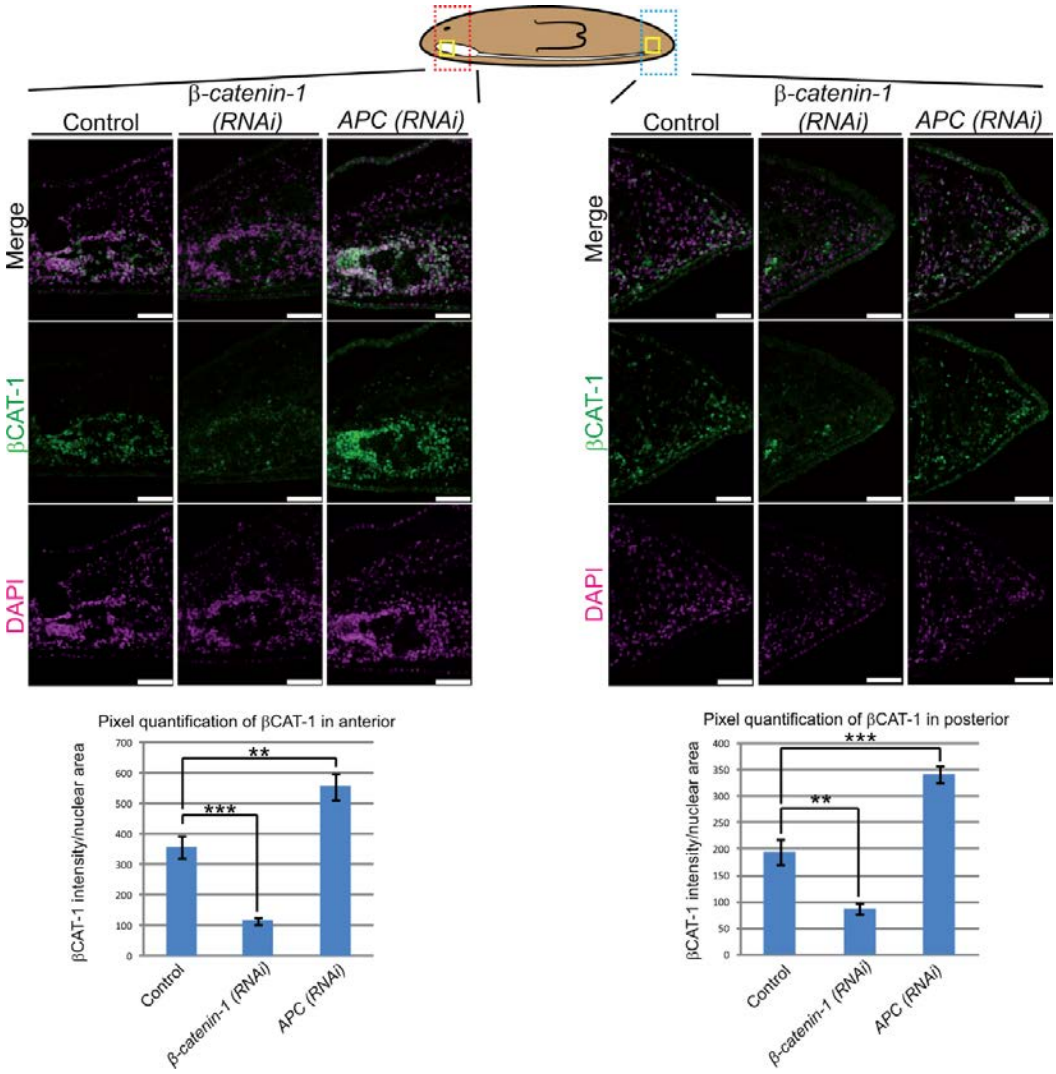

Supplementary figure 3. Specificity of the anti-βCAT-1 antibody by immunostaining. Anti-βCAT-1 immunostaining (green) of sagittal sections corresponding to the anterior and posterior region of *β-catenin-1 (RNAi), APC (RNAi)* and control uninjured planarians. Nuclei are stained with DAPI (magenta). *β-catenin-1 (RNAi)* animals show a decrease of β-CATENIN-1 signal compared with controls whereas *APC (RNAi)* animals show an increase. Doted square in the picture indicates the area shown in the images. Bar charts show the quantification of βCATENIN-1

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signal (yellow squares in the picture indicate the area used to quantify the signal) (n=6). Error bars indicate standard deviations, **p<0.005, ***p<0.0005. Dorsal, top; Anterior, left. Scale bar – 50 µm.

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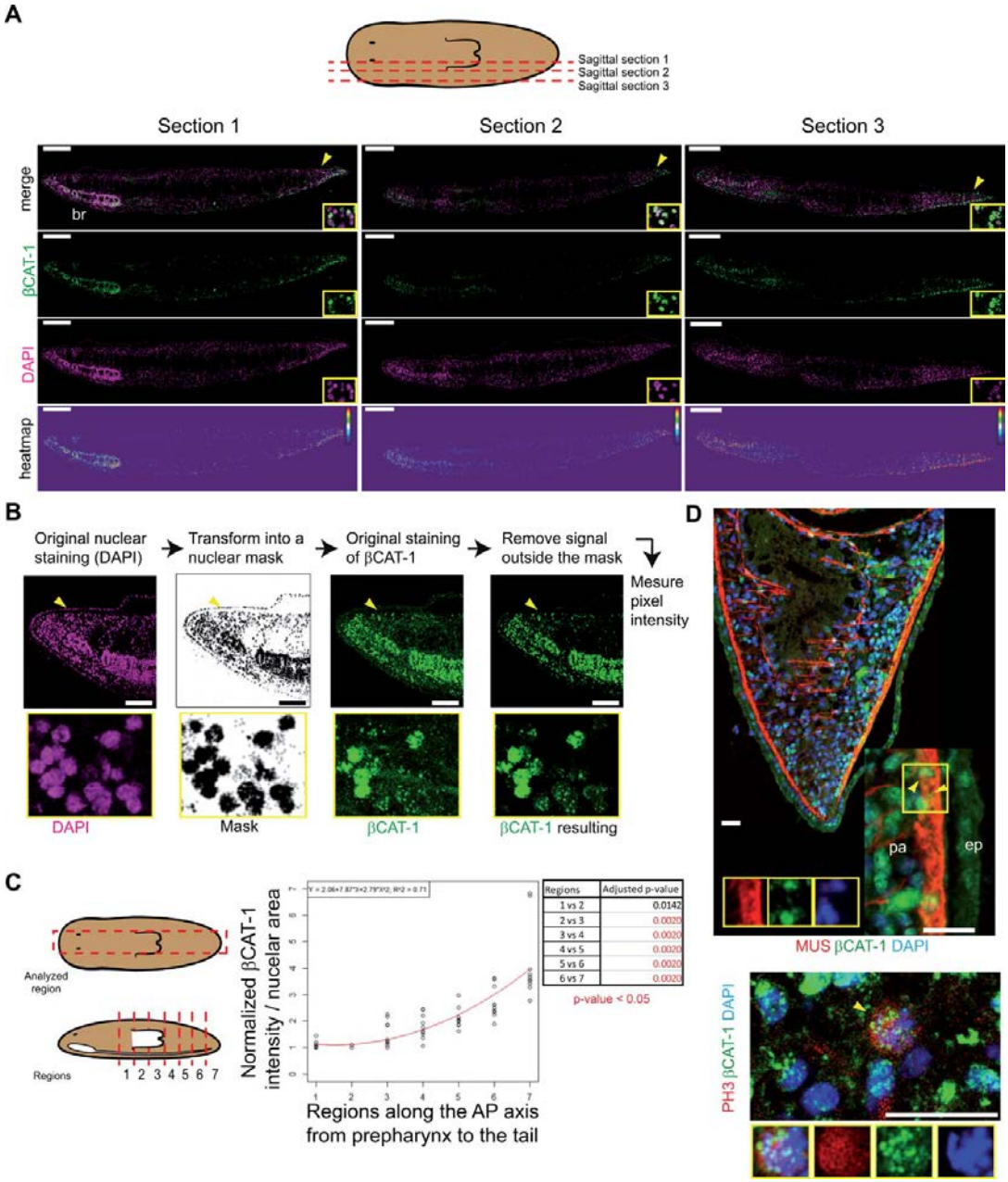

Supplementary figure 4. Immunostaining of sagittal sections of uninjured planarians with the anti-βCAT-1 antibody and treatment of confocal images to quantify nuclear β-CATENIN-1 signal. (A) anti-βCAT-1 immunostaining (green) of sagittal sections corresponding to the lateral region of uninjured planarians. Nuclei are stained with DAPI (magenta). A rainbow heatmap of anti-βCAT-1signal illustrates the intensity of the signal (red, maximum). Anterior, left. Arrowheads indicate areas shown

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at higher magnification (B) Scheme of the procedure applied to quantify the nuclear signal obtained with the anti-βCAT-1 antibody. Arrowheads indicate areas shown at higher magnification. (C) Quantification of parenchymal nuclear β-CATENIN-1 intensity on sagittal sections corresponding to the region around the midline normalized with the nuclear area of each section (red lines in the picture indicate the different regions analyzed along the AP axis, from the prepharynx region to the tail), excluding the pharynx (white area in the picture) and relative to the lower value (n=10 sections corresponding to 4 different animals). Values are represented using a polynomial regression and were analyzed using the Wilcoxon-signed-rank test. Table shows the p-values obtained comparing the different regions. p-values in red are significant. (D) Immunostaining on sagittal sections of uninjured animals labeled with anti-βCAT-1 (green), anti-MHC-A 6G10 (red) and DAPI (blue) [upper image] and anti-βCAT-1 (green), anti-PH3 (red) and DAPI (blue) [lower image]. A magnification of a muscular cell showing nuclear βCATENIN-1 is showed. Arrowheads indicate areas shown at higher magnification. br, brain; pa, parenquima; ep, epidermis. Scale bar – 200 µm (A), 50 µm (B), 20 µm (D).

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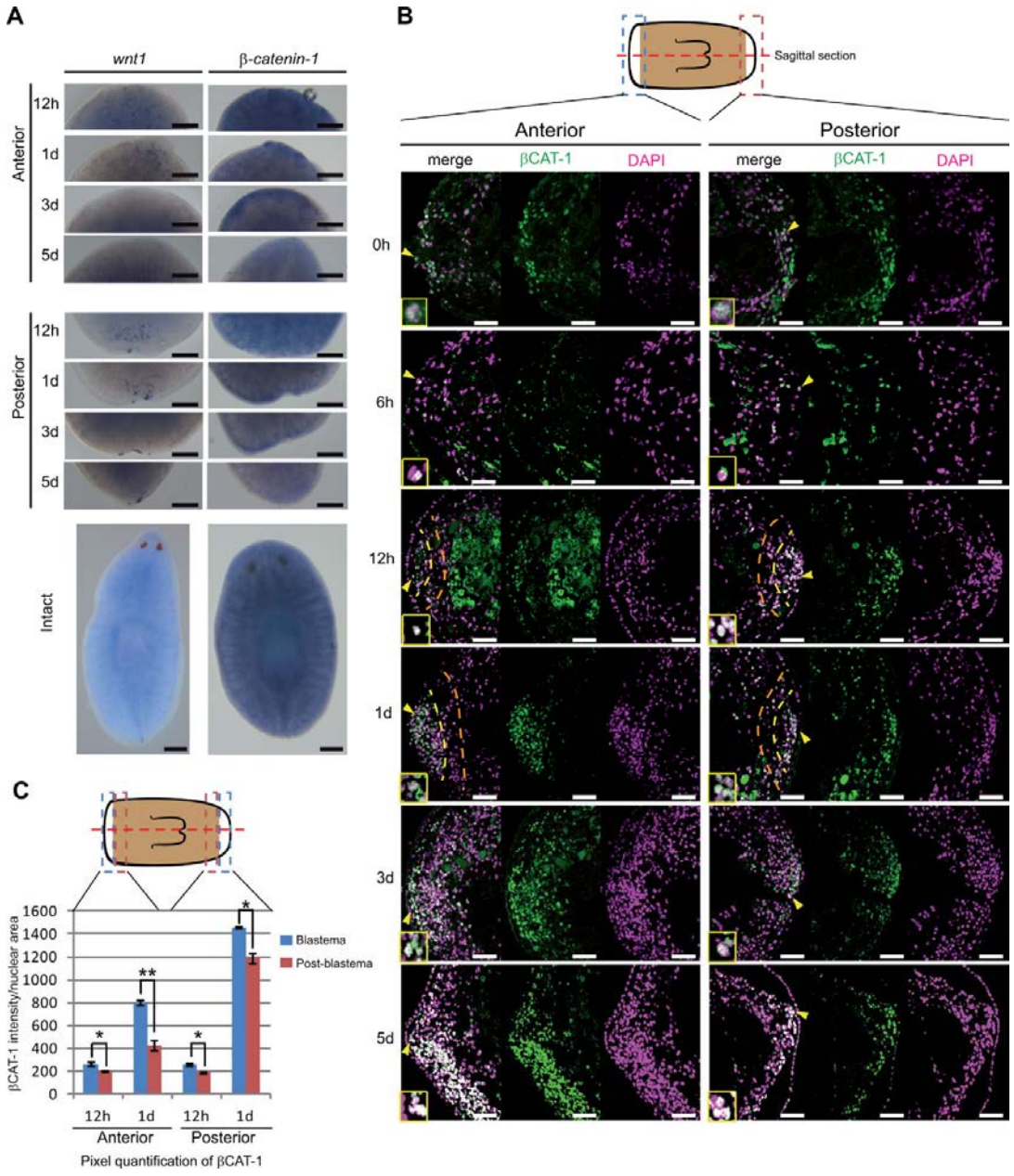

Supplementary figure 5. β-CATENIN-1 is localized to the nucleus in both anterior and posterior blastemas during regeneration. (A) *wnt1* and *β-catenin-1* detected by whole-mount *in situ* hybridization in regenerating trunks and uninjured animals. Animals labeled with *wnt1* are shown dorsally and animals labeled with *β-catenin-1* are shown ventrally. Anterior, top. (B) Trunk fragments were fixed at 0, 6 and 12 hpa, and

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1, 3 and 5 dpa, labeled with anti-βCAT-1 antibody (green) and DAPI (magenta). Sagittal sections corresponding to the regenerating regions are shown. Anterior, left; Dorsal, top. Arrowheads indicate the magnified regions. Yellow and orange dotted lines delimitate the blastema and adjacent pre-existent region analyzed in the bar chart on C. (C) Bar chart shows the quantification of β-CATENIN-1 signal in blastema and adjacent pre-existent region at 12 hours and 1 day after amputation in anterior and posterior wounds. The blastema and adjacent pre-existent region measured areas are equivalent. Blastema region is delimitated by the yellow dotted line in B and adjacent pre-existent region is delimitated by the yellow and orange dotted lines. *$p<0.05$, **$p<0.005$ (n=3). Blastema region shows a preferential accumulation of β-CATENIN-1 signal compared to the adjacent pre-existent region. Scale bar – 200 µm (A), 50 µm (B).

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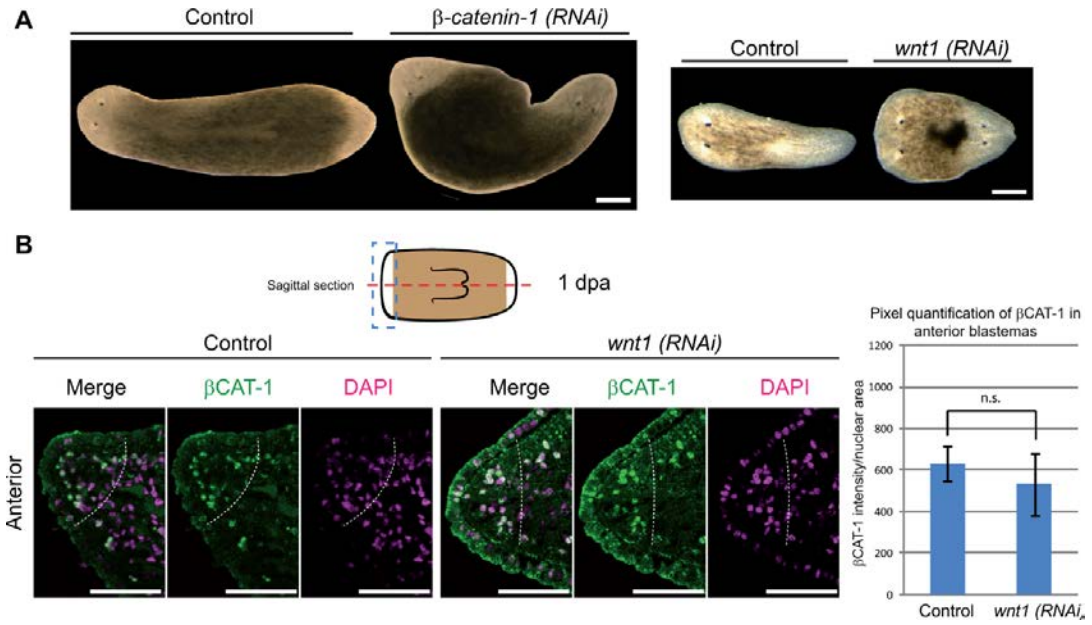

Supplementary figure 6. ***wnt1 (RNAi)*** **phenocopies** ***βcatenin-1 (RNAi)*** **and does not affect the β-CATENIN-1 nuclear localization in anterior blastemas during regeneration**. (A) Phenotype generated after *β-catenin-1* and *wnt1* RNAi silencing in *S. polychroa*. (B) Immunostaining on sagittal sections with anti-βCAT-1 antibody (green) and DAPI (magenta) of control and *wnt1 (RNAi)* posterior regenerating regions 1 day after amputation. Bar chart shows the quantification of β-CATENIN-1 signal in the blastema region (delimitated by the white dotted line). n.s. - no significance. *wnt1 (RNAi)* animals do not show differences in nuclear β-CATENIN-1 signal compared with controls (n=6). Dorsal, top; Anterior, left. Scale bar – 200 µm (A), 50 µm (B).

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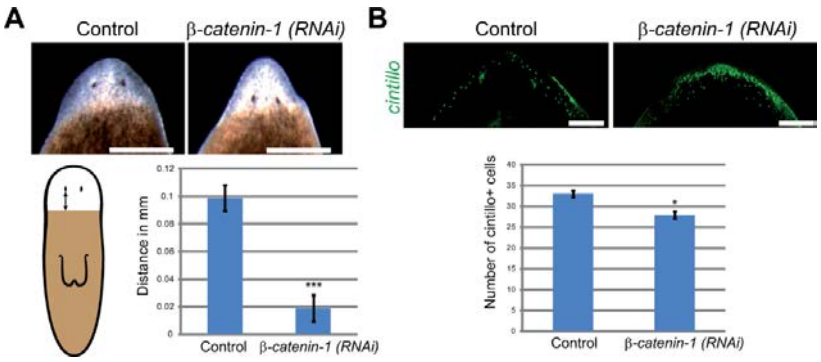

Supplementary figure 7. Anterior defects in *β-catenin-1 (RNAi)* regenerating planarians. (A) In vivo images of 8-day anterior regenerating control and *β-catenin-1 (RNAi)* trunks. Cartoon shows the measured distance (arrows) from the eye to the pre-existing pigmented region. Bar chart shows the quantification (n=7 per condition). (B) FISH to detect expression of *cintillo* (green) in 8-day anterior regenerating control and *βcatenin-1 (RNAi)* trunks. Bar chart shows the quantification of cintillo-positive cells (n=5 per condition). Error bars indicate standard deviations, *p<0.05, ***p<0.0005, two-tailed *t* test. Anterior, top. Scale bar – 500 µm (A), 200 µm (B)

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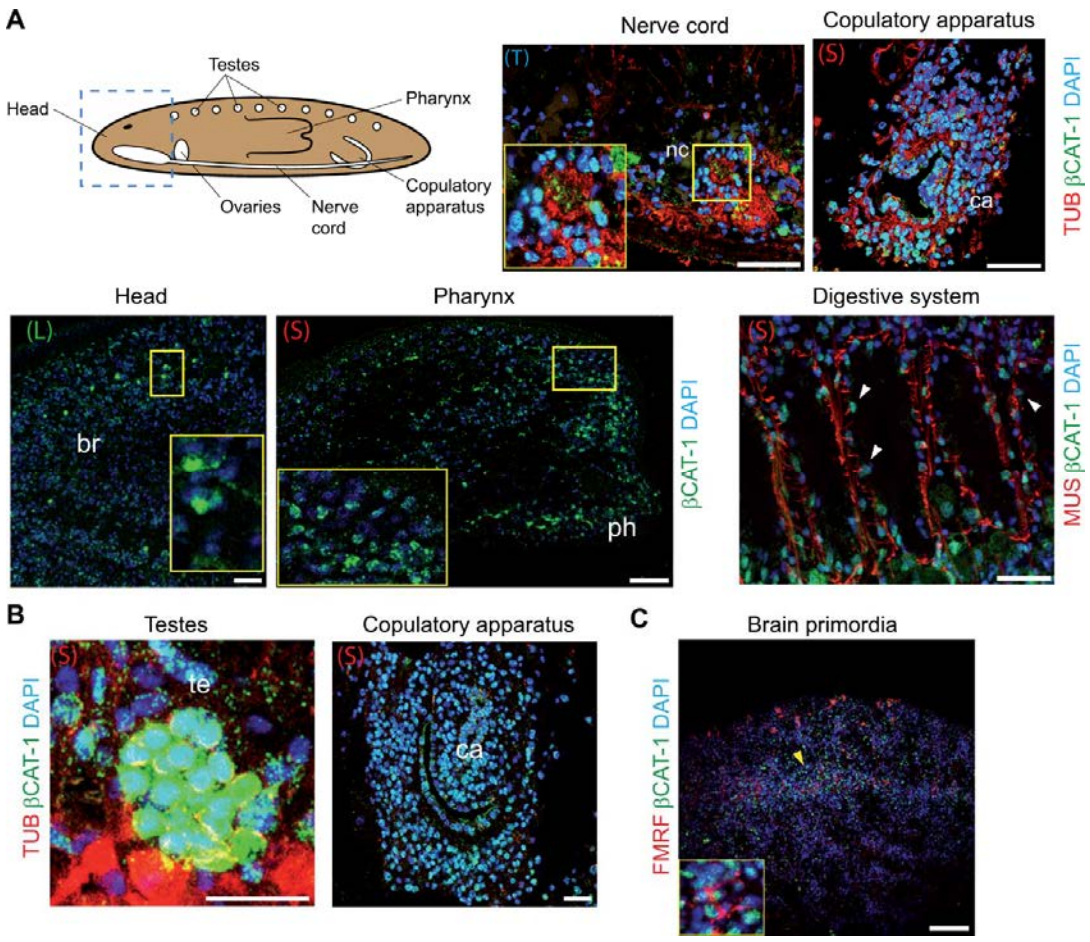

Supplementary figure 8. β-CATENIN-1 localization in different organs of uninjured and regenerating planarians (A) Immunostaining on sections of uninjured animals labeled with anti-βCAT-1 (green), anti-α-Tubulin (red) or anti-MHC-A 6G10 (red) and DAPI (blue). Yellow squares indicate images shown at higher magnification. Arrowhead indicates β-CATENIN-1 positive cells on the digestive system. (B) Immunostaining on sections of 3-day regenerating animals labeled with anti-βCAT-1 (green), anti-α-Tubulin (red) and DAPI (blue). (C) Immunostaining on whole-mount of a 6 day-old embryo (stage 5) with anti-βCAT-1 (green) and anti-FMRFamide (red). Nuclei are labeled with DAPI (blue). Yellow squares indicate images shown at higher magnification. br, brain; nc, nerve cord; ph, pharynx; te, testes; ca, copulatory apparatus. Scale bar – 50 µm (A), 20 µm (B), 100 µm (C).

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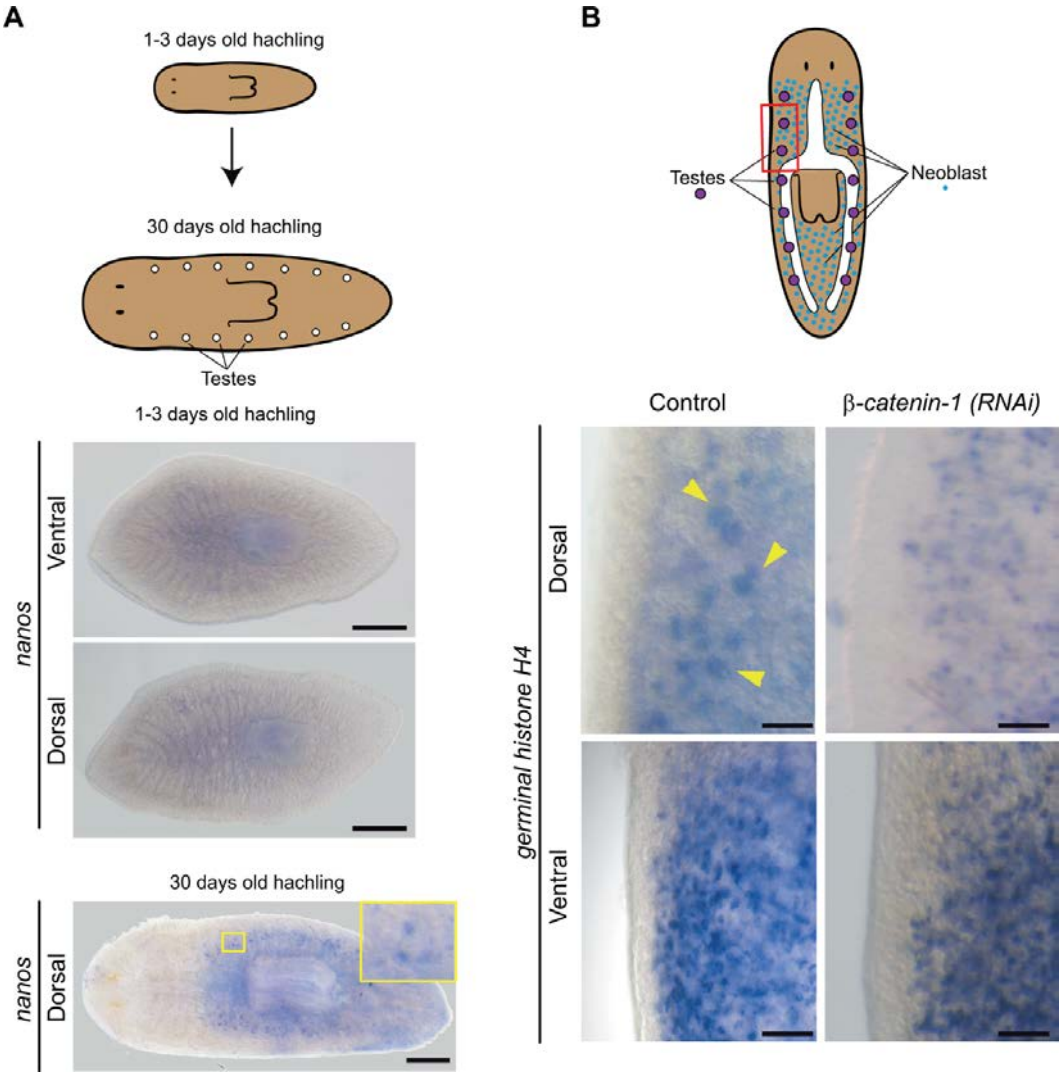

Supplementary figure 9. *nanos* expression in 1-3 day to 30 days old hatchlings and germinal histone H4 expression in controls and *β-catenin-1 (RNAi)* . (A) *In situ* hybridization of *nanos* showing that 1-3 day old hatchlings do not express this marker. 30 days old hatchlings show *nanos* in the dorsal part labeling the germ cells. Yellow square shows a magnification. Anterior, left. (B) Scheme representing the *germinal histone H4* expression pattern: it is expressed in testes (dorsal clusters, in purple) and neoblast (isolated cells in the parenchyma, in blue). Red square indicates the analyzed

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region. Analysis of the expression of *germinal histone H4* by whole-mount *in situ* hybridization in control and *β-catenin-1 (RNAi)* 4 week-old hatchling planarians shows that control animals present some clusters of testis labeled with *germinal histone H4* (yellow arrowhead) in the dorsal part (7/10) and neoblast labeling in the ventral part (10/10) while in *β-catenin-1 (RNAi)* animals *germinal histone H4* does not label testis clusters dorsally and only labels neoblasts in the dorsal and ventral part (10/10). Anterior, up. Scale bar – 200 µm (A), 50 µm (B).

Table S1. p-values corresponding to the analysis of the gradient using the Wilcoxon-signed-rank test.

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Regions along the AP axis

Regions	p-value	Adjusted p-value
1 vs 2	0.001953125	0.003645833
1 vs 3	0.001953125	0.003645833
1 vs 4	0.001953125	0.003645833
1 vs 5	0.001953125	0.003645833
1 vs 6	0.019531250	0.023777174
1 vs 7	0.193359375	0.208233173
1 vs 8	0.003906250	0.006076389
2 vs 3	0.001953125	0.003645833
2 vs 4	0.005859375	0.008634868
2 vs 5	0.019531250	0.023777174
2 vs 6	0.322265625	0.334201389
2 vs 7	0.009765625	0.013671875
2 vs 8	0.001953125	0.003645833
3 vs 4	0.695312500	0.695312500
3 vs 5	0.027343750	0.030625000
3 vs 6	0.003906250	0.006076389
3 vs 7	0.001953125	0.003645833
3 vs 8	0.001953125	0.003645833
4 vs 5	0.013671875	0.018229167
4 vs 6	0.003906250	0.006076389
4 vs 7	0.001953125	0.003645833
4 vs 8	0.001953125	0.003645833
5 vs 6	0.027343750	0.030625000
5 vs 7	0.001953125	0.003645833
5 vs 8	0.001953125	0.003645833
6 vs 7	0.001953125	0.003645833
6 vs 8	0.001953125	0.003645833
7 vs 8	0.001953125	0.003645833

Regions from the prepharynx to the tail

Regions	p-value	Adjusted p-value
1 vs 2	0.014266187	0.014266187
1 vs 3	0.001953125	0.002050781
1 vs 4	0.001953125	0.002050781
1 vs 5	0.001953125	0.002050781
1 vs 6	0.001953125	0.002050781
1 vs 7	0.001953125	0.002050781
2 vs 3	0.001953125	0.002050781
2 vs 4	0.001953125	0.002050781
2 vs 5	0.001953125	0.002050781
2 vs 6	0.001953125	0.002050781
2 vs 7	0.001953125	0.002050781
3 vs 4	0.001953125	0.002050781
3 vs 5	0.001953125	0.002050781
3 vs 6	0.001953125	0.002050781
3 vs 7	0.001953125	0.002050781
4 vs 5	0.001953125	0.002050781
4 vs 6	0.001953125	0.002050781
4 vs 7	0.001953125	0.002050781
5 vs 6	0.001953125	0.002050781
5 vs 7	0.001953125	0.002050781
6 vs 7	0.001953125	0.002050781

Articulo 3:

A C-terminally Truncated Form of β-catenin Acts as a Novel Regulator of Wnt/β-catenin Signaling in Planarians

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Resumen en castellano:

Una forma C-terminal truncada de β-catenina actúa como un nuevo regulador de la vía de señalización Wnt/β-catenina en planarias.

La β-catenina, el principal elemento de la vía Wnt/β-catenina, es una proteína multifuncional y conservada a lo largo de la evolución, la cual realiza funciones esenciales en diferentes procesos homeostáticos y del desarrollo. A pesar de su papel crucial, el mecanismo que regula su actividad dependiendo del contexto, es decir en el espacio y el tiempo, sigue siendo desconocido. La vía Wnt/β-catenina ha sido estudiada de forma extensa en las planarias, gusanos planos que poseen la habilidad de regenerar y remodelar el cuerpo entero, proporcionando un marco del desarrollo de un "animal completo" para abordar esta cuestión. En este articulo, hemos identificado una β-catenina truncada en la región C-terminal (β-catenina-4), generada por duplicación génica, la cual es necesaria para la especificación de las células fotoreceptoras de la planaria. Nuestros resultados indican que el rol de la β-catenina-4 es modular la actividad de la β-catenina-1 mediante su competición en la transducción de la señal de la vía Wnt en el núcleo, a través de competir por el factor de transcripción nuclear TCF-2. Esta forma dominante-negativa de β-catenina, expresada en tipos celulares específicos, proporciona un nuevo mecanismo para modular los niveles nucleares de β-catenina y su señalización. Análisis genómicos y ensayos "in vitro" sugieren que la existencia de una forma truncada en la región C-terminal de β-catenina podría ser un mecanismo conservado a lo largo de la evolución para lograr una regulación fina de la señal Wnt/β-catenina en contextos celulares específicos.

A C-terminally Truncated Form of β-catenin Acts as a Novel Regulator of Wnt/β-catenin Signaling in Planarians

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Keywords: β-catenin; Wnt signaling; genomic duplication; eye development; planarian

Short title: Regulation of Wnt signaling by a C-terminally truncated β-catenin

Abstract

β-Catenin, the core element of the Wnt/β-catenin pathway, is a multifunctional and evolutionarily conserved protein which performs essential roles in a variety of developmental and homeostatic processes. Despite its crucial roles, the mechanisms that control its context-specific functions in time and space remain largely unknown. The Wnt/β-catenin pathway has been extensively studied in planarians, flatworms with the ability to regenerate and remodel the whole body, providing a 'whole animal' developmental framework to approach this question. Here we identify a C-terminally truncated *β-catenin* (*β-catenin4*), generated by gene duplication, that is required for planarian photoreceptor cell specification. Our results indicate that the role of β-catenin4 is to modulate the activity of β-catenin1, the planarian β-catenin involved in Wnt signal transduction in the nucleus, through competition for binding to the nuclear transcription factor TCF-2. This dominant-negative form of β-catenin, expressed in specific cell types, would provide a novel mechanism to modulate nuclear β-catenin signaling levels. Genomic searches and *in vitro* analysis suggest that the existence of a C-terminally truncated form of β-catenin could be an evolutionarily conserved mechanism to achieve a fine-tuned regulation of Wnt/β-catenin signaling in specific cellular contexts.

Author summary

The Wnt signaling pathway is essential for proper intercellular communication in every developmental process since it controls basic cellular events as cell fate or proliferation. Thus, its malfunction leads to common diseases as cancer. The key element of the Wnt signaling is β-catenin, which controls the transcription of multiple genes in the Wnt receiving cell. A main level of regulation of the Wnt/β-catenin signaling occurs in the cytoplasm, where β-catenin protein levels depend on the activity of the β-catenin

destruction complex. However, once it reaches the nucleus β-catenin transcriptional activity requires a fine-tuned regulation to enable the multiple context-specific responses that it performs. These nuclear mechanisms that regulate the Wnt/β-catenin signaling remain poorly understood. Here we report the existence of C-terminal truncated forms of *β-catenin* in planarians (*β-cat3* and *4*), which do not show transactivation activity and compete with the canonical planarian *β-catenin* (*β-cat1*), thus acting as competitor inhibitors. Functional analysis in planarians indicates that β-cat4 acts as a negative regulator of nuclear β-cat1 in planarian eye photoreceptors and is essential for its proper specification. We provide evidence to suggest that this novel mechanism for the regulation of nuclear β-catenin activity could be conserved across animal evolution.

Introduction

The Wnt/β-catenin pathway is an evolutionarily conserved intercellular signaling pathway with essential roles in virtually every developmental process [1-3] and links to a wide range of human diseases [3-7]. Given its multiple, context-dependent roles, the pathway must be extensively regulated. A key element of this pathway is β-catenin, a bi-functional protein first discovered as a component of adherens junctions [8, 9]. β-catenin transduces the Wnt signal to the nucleus [5, 10] and is primarily regulated at the level of nuclearization. Binding of Wnts to their receptors (Frizzleds and LRP5/6) uncouples the β-catenin destruction complex (mainly composed of APC [adenomatous polyposis coli], Axin, CK-1 and GSK-3) and promotes β-catenin stabilization and its nuclear translocation [5, 11-14]. Inhibition of the ligand-receptor interaction through secreted inhibitory molecules (WIF, sFRP, DKK) also represents a common level of Wnt/β-catenin signal regulation [15-18]. Since β-catenin does not have a DNA binding domain, once it reaches the nucleus it must interact with a member of the DNA-binding T cell factor/lymphoid enhancer factor (TCF/LEF) family to regulate its downstream targets [19, 20]. Several β-catenin/TCF partners have been identified, which mainly target the C-terminal part of β-catenin, and which confer master regulatory properties to β-catenin since they are mainly involved in regulating chromatin structure and RNA polymerase II [21, 22]. Thus, the final activity of β-catenin relies not only on its nuclearization but also on its ability to bind to TCFs and their nuclear co-factors. Although an increasing number of factors have been reported to modulate the transcriptional activity of the β-catenin/TCF complex (e.g. ICAT, Groucho and Chibby) [20, 22-27], however, the regulation of β-catenin activity once it reaches the nucleus remains poorly understood.

Planarians, flatworms with an almost unlimited ability to regenerate and remodel their tissues during their whole life span [28-30], have become a robust model to study the function of Wnt/β-catenin signaling

in different developmental contexts [31-39]. Although most organisms have a single bi-functional β-catenin protein, gene duplication and functional specialization have led to the generation of two β-catenins in planarians: Smed-β-catenin1 (β-cat1) and Smed-β-catenin2 (β-cat2) [40]. β-cat1 is the intracellular effector of Wnt/β-catenin signaling but exerts no role in cell adhesion, whereas β-cat2 is exclusively found in cell-cell junctions [40]. This functional diversification provides a unique scenario in which to study the Wnt signaling properties of β-catenin. A functional specialization of β -catenins has also been found in the nematode *C. elegans*. However, their specific role in nuclear signaling appears extremely complex and divergent [41, 42]. Thus, although genetic tools to generate cells that exclusively lack canonical Wnt pathway activity have been reported in mouse [43], planarians represent a unique scenario in which to study the signaling properties of β-catenin in vivo without interference of the cell adhesion properties.

Functional analysis of *β-cat1* and the main elements of the Wnt/β-catenin signaling pathway demonstrate an essential role for this pathway in the specification of the antero-posterior (A-P) axis during planarian regeneration and homeostatic cell turnover [31-34, 44]. *β-cat1* silencing generates a range of anteriorized phenotypes, from “tailless” to “radial-like hypercephalized” planarians [32, 45]. Recently, novel functions for *β-cat1* have been reported in planarian brain and eye regeneration, and in gonad development [34, 36, 46]. Importantly, analysis of β-cat1 protein localization reveals that it is present in the nucleus of posterior cells, according to its role in A-P axial identity specification, and also in the main planarian tissues [45]. Thus, given that it has both activity- and context-dependent effects, nuclearization alone cannot account for its regulation. This makes planarians an excellent model to further understand how the transcriptional activity of β-catenin might be regulated once it is in the nucleus.

Here we report the existence of two new planarian β-catenins, *β-catenin3* (*β-cat3*) and *β-catenin4*

(*β-cat4*), which have a truncated C-terminal transactivation domain and are expressed primarily in the nervous system. β-cat3 and β-cat4 can bind to TCF but do not activate the Wnt signal in vitro. Functional analysis in planarians indicates that β-cat4 acts as a negative regulator of nuclear β-cat1 in planarian eye photoreceptors by competing for binding to TCF-2, a new TCF found in planarian photoreceptor cells. We provide evidence to suggest that this novel mechanism for the regulation of nuclear β-catenin activity could be conserved across animal evolution.

Results

Smed-β-catenin3 and 4 are two new β-catenin homologs

A search for *β-catenin* family members in the *Schmidtea mediterranea* transcriptomes revealed two new genes with protein sequences indicating that they were *β-catenin* homologs (S1 Fig). We named them *Smed-β-catenin3* (*β-cat3*) and *Smed-β-catenin4* (*β-cat4*), since two *β-catenin* paralogs had been already reported in this species [31-33, 40]. β-cat3 and 4 proteins conserve the GSK3 phosphorylation sites in the N-terminal region, and their armadillo repeats contain the interacting amino acids for multiple β-catenin-binding proteins, including APC, Axin, TCF and E-cadherin (Fig 1A and S1). The α-catenin binding sites are conserved in β-cat4 but not in β-cat3 (Fig 1A and S1). Importantly, the C-terminal transactivation domain, which interacts with crucial chromatin‐dependent factors, is lost in both β-catenins (Fig 1A and S1). The finding that the Wnt signaling domains but not the transactivation domain are conserved suggests that β-cat3 and 4 could function as dominant-negative forms of β-cat1, which is the β-catenin homolog involved in signaling to the nucleus in planarians [37, 40].

β-cat3 and 4 inhibit β-cat-dependent Wnt signaling *in vitro*

Since β-cat3 and 4 contain several conserved domains or residues involved in Wnt signaling and cell

adhesion, we further tested the interaction of β-cat3 and 4 with the main components involved in these two processes in mammalian cell lines. Co-immunoprecipitation experiments indicated that β-cat4 strongly interacts with the cell-cell adhesion elements E-cadherin and α-catenin (S2A Fig), whereas β-cat3 showed interaction with E-cadherin but not α-catenin (S2B Fig). This observation is consistent with the conservation of their functional protein domains (Fig 1A and S1). In this experiment, β-cat1 was used as a control, since it retains the essential residues for the interaction with E-cadherin, but not the conserved α-catenin binding domain [40]. Interestingly, both β-cat3 and 4 were able to interact with the elements involved in Wnt signaling, β-Trcp and TCF (Fig 1B). In this experiment, planarian β-cat2 was used as a control and, according to its reported role in cell adhesion but not in the Wnt cascade [40], it showed no binding to β-Trcp or TCF. Moreover, immunofluorescence assays revealed co-localization of β-cat3 and 4 with Axin, the core element of the β-catenin destruction complex, which is also consistent with their protein sequence analysis (Fig 1C).Thus, these results indicate that β-cat3 and 4 are under the control of the β-catenin destruction complex, and have the potential to bind to their nuclear co-factor TCF.

Considering the loss of their C-terminal transactivation domain, our results further support the hypothesis that β-cat3 and 4 could act as dominant negative forms of β-cat1. To test this hypothesis, we used the Super-TOPflash reporter system in HEK293T cells [47] to analyze the potential of β-cat3 and 4 to activate Wnt/β-catenin signaling. Whereas β-cat1 activated the reporter significantly, consistent with its reported role in Wnt signal transduction [40], β-cat3 and 4 had no effect on the reporter, even after increasing the dosages (Fig 1D). Consistent with its specific role in cell adhesion [40], the β-cat2 paralog was also not able to activate the Super-TOPflash reporter. Strikingly, when β-cat1 was co-transfected together with β-cat3 or 4, the levels of reporter activity decreased in a dose-dependent manner (Fig 1E). The same result was obtained when analyzing the axial induction capability of planarian *β-catenin*s in *Xenopus*

β-cat4 RNAi animals show highly reduced levels of *β-cat4* but not of *β-cat1, 2* and *3* mRNA (S3E Fig). Thus, we focused on the study of *β-cat4* function specifically in the eye.

Planarian eyes are simple structures comprising two main, well-characterized cell types: photoreceptors and pigment cells [50, 51]. The number of each cell type was quantified during regeneration of *β-cat4* (RNAi) planarians by analyzing the expression of *opsin* and *tph,* specific markers of photoreceptor and pigment cells, respectively [52] (Fig 2B and S4). Remarkably, RNAi of *β-cat4* resulted in reduced photoreceptor cells early in regeneration (R4d), whereas pigment cells did not show significant differences at this stage (Fig 2B and S4). As regeneration progressed, *β-cat4* (RNAi) animals showed a significantly reduced number of pigment cells compared to control (Fig 2B and S4), possibly due to a non-autonomous effect [53]. Consistent with the effects on photoreceptor cells, *β-cat4* (RNAi) animals did not show the proper negative phototaxis behavior (Fig 2C). When exposed to a light gradient, all control animals moved away from the light and remained in the darkest zone (zone 3). Conversely, although *β-cat4* (RNAi) organisms seemed to move normally, most of them remained in the clearest zone and did not reach the darkest zone in the same time period (Fig 2C and Supplemental movies 1 and 2). These data show that *β-cat4* silencing causes a reduction of photoreceptor cells, followed by a reduction of pigment cells, and influences their normal behavioral responses to light, suggesting its role in photoreceptor specification.

Since planarians continuously remodel their tissues [28], we analyzed whether *β-cat4* is also required for eye cell maintenance during normal planarian homeostasis. Injection of *β-cat4* dsRNA over a period of 5 weeks produced a decrease in eye size and in the photoreceptor area (S5A Fig). Quantification of photoreceptor and pigment cells through *opsin* and *tph* FISH over the 5 weeks of the experiment revealed that *β-cat4* (RNAi) animals always have fewer photoreceptor cells (S5A Fig). The gradual reduction of

photoreceptor cell number observed in control animals (S5A Fig) is due to shrink age of the animals, which remained starved over the 5-week period. According to the phenotype, *β-cat4* (RNAi) animals showed a defective negative phototaxis response that got worse as the experiment progressed (S5B Fig). Thus, these data demonstrate that *β-cat4* is required for photoreceptor maintenance during homeostasis.

Planarian photoreceptor and pigment cells differentiate from progenitor cells that are located as a trail of cells extending caudally from the eye and express the pan-eye marker *ovo* [54]. A small number of eye progenitor cells co-express *ovo* with stem-cell specific markers (*h2b*) and correspond to the specialized eye stem-cells [54]. Eye stem cells acquire the expression of specific determinants that direct their final fate to photoreceptor or pigment cells [52, 55]. In order to understand the mechanism by which *β-cat4* influences eye regeneration, *β-cat4* expression was further studied with FISH. Besides its expression in eye photoreceptor cells, *β-cat4* was found to be expressed in the trail of eye precursors posterior to the eye both in intact animals and during regeneration (Fig 2D and S5C). Double FISH analysis of *β-cat4* with *ovo* and *sp6-9,* a pigment cell determinant, revealed that *β-cat4* is exclusively expressed in photoreceptor but not pigment-cell progenitors, since it was always co-expressed with the eye marker *ovo* but never with the pigment-specific marker *sp6-9* (Fig 2E and S5D). In addition, a few isolated *β-cat4*+ cells were also found to be *h2b*+ (Fig 2F), indicating that *β-cat4* is already expressed in the eye stem cell.

To test whether *β-cat4* is required for specification of photoreceptor cells from the common eye stem cell precursor, we quantified the number of photoreceptor and pigment-cell progenitors in the eye trail of *β-cat4* (RNAi) animals using the specific markers *otxA* and *sp6-9*, which label differentiating photoreceptor and pigment cells, respectively [52, 54, 55]. As expected, *β-cat4* (RNAi) animals had a reduced number of *otxA*+ cells in the eye from early regeneration stage and a later decrease in *sp6-9*+ cells (S6 Fig). Importantly, the same result was observed when quantifying *otxA*+ and *sp6-9*+ cells in the trail; *β-cat4*

(RNAi) resulted in failure of photoreceptor progenitor-cell specification from the early regeneration stage (R3d), whereas pigmented cells appeared reduced at a later stage (R7d) (Fig 2G and S6). Thus, *β-cat4* is required for photoreceptor progenitor-cell specification from the common eye stem cell (Fig 2H).

β-cat4 specifies photoreceptor cells through _β-cat1_ inhibition

Considering that β-cat4 inhibits TCF-mediated β-cat1 activity in cell cultures and that it has lost the C-terminal transactivation domain, β-cat4 could inhibit Wnt signaling in photoreceptors by competing with β-cat1 for TCF binding in the nucleus. To test whether this molecular mechanism could be functional in planarians, we first analyzed *β-cat1* and *β-cat4* expression in the planarian eye field. FISH for *β-cat1* followed by immunostaining of VC-1, which labels the rhabdomeres of photoreceptor cells [52, 56, 57], showed that *β-cat1* was expressed in regenerating photoreceptor cells (S7A Fig). Moreover, double FISH for both *β-cat1* and *β-cat4* mRNA revealed that both are found in photoreceptor cells (S7A Fig). Importantly, immunostaining with a β-cat1-specific antibody [45] revealed that it is localized in the nucleus of photoreceptor cells in *S. polychroa*, the sister species of *S. mediterranea* (Fig 3A). Using a specific antibody generated in this study (S7B Fig) we could demonstrate that β-cat4 is also localized in the nucleus of photoreceptors (Fig 3A). Thus, our results show that both β-cat1 and β-cat4 are localized in the nucleus of photoreceptor cells, which is consistent with their nuclear interaction.

Next, we performed RNAi experiments to analyze the functional relationship between the two *β-catenin*s. Planarians were decapitated after *β-cat1* dsRNA injection and allowed to regenerate. The efficiency of the inhibition was tested by qPCR (S7C Fig). Newly formed heads showed “slanted eyes” with a very thin and elongated periglobular unpigmented epidermis and pigmented cup (Fig 3B and Supplemental movies 3 and 4).The observed phenotype is very similar to that reported for *β-cat1* (RNAi) [38]. FISH with eye-specific markers confirmed that *β-cat1* (RNAi) led to a disordered eye structure, in

which photoreceptor and pigment cells formed larger eyes and in which ectopic eye cells appeared (S7D Fig). Quantification of *opsin*+ and *tph*+ cells present in the eye structure (S7D Fig) showed an increase with respect to control animals (Fig 3B). This result is the opposite of that found in *β-cat4* (RNAi) planarians, which had a decrease in the number of photoreceptor cells, thus supporting the opposing role of these *β-catenin*s.

Since techniques for overexpression are currently unavailable in planarians, we took an indirect approach to up-regulate β-cat1 through silencing *APC-1*, the *APC* homolog in planarians [31, 34]. *APC-1* (RNAi) leads to β-cat1 up-regulation and nuclear accumulation [45], resulting in the regeneration of a tail at anterior wounds [31, 34, 45]. In order to analyze the eye field in *APC-1*-knockdown planarians, we performed RNAi experiments in intact animals, since it is known that *APC-1* silencing during two weeks does not lead to tail-head transformation [45]. We injected dsRNA for *APC-1*, *β-cat4*, and *APC-1*;*β-cat4* for 2 weeks. The efficiency of the inhibition was analyzed by qPCR (S7E Fig). As expected, *β-cat4* (RNAi) caused smaller eyes with a decrease in both photoreceptor and pigment cell numbers compared to controls (Fig 3C). Importantly, *APC-1* (RNAi) generated the same phenotype (smaller eyes with fewer photoreceptor and pigment cells) (Fig 3C). Co-silencing *APC-1* and *β*-cat4 caused an even more severe decrease in the number of photoreceptor cells (Fig 3C). This last result is consistent with the hypothesis that the truncated *β*-cat4 competes with *β*-cat1 in the nucleus inhibiting its transcriptional activity. However, it should also be considered that, since both *β*-catenins seem to be regulated by the destruction complex, in *APC* RNAi animals not only *β*-cat1 but also *β*-cat4 could be stabilized.

Overall, *β-cat1* gain of function through *APC-1* (RNAi) results in the same phenotype of diminished eye cell numbers as *β-cat4* (RNAi), whereas *β-cat1* loss of function causes the opposite effect. Thus, β-cat1, as a key downstream transcriptional co-activator in Wnt signaling, plays a negative role in planarian

photoreceptor development. Our results indicate that the activity of β-cat1 in the eyes is not only controlled by the elements of the β-catenin destruction complex, like APC-1, but also by β-cat4, which could act as a competitor of β-cat1 in the nucleus of photoreceptor cells and their precursors.

Smed-TCF-2 mediates *β-cat4* and *β-cat1* activity during photoreceptor differentiation

To gain further insight into the hypothesis that β-cat4 specifies photoreceptor differentiation by competitive inhibition of β-cat1, we searched for the TCF transcription factor that acts as a competitive target to modulate photoreceptor differentiation. Although most invertebrate genomes contain a single TCF/LEF ortholog, we identified three TCF orthologs (Smed-TCF-1 to -3) in the *S. mediterranea* transcriptome database Planmine [58] (S8A Fig). The corresponding homologs were found in five more planarian species in the same database (S8A Fig). The phylogenetic analysis suggests that the duplications found in planarians arise from Platyhelmintes and are independent of the vertebrate TCF expansion (S8A Fig). Protein sequence analysis of the three *S. mediterranea* TCFs demonstrated that TCF-2 is the only *S. mediterranea* TCF that conserves all functional domains required to bind to β-catenin, Groucho and DNA (Fig 4A and S9) [59]. TCF-1 has lost the β-catenin binding domain and TCF-3 does not conserve the Groucho binding sites (S9 Fig). Analysis of their expression pattern in planarians showed that *TCF-1* was expressed specifically in planarian brain, as previously reported [60] (S8B Fig). *TCF-2* and *-3* are also mainly expressed in the CNS and, importantly, *TCF-2* is found in photoreceptors (Fig 4B), strongly resembling the *β-cat4* expression pattern. Thus, TCF-2 is a candidate to function as a β-cat1 and β-cat4 target during photoreceptor development.

The eyes of *TCF-2* RNAi animals were analyzed to understand its possible function. The efficiency of the inhibition was analyzed by qPCR (S8C Fig). Analysis of *TCF-2* RNAi regenerating animals revealed that their eyes were bigger than in controls (Fig 4C and S8D). The brain of *TCF-2* RNAi animals appeared

normal (S8E Fig), which suggests that the larger eye phenotype is eye specific. Quantification of the different eye populations demonstrated that *TCF-2* silencing results in an increased number of photoreceptor cells (Fig 4C and S8D). The number of pigment cells also increased (Fig 4C and S8D), probably due to the cellular relationship between the two compartments, as shown earlier in *β-cat4* RNAi planarians. The *TCF-2* (RNAi) phenotype in the eyes phenocopies the *β-cat1* (RNAi) phenotype with respect to the number of photoreceptor cells and is consistent with our hypothesis that Wnt/β-cat1 signal inhibition is required for correct planarian photoreceptor specification. To note, *TCF-2* RNAi animals showed bigger eyes but not the appearance of ectopic eye cells, suggesting that this defect is caused in a *TCF-2* independent manner.

To analyze whether *β-cat4* function in photoreceptor specification also depends on *TCF-2*, we performed a double RNAi assay to inhibit *β-cat4* and *TCF-2* simultaneously during planarian regeneration. The efficiency of the inhibition was tested by qPCR (S8F Fig). As expected, *β-cat4* (RNAi) planarians had smaller eyes with a reduced number of photoreceptor cells, whereas *TCF-2* (RNAi) resulted in larger eyes with an increased number of photoreceptor cells. Remarkably, the size of the eyes in double *β-cat4* and *TCF-2* RNAi animals, and the number of photoreceptor and pigment cells, resembled the phenotype observed with *TCF-2* (RNAi) alone (Fig 4D). This observation is consistent with a role for TCF-2 as the transcription factor downstream of β-cat4. Above all, the data suggest a model in which β-cat4, which lacks the C-terminal transactivation domain, competes with β-cat1 to bind to TCF-2 and modulates Wnt signaling to an appropriate level for the correct photoreceptor cell differentiation (Fig 5).

Plakoglobin and Neural Arm act as β-catenin and Arm inhibitors, respectively, in vitro

To determine whether competitive inhibition of TCF is a conserved mechanism for the regulation of Wnt

signaling, we investigated the existence of C-terminally truncated β-catenins in other organisms. The existence of *β-catenin* paralogs is not exclusive to planarians. The *β-catenin* family has undergone a vertebrate-specific subphylum duplication (*β-catenin* and *plakoglobin*) [61], two nematode-specific phylum duplications (4 *β-catenins* in *C. elegans*) [41], and multiple species-specific duplications in the Arthropoda phylum [61] (S10 Fig). Phylogenetic analysis of the *β-catenin* family members of different Lophotrocozoa species shows the existence of a unique bi-functional *β-catenin* in all of them except for Platyhelmintes (S10 Fig). The analysis suggests that *β-catenin* underwent two phylum-specific duplications in Platyhelmintes to generate *β-cat1*, *2* and *3/4* classes, and that in Triclads a third duplication produced the *β-catenin3* and 4 orthologs (S10 Fig). Furthermore, a genus-specific duplication occurred in the *β-cat3* class (S10 Fig). Thus, a *β-cat3*/4 ortholog that retains the signaling domains but not the C-terminal transactivation domain (S11 Fig) exists in all Plathyhelminthes.

Taking into account the number of β-catenin duplications found across evolution, we hypothesized that the existence of an inhibitory β-catenin to regulate β-catenin-dependent Wnt signaling could be a common mechanism throughout evolution as a result of convergent evolution. A protein sequence analysis of different *β-catenin* orthologs found across the animal phyla shows the shortening of the C-terminal end in several cases, for instance in one of the two β-catenins found in the sponge *A. quensatlantica* or the vertebrate β-catenin duplication Plakoglobin (S11 Fig). Although Plakoglobin shows a high protein sequence conservation with β-catenin in the central armadillo repeats, and shares the binding domains for α-catenin, Cadherins and TCF, it has a very low amino acid sequence conservation in the C-terminal transcriptional transactivation domain (15%) (Fig 6A) [62]. Accordingly, it has been shown that Plakoglobin has limited transactivation ability compared to β-catenin [63]. Furthermore, although a unique *β-catenin* is found in the genome of *D. melanogaster (Armadillo, Arm)*, a C-terminally truncated

Arm named *Neural Armadillo* (*NArm*) has been reported to occur through alternative splicing [64] (Fig 6B and S11). It is known that *NArm* is expressed in the brain from larval stage [64] but no functional studies have been reported. To analyze whether vertebrate Plakoglobin or *Drosophila* NArm could have an inhibitory function, we designed *in vitro* Super-TOPflash experiments. Results showed that while β-catenin (S37A), a stabilized β-catenin that cannot be captured by the cytoplasmic destruction complex, could highly activate Wnt signaling, Plakoglobin activity was very low (Fig 6A). Interestingly, Plakoglobin co-transfection with β-catenin (S37A) decreased the reporter signal in a dose-dependent manner (Fig 6A). Similarly, activation of Wnt signaling by NArm was very weak compared to that induced by Arm. Importantly, co-transfection of both plasmids showed that NArm suppresses the Arm induced TCF-reporter activity in dosage dependent manner (Fig 6B).

Our results demonstrate that Plakoglobin and NArm inhibit the Wnt signal activated by β-catenin/Arm through a TCF transcription factor. This result, together with the presence of β-catenin paralogs in several species, indicates that the existence of an inhibitory β-catenin could be a conserved mechanism to fine tune β-catenin-dependent Wnt signaling. The phylogenetic relationship between the β-catenin family members and the existence of splice variants indicates that the inhibitory form of β-catenin would not have evolved from a common ancestor but appeared during evolution as a product of unrelated events (species- or phylum-specific genome duplications or alternative splicing), thus representing an example of convergent evolution.

Discussion

Here we show a new role for the Wnt/β-catenin pathway in planarian eyes and demonstrate the existence of a novel mechanism to regulate its activity in a context-specific manner. We have identified *β-cat4*, a C-terminally truncated *β-catenin* generated by gene duplication within the planarian group, and we

demonstrate that it modulates β-cat1 activity during eye photoreceptor cells specification but not during axial patterning. Wnt/β-catenin activity is modulated through a novel mechanism in which β-cat1 and β-cat4, which lacks the transactivation domain, compete for binding to the TCF-2 transcription factor. The existence of this new Wnt/β-catenin activity regulatory mechanism would ensure the appropriate fine-tuning of nuclear β-catenin/TCF activity in specific cell types. Genome searches and *in vitro* assays indicate that use of an inhibitory form of β-catenin could be a conserved mechanism to regulate β-catenin activity in specific contexts.

The inhibitory role of the C-terminally truncated planarian β-catenin4 represents a novel mechanism for modulation of nuclear β-catenin activity.

The multiple roles and complexity of the Wnt/β-catenin signaling necessitate regulation at different levels. Extracellularly, secreted proteins interact with Wnts or their receptors (e.g. DKK1, SFRP, WIF1, notum) to inhibit the ligand-receptor interaction [65, 66], and in the cytoplasm, regulation of the β-catenin destruction complex determines the amount of β-catenin that will escape phosphorylation and degradation and reach the nucleus [5]. However, the modulation of β-catenin activity once reaches the nucleus remains poorly understood. We used planarians to approach this question since, although β-cat1 exerts multiple functions, it is primarily localized to the nucleus [31-33, 36, 44-46, 48], suggesting that mechanisms must be available to modulate β-cat1 nuclear activity.

Here we found two new β-catenins (β-cat3 and 4) in planarians, after the two β-catenins (β-cat1 and 2) already described [40], which 1) showed a C-terminal truncated transactivation domain, while conserving the TCF binding amino acid residues, and 2) were mainly expressed in the nervous tissues. Those findings lead to hypothesize that β-cat3 and 4 could be acting as inhibitors of the canonical β-cat1 in the nucleus and in a tissue-specific manner. Indeed, we could demonstrate that *β-cat4* is required for

normal specification of photoreceptors from the common eye stem cell and that its function relies on the inhibition of β-cat1 activity. Importantly, *β-cat4* has no role in A-P axial polarity, in contrast to *β-cat1.*

Our RNAi experiments indicate that inhibition of *β-cat4* or *APC*, which in planarians is demonstrated to increase β-cat1 activity [31, 34, 45], produces a decrease in the number of photoreceptor cells, whereas inhibition of *β-cat1* produces the opposite phenotype. Furthermore, our results strongly indicate that the inhibition of β-cat1 by β-cat4 takes place in the nucleus, and that it is achieved by the competition of β-cat4 for the β-cat1 binding to the TCF-2 transcription factor, the TCF factor identified in the present study. Two results support this model: 1) the localization of both β-cat1 and β-cat4 in the nucleus of photoreceptor cells (although β-cat1 expression analysis was performed in a *S. mediterranea* sister species due to technical limitations [45]; and 2) the finding that *TCF-2* RNAi inhibition leads to a decrease in the number of photoreceptor cells, as observed after *β-cat1* RNAi. Furthermore, the result showing that RNAi inhibition of *β-cat4* together with *TCF-2* leads to the same phenotype as *TCF-2* RNAi alone strongly supports that TCF-2 is the downstream effector of the β-cat4 action. Although we have not directly demonstrated the binding of β-cat1 or β-cat4 to TCF-2, we do demonstrate that both planarian β-catenins conserve the TCF binding sites and bind to TCF-1 in co-immunoprecipitation experiments. Furthermore, the '*in vitro*' TOP-flash assays demonstrate that β-cat1 and β-cat4 compete for binding to TCF-1 and that β-cat4 inhibits the TCF mediated β-cat1 transcriptional activity. Thus, although we cannot rule out the possibility that β-cat4 and β-cat1 could also interact in the cytoplasm, since β-cat4 also conserves the important domains to interact with cytosolic β-catenin destruction elements (GSK3 phosphorylation and Axin binding sites), our results strongly support the competition of β-cat1 and β-cat4 at nuclear level. Altogether, our data supports the existence in planarians of a novel level of Wnt/β-catenin signaling modulation in the nucleus through the action of β-catenins with different transactivation capacity.

Competition of those different β-catenins for TCFs binding would result in the modulation of the transcriptional rates of downstream Wnt target genes and Wnt-responsive activities. It also exist the possibility that β-cat4/TCF-2 could exert a direct repressive transcriptional activity through an alternative TCF-binding domain, as described in *Drosophila* [67, 68]. In this case, the binding of β-cat4 to TCF-2 would lead to the direct repression of specific targets, instead to the inhibition of β-cat1 targets. Although the result showing that *TCF-2* RNAi and *β-cat1* RNAi produce a similar increase in the number of photoreceptors, supporting the hypothesis that β-cat4 main function is to inhibit β-cat1 targets, the analysis of the specific targets would be required to solve this question.

Regulation of β-catenin/TCF transcriptional activity is achieved through nuclear factors that regulate β-catenin binding properties (e.g. Chibby and ICAT) [20, 22-27], or regulation of TCF transcriptional and binding properties [20, 22-27]. Accordingly, the existence of TCFs that lack the β-catenin-binding domain and act as endogenous dominant negative forms [59], and also post-translational modifications of TCFs that modulate their activity have been extensively reported [69-74]. The C-terminal truncated inhibitory β-cat4 found in this study represents a novel mechanism to modulate β-catenin/TCF activity that also targets to TCF. As reported for the endogenous dominant negative forms of TCFs, in which alternative splicing and alternative promoters leads to the expansion of the isoforms [59], this level of regulation would allow the refinement of Wnt signaling in time and space.

A C-terminal truncated form of β-catenin could be an evolutionarily conserved mechanism to achieve the multiple context-specific roles of Wnt/β-catenin signaling

Here we have found a new planarian β-catenin (β-cat4) which represents a novel mechanism to fine tune the activity of nuclear β-catenin in a context-specific manner. Not surprisingly, this mechanism acts in

neural tissues, where *β-cat3* and *4* are mainly expressed, the complexity of which requires a more sophisticated regulation. Phylogenetic analysis of the β-catenin family in platyhelminthes supports the hypothesis that a *β-cat1*, *β-cat2* and *β-cat3/4* ortholog was present in their common ancestor. Although the expression pattern and functional analysis of the *β-cat3/4* ortholog in other platyhelminth species should be done, our data predicts that the *β-cat3/4* ortholog would modulate β-cat1 activity in Platyhelminthes.

Importantly, although the presence of a unique *β-catenin* with dual adhesion and signaling functions has been proposed to be present in the last common animal ancestor [75], genomic duplications in the *β-catenin* family are more common than previously thought. In vertebrates, *plakoglobin* is a genomic duplication of *β-catenin* that has suffered a functional specialization, since it is predominantly involved in desmosomes [76, 77]. However, Plakoglobin shows limited capacity to activate Wnt signaling [63, 78] and, importantly, its nuclear accumulation down-regulates β-catenin activity in a dose-dependent manner, as demonstrated by our TOPflash reporter assays (Fig 6A). Thus, Plakoglobin could be acting as a competitor of β-catenin in vertebrates. Since Plakoglobin shows much lower transactivation properties than β-catenin, its presence results in a decrease of the transcriptional activation promoted by β-catenin. The real existence and the importance of this regulatory mechanism is further supported by recent studies demonstrating that an increase of Plakoglobin nuclear translocation is associated with diseases such as arrhythmogenic right ventricular cardiomyopathy or head neck cancer and leads to a suppression of the β-catenin mediated TCF/LEF transcriptional activity [79, 80]. Taking into account that the C-terminal part of Plakoglobin is the one showing less conservation with respect to β-catenin (Fig 6A), the inhibitory function could be associated with the lack of conservation of the transactivation domain, as described in the present study for planarian β-cat4.

In invertebrates, the *β-catenin* family has undergone several species- and phylum-specific duplications [81]. The nematode *C. elegans* has four β-catenins with different roles in cell adhesion and nuclear signaling [42], and several insects have two β-catenins, which have also undergone partial subfunctionalization between the cell adhesion and the centrosome separation functions [81]. Importantly, although in *Drosophila* a unique *β-catenin* is found (*Arm*), an alternative splicing which occurs mainly in neural cells has been reported, which is known as *Neural Armadillo* (*NArm*) [81]. Interestingly, this alternative splicing deletes exon 6 and results in a C-terminal truncated Arm isoform that, according to our TOPflash reporter assay, exerts an inhibitory role of Arm in a dose dependent manner, as described for planarian *β-cat4* towards *β-cat1*. Although the role of NArm as modulator of Arm should be demonstrated '*in vivo*', it should be stressed that the presence of the alternative splicing isoform deleting exon 6 is conserved across all insect homologs [81], supporting the evolutionarily pressure to maintain it and thus its biological implications. Overall, the present data supports the hypothesis that modulation of nuclear β-catenin activity through competition for TCF binding by a C-terminally truncated β-catenin could be a conserved mechanism to regulate the Wnt/β-catenin pathway. Interestingly, the C-terminally truncated *β-catenin* of different species did not arise from any homolog of a common ancestor but originated in different animal groups from different mechanisms of gene diversification, as gene duplication or alternative splicing. Thus, the presence of a β-catenin with limited transactivation activity, which could compete with the canonical β-catenin, in different species represents an example of convergent evolution and supports its importance as a regulatory mechanism.

The existence of inhibitory β-catenins and its expression in specific cell types provides a new answer to the important question of how the same transcription factor elicits so distinct responses. It is assumed that the different transcriptomic/proteomic context of each cell could result in differential activation of

β-catenin target genes. However, few tissue-specific Wnt/β-catenin regulators have been found, leading to the idea that not only Wnt/β-catenin regulators but the interplay with the other conserved signaling pathways as BMP would be essential for their complex regulation [82]. Our results provide evidences that the existence of inhibitory β-catenins is not only a new mechanism to regulate Wnt signaling but to modulate it in specific cell types, for instances in specific subsets of neuronal types.

Eye development and Wnt/β-catenin signaling

Our results demonstrate that *β-cat4* is a new essential element for photoreceptor cell specification in planarians. Planarian eyes are true cerebral eyes and their simplicity makes them an excellent system to study eye development. They are composed by two cell types: rhabdomeric photoreceptor neurons, which express evolutionarily conserved photoreceptor genes such as *otxA* or *opsin* [49, 55], and pigment cells, which express *Tryptophan hydroxylase (tph)*, an enzyme involved in the production of melanin, the pigment found in planarian eyes. Several studies demonstrate that both cell types arise from a common eye stem cell that express stem cell markers (*h2b*) and eye determinants as the transcription factors *ovo*, *eya* and *six1/2* [54, 55, 83]. Among them *ovo* is the only factor exclusively found in the eye cell and specifically essential for eye regeneration and maintenance [54]. The expression of *sp6-9* and *dlx* in the common eye progenitor determines the pigment fate, while *otxA* specifies photoreceptors [52, 55]. We demonstrate that *β-cat4* is a new factor that specifies the photoreceptor fate and that it is expressed in the stem cell precursors (*h2b+/ovo+*), a fact that has not been yet demonstrated for *otxA*. Inhibition of *β-cat4* results in a decrease in number of photoreceptor progenitor and differentiated cells from the earliest time points analyzed (3-4 days of regeneration), while the number of progenitors and differentiated cells of the pigment lineage only decrease several days after. This observation agrees with the already described mutual dependence of both cell populations in planarians [53]. Thus, although *β-cat4* is specifically

required for photoreceptor determination, according to its expression in the photoreceptor lineage, the reduced number of photoreceptor cells would lead to the subsequent decrease of the pigment lineage. Whether the origin of this mutual dependence is at the level of the common stem cell progenitors, or at the level of the differentiated cells in the optic cup, remains to be studied. Although not much is known about the mutual dependence between photoreceptor and pigment cells in vertebrates, some studies suggest that it also exists [84].

To date, the main focus on the role of the Wnt/β-catenin signaling pathway in planarians has been in its essential role for axial patterning and posterior identity specification [31-33]. Our results highlight the importance of the regulation of the Wnt/β-catenin pathway in a different context, namely during planarian photoreceptors regeneration and maintenance. The finding is not surprising, since appropriate Wnt/β-catenin signaling levels are required for retina progenitor differentiation in several animal models [85-90]. For example, during chicken embryo development, β-catenin overexpression in the central neural retina inhibits the differentiation of retinal neurons, while loss of β-catenin leads to neural retina enlargement [86]. In zebrafish the ectopic expression of the Wnt antagonists DKK1 or SFRP1 causes expansion of the embryonic retina [91, 92], whereas activation of the Wnt/β-catenin pathway leads to lack of expression of eye markers [93]. Here we show that in planarians *β-cat1* inhibition results in larger eyes, while *APC-1* silencing causes a reduction of photoreceptor cell number. The simplicity and approachability of planarian eyes has allowed the identification of a new mechanism to fine regulate Wnt/β-catenin pathway activity in the eyes. Thus, the truncated β-cat4 that competes with the canonical β-cat1 for binding to TCF-2 allows the required fine-tuning of the Wnt/β-catenin pathway in planarian photoreceptor cells. Since the *in vitro* experiments showed that β-cat4 could bind to E-cadherin and α-catenin, a role of β-cat4 in cell adhesion should be considered. However, β-cat4 protein was only found in the nucleus of

photoreceptors (Fig 3A), and *β-cat4* RNAi eyes appeared properly patterned with no apparent defects in cell adhesion.

The new described mechanism to fine-tune the Wnt/β-catenin signal in specific cell types implies the existence of Wnt elements specifically expressed in photoreceptor and not in pigment cells. The finding that the phenotype of *βcat1* RNAi animals, which show ectopic differentiation of eye cells, differs from the *TCF-2* RNAi, which show bigger eyes but properly patterned, indicates that β-cat1 could exert a TCF-2 independent role during eye regeneration. The cause of this defect could be a non-autonomous role of β-cat1, which is broadly expressed in planarians [45], or a role in pigment cells. This finding emphasizes the requirement of a cell-specific regulation of the Wnt/β-catenin signal during development. It will be interesting to study whether in other animal species eye-population specific Wnt elements (or isoforms) are found and to investigate if this novel mechanism of β-catenin regulation also takes place during eye development.

Conclusions

Our finding that planarian β-cat4 functions as a modulator of β-cat1 activity in the nucleus by competing for TCF-2 binding has two deep implications: 1) considering its specific role in modulating β-cat1 activity during planarian photoreceptor specification, and not in other Wnt/β-catenin dependent processes such as axial patterning, it appears as a tissue-specific manner to fine-tune nuclear β-catenin activity; and 2) the finding of C-terminal truncated/non-conserved β-catenin forms in other species (NArm or Plakoglobin), which inhibit β-catenin activity in TOPflash experiments, strongly suggests that an inhibitory β-catenin could be an evolutionarily conserved mechanism to regulate the Wnt/β-catenin signaling. The identification of a novel mechanism of Wnt/β-catenin signaling regulation has important implications in

view of the complex and essential roles of this pathway in development and diseases. Thus, the present research represents a starting point to design further studies: 1) to demonstrate whether the C-terminal truncated/non-conserved β-catenins found in *Drosophila* (NArm) and vertebrates (Plakoglobin) can compete for Arm or β-catenin binding, respectively, to the TCF co-factor 'in vivo' and assess whether this mechanism provides a tissue-specific manner to modulate β-catenin/Arm once reaches the nucleus; 2) to study the existence and function of possible β-catenin isoforms present in other animal species, as a product of gene duplication or alternative splicing, and understand their real contribution to the regulation of the Wnt/β-catenin signal; and 3) to test whether new drugs mimicking the inhibitory action of β-cat4 could be used to fine tune the activity of nuclear β-catenin in human diseases.

Materials and Methods

Planarians

The planarians used in this study belong to an asexual clonal strain of *S. mediterranea* BCN-10 biotype. The animals were maintained at 20℃ in PAM (1X) water [94]. Animals were fed with organic veal liver and starved for at least a week before all experiments. A sexual strain of *S. polychroa* collected from Sant Celoni (Barcelona, Spain) was used in Fig 3A.

Gene identification and phylogenetic analysis

β-Catenin sequences from Planarian species were identified in the Planmine database [58]. *β-Catenin* sequences from *Echinococcus multilocularis* and *Kronborgia cf. amphipodicola* were found in the available databases [95, 96]. The rest of the sequences were found in the NCBI. β-Catenin protein sequences from different species were aligned using the MAFFT server (http://mafft.cbrc.jp/alignment/server/). Neighbor joining distance-based analyses were conducted using

MEGA version 6 [97], and the support given by bootstrap percentiles of 1000 replicates. BioEdit was used to edit the protein alignments.

Luciferase reporter assay

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. To obtain pCS2+-6Myc-Smed-β-catenin3 and 4, the full length Smed-β-catenin3 and 4 was amplified by PCR and inserted into pCS2+-6Myc vector at StuI/XbaI sites. HEK293T cells were seeded in 96-well plates and transfected in triplicates with pCS2+-6Myc-Smed-β-catenin1/2/3/4 plasmids, together with Super-TOPflash and pRL-TK as the internal control. Firefly and Renilla luciferase activities were measured 36h after transfection using the Dual-Luciferase assay kit (Promega). TOPflash luciferase activity was normalized to that of Renilla. 15 ng of Super-TOPflash and 0.5 ng of pRL-TK reporter plasmids were added per well. pCS2+ empty vector was used to adjust the total DNA amount to 150 ng/well. All experiments were repeated at least three times.

Immunofluorescence in cell cultures

HeLa cells were cultured in DMEM with 10% fetal calf serum and grown on glass coverslips. Thirty-six hours post transfection of pCS2+-6Myc-Smed-β-catenin3/4 and pCS2+-HA-mAxin, cells were fixed with4% paraformaldehyde/PBS for 20 min, permeabilized with 0.2% Triton X-100/PBS for 10 min and then blocked with 3% BSA/PBS for 30 min before primary antibodies were applied. A PBS rinse for 5 min between each step was performed. The primary antibodies anti-Myc (mouse, Santa Cruz) and anti-HA (rabbit, Santa Cruz) were incubated for 1h at RT diluted in 1%BSA, 0.1%Tween 20/PBS at 1:100. After washing 5min X 3 times in 3%BSA, 1%TritonX-100/PBS, the fluorophore-conjugated secondary antibodies diluted at 1:400 in 3%BSA/PBS were incubated for 1h. Donkey anti mouse–Alexa Fluor 568 (Molecular Probes) was used to visualize β-catenin3/4, and goat anti rabbit–Alexa Fluor 488 (Molecular

Probes) to visualize Axin. Cell nuclei were visualized with DAPI staining. Cells were mounted in Prolong Gold antifade reagent (Thermo Fisher Scientific) and stored at 4℃ before imaging. Images were recorded using a Zeiss LSM710 confocal microscope.

Co-immunoprecipitation assay and western blot

HEK293T cells were seeded into 6-well plates and the following plasmids transfected the following day: pCS2+-6Myc-Smed-β-catenin1/2/3/4, pCS2+-flag-xTCF1, pcDNA3.1-flag-β-Trcp, pcDNA3.1-flag-E-cadherin and pcDNA3.1-flag-α-catenin. Forty-eight hours after transfection, cells were lysed and sonicated in 400 μl of lysis buffer/well (50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM EDTA , pH 8.0, 1% NP-40) containing protease inhibitor mixture (Roche Applied Science) at 4℃. After centrifugation at 14000 rpm, 4℃ for 15min, 40 μl of supernatant was mixed with 10 μl 5x SDS-loading buffer and treated at 95℃ for 5min. The remaining supernatant for each well was incubated with 10 μl of FLAG-M2 beads (Sigma) at 4℃ for 6h. The beads were then washed three times with lysis buffer at 4℃ for 10min each, and bound proteins were eluted with 40 μl of 2x SDS loading buffer at 95℃ for 5min. Immunoprecipitates and total lysates were separated by SDS-PAGE and analyzed by immunoblot with anti-Myc or anti-Flag specific antibodies.

***Xenopus* embryo assays**

Xenopus embryos were cultured under standard conditions. mRNA was synthesized using a mMESSAGE mMACHINE SP6 kit (Ambion, Austin, TX) according to the manufacturer's instructions. Synthetic mRNAs were microinjected into embryos cultured in 2% Ficoll 400 in 0.3× MMR (1× MMR: 100 mM NaCl, 2 mM KCl, 2 mM CaCl2, 1 mM MgCl2) at 8-cell stage, and fixed at stage 20.

***In situ* hybridization in whole-mount planarians**

Colorimetric whole-mount *in situ* hybridization (WISH) and fluorescent *in situ* hybridization (FISH) were

performed as elsewhere described [98, 99]. The following DIG- (Roche), FITC- (Roche), or DNP- (Perkin Elmer) labeled riboprobes were synthesized using an *in vitro* transcription kit (Roche): *Smed-β-catenin1/3/4*, *Smed-TCF1/2/3*; *Smed-opsin*, *Smed-otxA*, *Smed-sp6/9* [52]; *Smed-tph* [100]; *Smed-ovo* [54]; *Smed-h2b* [101]; *Smed-th* (tyrosine hydroxylase), *Smed-tbh* (tryptophan hydroxylase) [102], *Smed-pc2* (*prohormone convertase*) [103]. Primers used for their synthesis are indicated (Supp Table 1). Riboprobes were finally diluted to 250 ng/µL in pre-hybridization solution, stored at -20℃, and were used at 1:500 in hybridization solution, except for *sp6/9* (1:200). Samples were observed through Leica MZ16F (Leica Microsystems, Mannhiem, BW, Germany), Zeiss Stemi SV6 stereomicroscopes and a Zeiss Axiophot microscope (Zeiss, Jena, TH, Germany); images were captured with a ProgRes C3 camera from Jenoptik (Jena, TH, Germany), sCMEX 3.0 camera (Euromex, Arnhem, The Netherlands) and Leica DFC300FX camera (Leica Microsystems, Heerbrugg, CH, Switzerland). Confocal laser scanning microscopy was performed with a Leica TCS-SP2 (Leica Lasertchnik, Heidelberg, BW, Germany) adapted for an inverted microscope.

Immunohistochemistry in planarians

After FISH, samples were rinsed with TNTx 5min, 50%PBSTx, 50%TNTx (0.1M Tris•HCl pH7.5, 0.15M NaCl, 0.3%TritonX-100) 10min, then PBSTx 10min. 1% BSA or 10% Goat serum were used as blocking reagent for 2 hours, followed by anti-*β-cat4* (1:200, diluted in 10% goat serum) or anti-VC-1 (1:15000, diluted in 1% BSA, kindly provided by Hidefumi Orii, Himeji Institute of Technology, Hyogo, Japan) antibody incubation. PBSTx (PBS with 3% TritonX-100) wash for 15min x3. Goat-anti-mouse-488 conjugated antibody (1:400, Molecular Probes) and Goat-anti-rabbit-HRP conjugated antibody (1:500, Pierce) were used as secondary antibody. HRP signal was developed with a tyramide signal amplification kit following manufacturer's recommendations (Perkin Elmer). anti-β-cat1 immunohistochemistry was

performed as previously described [45]. Nuclei were counterstained with DAPI (Sigma, 1:5000). The polyclonal anti-β-cat4 antibody was generated against 34 amino acids of the N-terminal part of the β-cat4 protein (indicated in S1 Fig) (GeneCust, Luxembourg).

RNAi Silencing

Double-stranded RNAs (dsRNA) for *Smed-β-catenin1/3/4*, *Smed-TCF2* and *Smed-APC-1* were synthesized and delivered as described elsewhere [49]. Primers used for their synthesis are indicated (Supp Table 1). Control animals were injected with dsRNA for GFP [53]. dsRNA was diluted to 1 µg/µl in water. Microinjections were performed as described elsewhere [49] following the standard protocol of a 32 nl injection of dsRNA on three consecutive days. On the next day planarians were amputated pre- and post-pharyngeally, and the head, trunk, and tail pieces were allowed to regenerate. When injecting *Smed-β-catenin3/4* the same procedure was performed during 2 weeks to improve the penetrance of the phenotype. For experiments in intact planarians, animals were injected 3 consecutive days per week for 5 weeks.

Quantitative real-time PCR (qPCR)

Total RNA was extracted from a pool of 4 planarians each for every RNAi condition. Quantitative real-time PCR was performed as previously described [104], and data was normalized based on the expression of URA4 as internal control. All the experiments were performed using three biological replicates. The primers for qPCR are indicated in Supp Table 1. Statistical significance was measured by Student's T test by comparing values from each sample to their respective control sample.

Phototactic assay

Phototactic assay was carried out as described previously [53]. The behavior analysis software SMART v.2.5.21 was used to quantify the numbers of worms in each of the three virtual subdivisions of a

60x30x10 mm transparent container filled with 10 ml planarian water after 2 minutes of positioning the worms at the indicated beginning point in the light zone.

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Author contributions

H.S., M.S-G, and T. A. designed the experiments. H.S., M.S-G., N.R-L. and M.G. carried out experiments in planarians. H.S. performed the experiments in human cell lines. J.X. carried out the *Xenopus* embryo assay. T.A performed the phylogenetic analysis. H.S., M.S-G, W.W. and T. A. analyzed the data. W.W. and T. A. acquired funding. T.A. supervised the research. H.S., M.S-G, and T. A. drafted, reviewed and edited the manuscript. All authors proofread and approved the final version of the manuscript.

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Figure 1

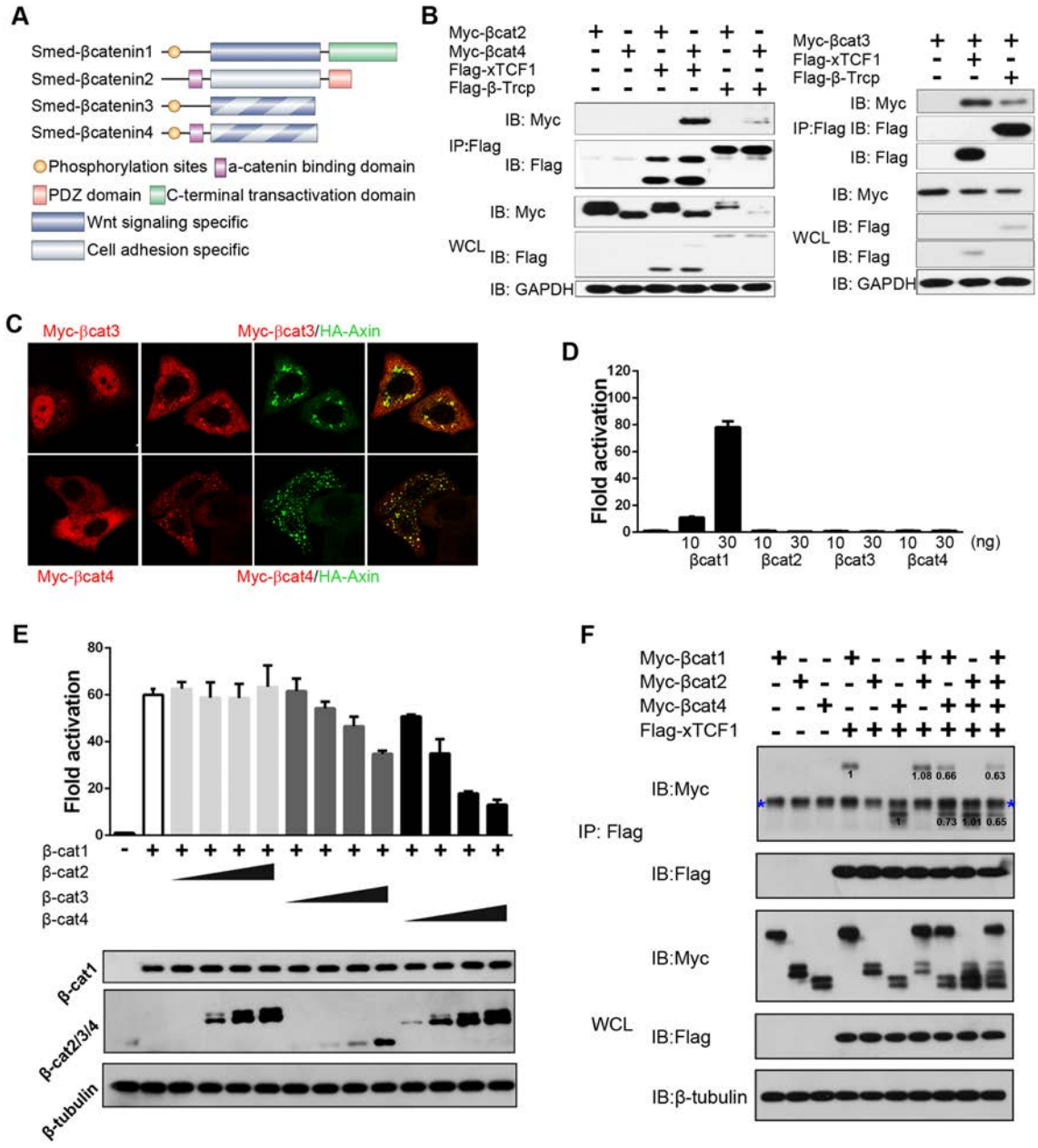


Fig 1. β-cat3 and 4 inhibit β-cat1 dependent Wnt signaling.

(A) Schematic of the four β-catenin homologs in *S. mediterranea*. β-cat1 and β-cat2 are structurally segregated. β-cat1 conserves the N-terminal GSK3 phosphorylation sites, the binding surface for Wnt signaling components in the central armadillo repeats, and the C-terminal transactivation domain. β-cat2 conserves the N-terminal α-catenin binding motif, the interacting platform for the cadherin complex in the central armadillo repeats but not for Wnt signaling elements, and the C-terminal PDZ domain. The newly identified β-cat3 and 4 conserve the GSK3 phosphorylation sites, the binding surface for Wnt signaling components and a partial conservation of the cell adhesion elements, whereas they have lost the C-terminal transactivation domain. (B) HEK293T cells were transfected with the indicated plasmids, and lysates were immunoprecipitated (IP) with FLAG-M2 beads. Western blotting (IB) with anti-Myc revealed co-IP of β-cat3 and 4 following FLAG-M2 immunoprecipitation, indicating that both of them interact with TCF and β-Trcp. β-cat2 was analyzed as a negative control. (C) Localization of β-cat3 and 4 alone or co-expressed with Axin in transfected HeLa cells. In HeLa cells, β-cat3, which localized mainly in the nucleus alone, was recruited to the cytoplasm when co-transfected with Axin. β-cat4, which was more widely dispersed in the cytoplasm, was recruited by Axin and had a punctate distribution. Scale bar=20 μm. (D) TOPflash reporter assay following co-transfection

of HEK293T cells with β-cat1, β-cat2, β-cat3, β-cat4 and reporter plasmids.10 or 30 ng were transfected of each β-catenin. Just β-cat1 but not β-cat3 or 4 activated Wnt/β-catenin signaling. β-cat2 shows also not Wnt reporter activity, as reported [40]. (E) TOPflash reporter assay following co-transfection of HEK293T cells with β-cat1 (20 ng), β-cat2 (10, 20, 30 or 60 ng), β-cat3 (10, 20, 30 or 60 ng), β-cat4 (10, 20, 30 or 60 ng) and reporter plasmids. The co-transfection of β-cat3 or 4 inhibited β-cat1 reporter activation in a dosage dependent manner. Immunoblot analysis shows the protein expression level of β-cat1/3/4 in each line. (F) HEK293T cells were transfected with the indicated plasmids, and lysates were immunoprecipitated (IP) with FLAG-M2 beads. Co-immunoprecipitated β-catenins were detected with anti-Myc antibody. Upon co-transfection of β-cat1 and β-cat4, the amount of immunoprecipitated β-catenins by TCF were less than those during sole transfection of each, supporting that they compete with each other for TCF binding. The protein levels of β-catenins immunoprecipitated by TCF were quantified and normalized against tubulin, and the values are shown below the bands. β-cat2 was analyzed as a negative control. * indicates non-specific bands.

Figure 2

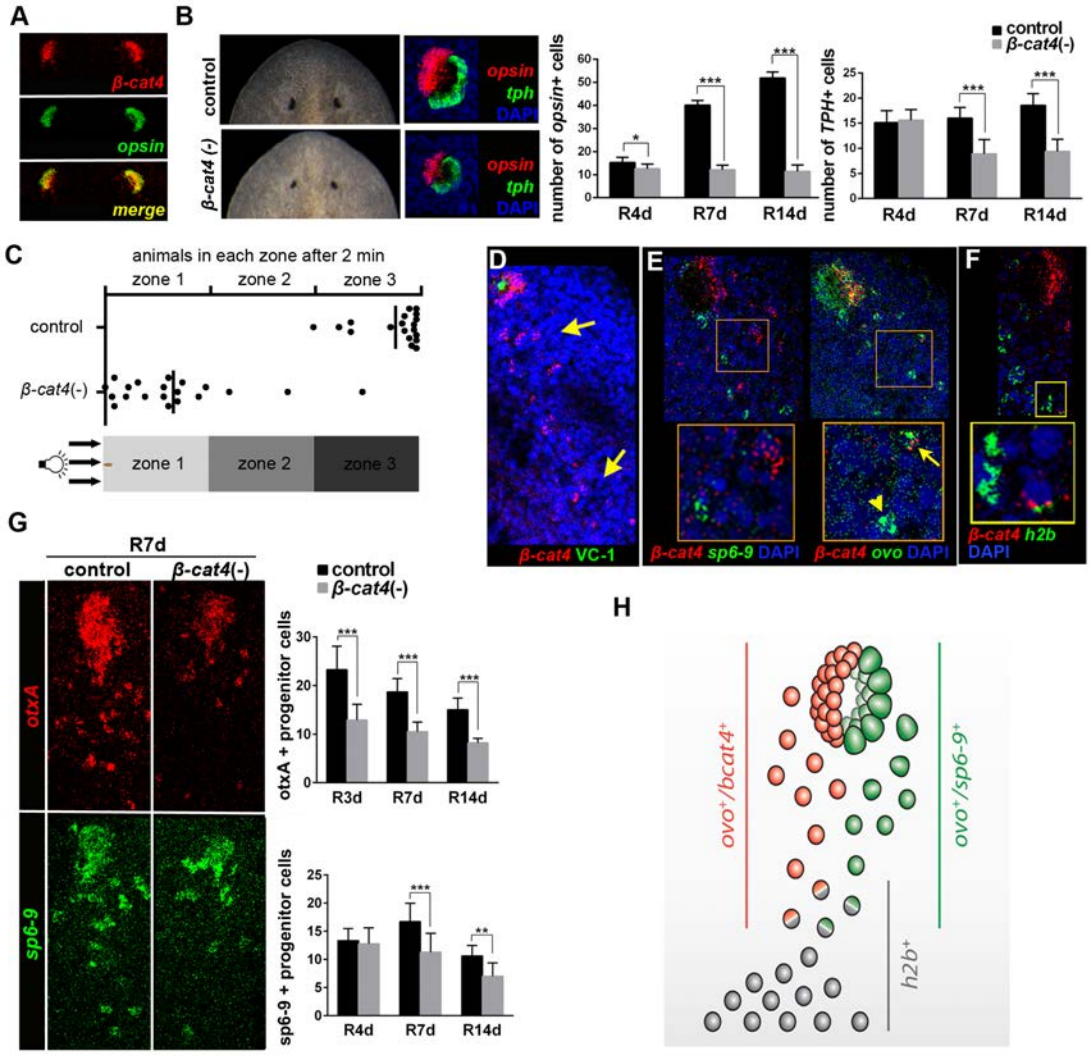


Fig 2. Smed-β-cat4 is required for planarian photoreceptor specification.

(A) Double FISH assay with *β-cat4* (red) and *opsin* (green), a marker of photoreceptor cells, in intact animals, showing their co-localization. (B) Left, live and FISH [*opsin* (red) and *tph* (green)] images of control and *β-cat4 (RNAi)* planarian eyes at 12 days of regeneration. Right, quantification of the number of photoreceptor (*opsin*+) and pigment (*tph*+) cells per eye at 4, 7 and 14 days of regeneration in control and *β-cat4 (RNAi)* planarians. *opsin*+ cells in control R4d ,15.25±2.19 (SD; n= 8 eyes); *β-cat4 (RNAi)* R4d,12.5±2.07 (SD; n=8 eyes); control R7d,40.11±2.03 (SD; n=9 eyes); *β-cat4 (RNAi)* R7d,12.08±1.98 (SD; n=12 eyes); control R14d,51.92±2.50 (SD; n=12 eyes); *β-cat4 (RNAi)* R14d,11.38±2.83 (SD; n=8 eyes). *tph*+ cells in control R4d, 15.13±2.36 (SD, n=8 eyes); *β-cat4 (RNAi)* R4d, 15.63±2.07 (SD; n=8 eyes); control R7d, 16.00±2.12 (SD; n=9 eyes); *β-cat4 (RNAi)* R7d, 8.92±2.81 (SD; n=12 eyes); control R14d, 18.58±2.27 (SD; n=12 eyes); *β-cat4 (RNAi)* R14d, 9.38±2.45 (SD; n=8 eyes). *p<0.05, ***p<0.001 (t test). (C) Phototaxis assay of *β-cat4 (RNAi)* animals. Graphical representation of the percentage of control and *β-cat4 (RNAi)* planarians found in the different regions in 2 minutes (n=20). The schematic of the container indicates the clearest and the darkest zone. (D) *β-cat4* (red) FISH followed by immunohistochemistry with VC-1 (anti-arrestin) antibody (green), to label the visual axons, shows the expression of *β-cat4* in a trail of isolated cells posterior to the eyes (yellow arrows) in addition to expression in the photoreceptors in the eyes. Animals were analyzed at 4 days of regeneration. (E) Double FISH of *β-cat4* (red) and *ovo* (green), a pan-eye cell marker, or *sp6-9* (green), a pigment progenitor cell marker, shows that among all *ovo*+ cells some of them co-express with *β-cat4* (*ovo*+/*β-cat4*+, yellow arrow) and some of them do not (*ovo*+/*β-cat4*-, yellow arrowhead). *β-cat4* was never co-expressed with *sp6-9*. Insets show magnifications of the selected areas. Animals were analyzed at 7 days of regeneration. (F) Double FISH of *β-cat4* (red) and *h2b* (green) shows that *β-cat4* is expressed in stem cells. Animals were analyzed at 7 days of regeneration. (G) FISH of *otxA* (red), a photoreceptor progenitor cells marker, and *sp6-9* (green), in control and *β-cat4*

(RNAi) animals, at 7 days of regeneration. The quantification of *otxA*+ and *sp6-9*+ cells in the trail posterior to the eyes at different regeneration time points is shown. *otxA*+ cells in control R3d, 23.25±4.81 (SD; n= 13 eyes; *β-cat4 (RNAi)* R3d, 12.89±3.26 (SD; n= 9 eyes); control R7d, 18.69±2.72 (SD; n= 13 eyes); *β-cat4 (RNAi)* R7d, 10.50±1.96 (SD; n= 10 eyes); control R14d, 15.00±2.39 (SD; n= 8 eyes); *β-cat4 (RNAi)* R14d, 8.20±0.92 (SD; n= 10 eyes). *sp6-9*+ cells in control R4d, 13.33±2.16 (SD; n= 6 eyes); *β-cat4 (RNAi)* R4d, 12.75±2.87 (SD; n= 8 eyes); control R7d, 16.71±3.25 (SD; n= 7 eyes); *β-cat4 (RNAi)* R7d, 11.29±3.35 (SD; n= 7 eyes); control R14d, 10.63±1.85 (SD; n= 8 eyes); *β-cat4 (RNAi)* R14d, 7.00±2.40 (SD; n= 9 eyes). **p<0.01, ***p<0.001 (t test). (H) Schematic of *β-cat4* expression in the eyes. Stem-cells (*h2b*+) acquire expression of *ovo* to become eye progenitors. Eye progenitors became photoreceptor or pigment cells when acquiring the expression of *β-cat4* or *sp6-9*, respectively. In all images anterior is to the top. Scale bars, 50 μm (A-F), and 10 μm (E and F insets).

Figure 3

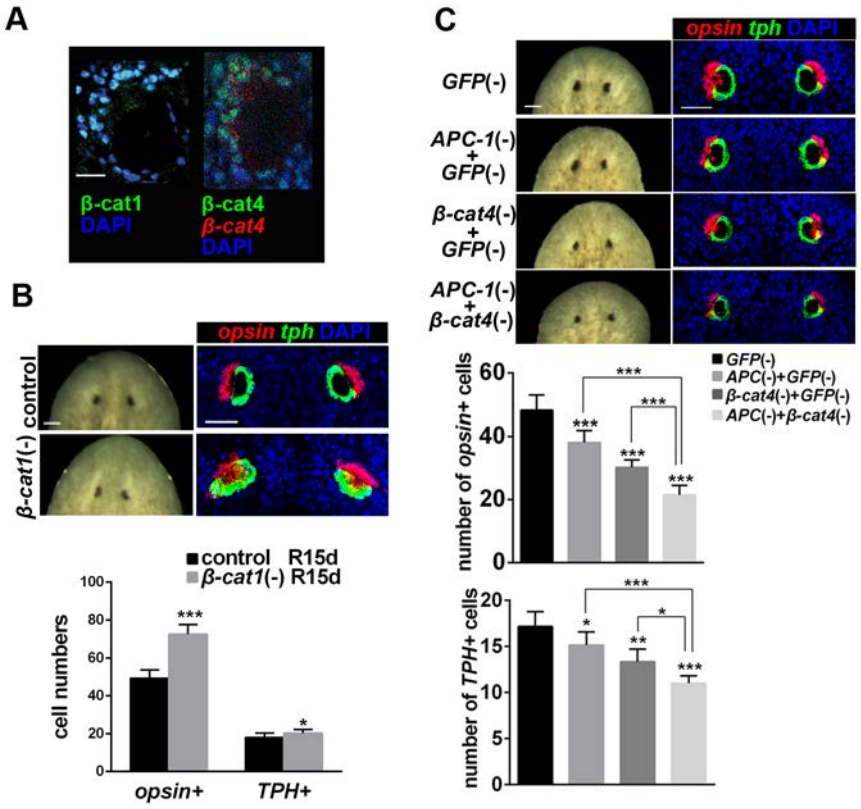


Fig 3. β-cat4 specifies photoreceptor cells through β-cat1 inhibition.

(A) β-cat1 protein (green) localizes to the nucleus of photoreceptors. β-cat4 protein (green) and *β-cat4* mRNA (red) colocalize in photoreceptor cells, where the protein is found in the nucleus. β-cat1 and β-cat4 images correspond to intact *S. polychroa* and *S. mediterranea* animals, respectively (B) Eye phenotype of *β-cat1 (RNAi)* animals at 15 days of regeneration. Live images and FISH of *opsin* (red) and *tph* (green) with the respective quantification of *opsin*+ and *tph*+ cells per eye. *opsin*+ cells in control R15d, 49.25±4.37 (SD; n= 12 eyes); *β-cat1 (RNAi)* R15d, 72.50±5.21 (SD; n= 6 eyes). *tph*+ cells in control R15d, 18.00±2.26 (SD; n= 12 eyes); *β-cat1 (RNAi)* R15d, 20.33±1.86 (SD; n= 6 eyes). *p<0.05, ***p<0.001 (t test). (C) Double knockdown of *APC-1 (RNAi)* and *β-cat4 (RNAi)* in intact animals. Live images and FISH of *opsin* (red) and *tph* (green) after the indicated RNAi treatments with the respective quantification of *opsin*+ and *tph*+ cells per eye. *opsin*+ cells in *GFP (RNAi),* 48.17±4.80 (SD; n= 6 eyes); *APC-1;GFP (RNAi)*, 38.13±3.76 (SD; n= 8 eyes); *β-cat4;GFP (RNAi)*, 30.17±2.40 (SD; n= 6 eyes); *APC-1;β-cat4 (RNAi)*, 21.50±2.35 (SD; n= 6 eyes). *tph*+ cells in *GFP (RNAi)*, 17.17±1.60 (SD; n= 6 eyes); *APC-1;GFP (RNAi)*, 15.13±1.46 (SD; n= 8 eyes); *β-cat4;GFP (RNAi)*,13.33±1.37 (SD; n= 6 eyes); *APC-1;β-cat4 (RNAi),* 10.83±0.75 (SD; n= 6 eyes). *p<0.05, **p<0.01, ***p<0.001 (t test). *APC-1 (RNAi)* and *β-cat4 (RNAi)* caused smaller eyes than control. Notice that double RNAi of *APC-1* and *β-cat4* resulted in a more severe phenotype than each one alone. Anterior is to the top. Scale bar=20 μm (A), 50 μm (C, B).

Figure 4

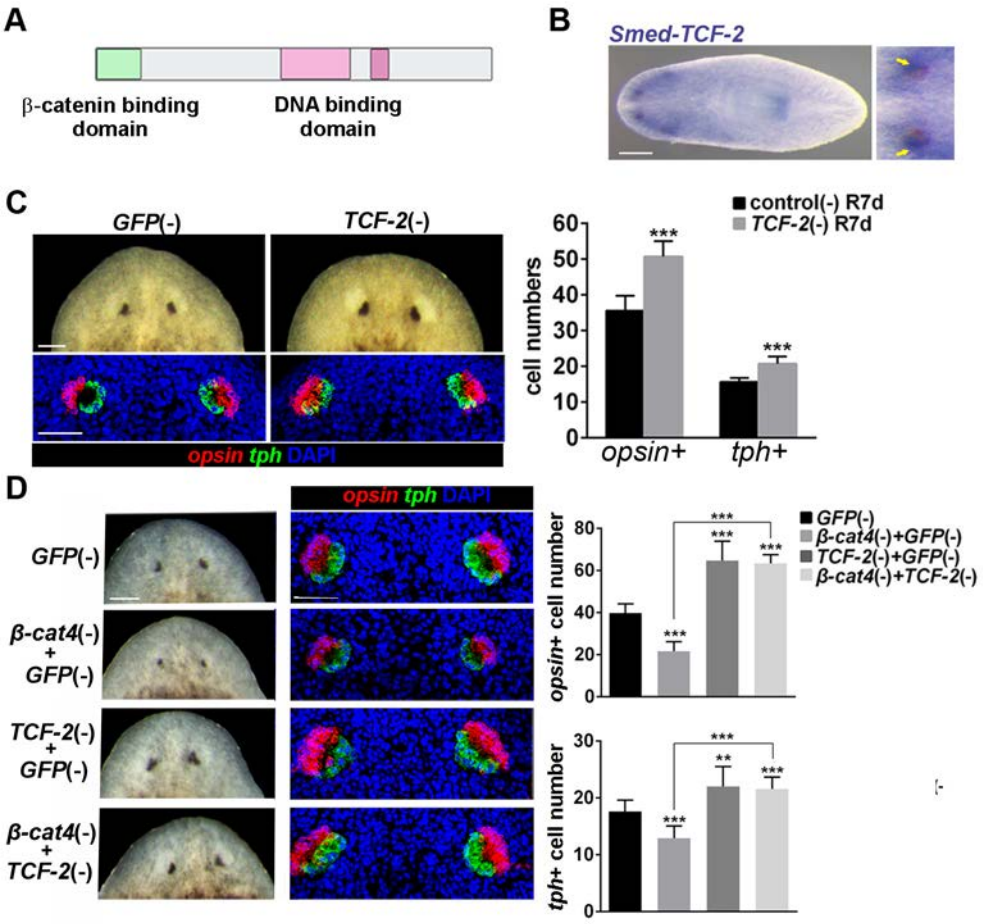


Fig 4. TCF-2 mediates β-cat1 and β-cat4 signaling.

(A) TCF-2 conserves the characteristic domains of β-catenin and DNA binding (HMG domain). (B) Expression of *TCF-2* in the CNS and in the photoreceptors (yellow arrows in the magnification) (C) Live images and FISH of *opsin* (red) and *tph* (green) of *TCF-2 (RNAi)* and *GFP (RNAi)* planarians at 7 days of regeneration, with the respective quantification of the *opsin*+ and *tph*+ cells per eye. *opsin*+ cells in control R7d, 35.60±4.17 (SD; n= 10 eyes); *TCF-2 (RNAi)* R7d, 50.8±4.21 (SD; n= 10 eyes). *tph*+ cells in control R7d, 15.7±1.06 (SD; n= 10 eyes); *TCF-2 (RNAi)* R7d, 20.70±2.06 (SD; n= 10 eyes). ***p<0.001 (t test). Anterior is to the top. (D) Double knockdown assay of *β-cat4 (RNAi*) and *TCF-2 (RNAi)*. Live images and FISH of *opsin* (red) and *tph* (green) to show planarian regenerated eyes after the indicated RNAi treatment. The respective quantification of *opsin*+ and *tph*+ cells is shown. *opsin*+ cells in *GFP (RNAi)*, 39.70±4.47 (SD; n= 10 eyes); *β-cat4;GFP (RNAi)*, 21.60±4.48 (SD; n= 10 eyes); *TCF-2;GFP (RNAi)*, 64.64±8.68 (SD; n= 10 eyes); *APC-1;β-cat4 (RNAi)*, 63.50±4.32 (SD; n= 12 eyes). *tph*+ cells in *GFP (RNAi)*, 17.60±2.01 (SD; n= 10 eyes); *β-cat4;GFP (RNAi)*, 12.89±2.15 (SD; n= 10 eyes); *TCF-2;GFP (RNAi)*, 22.00±3.49 (SD; n= 10 eyes); *APC-1;β-cat4 (RNAi)*, 21.58±2.27 (SD; n= 12 eyes). **p<0.01, ***p<0.001 (t test). Scale bars, 250 μm (B), 50 μm (C, D).

Figure 5

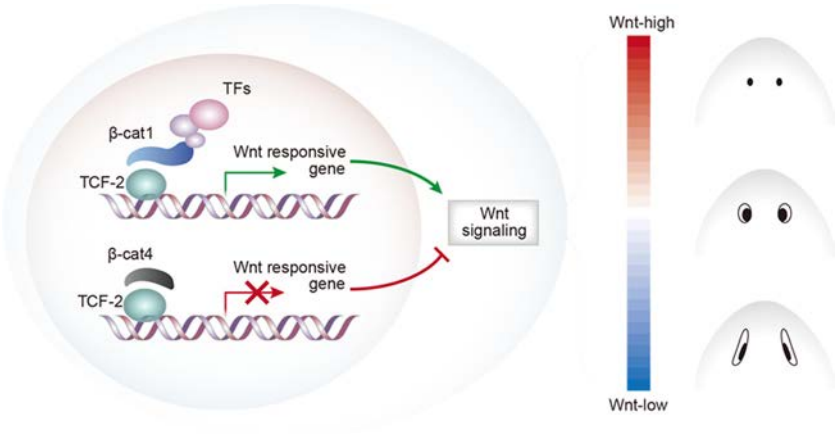


Fig 5. Proposed Model for Wnt/β-catenin activity modulation in planarian eyes

During planarian eye regeneration or maintenance, β-cat4/TCF-2 antagonizes β-cat1/TCF-2, modulating Wnt signaling to an appropriate level to ensure correct differentiation of photoreceptors.

Figure 6

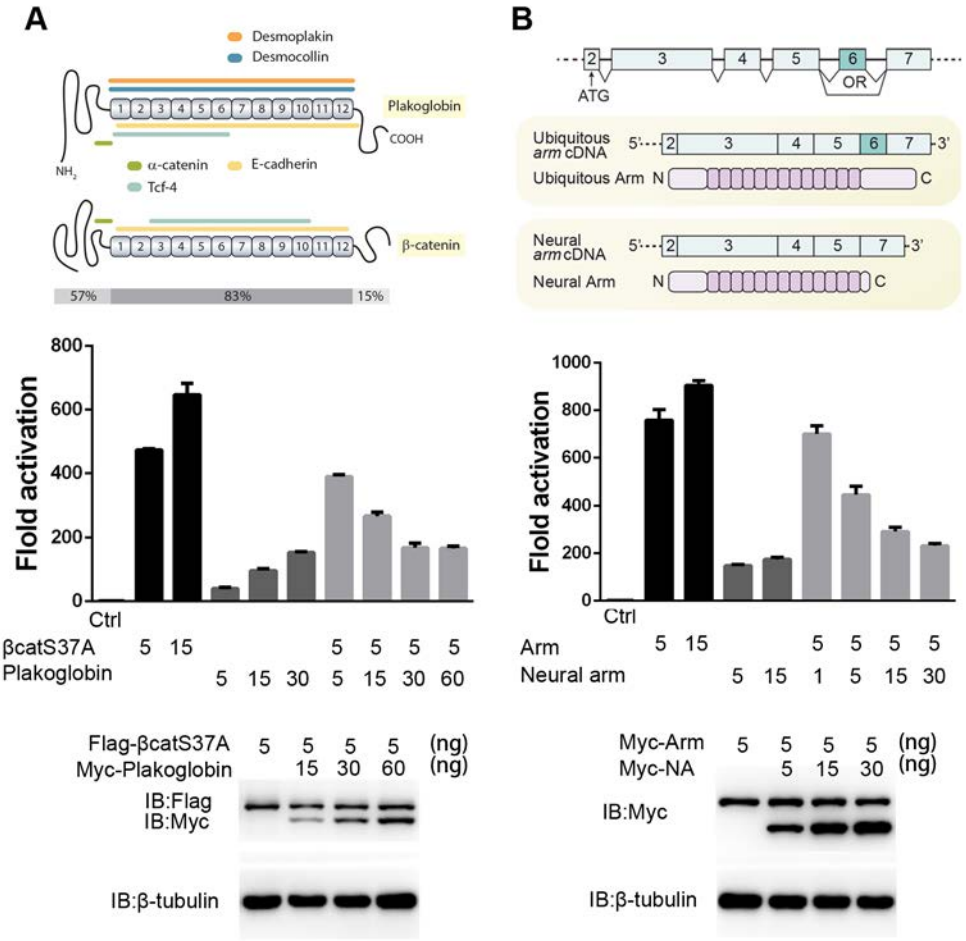
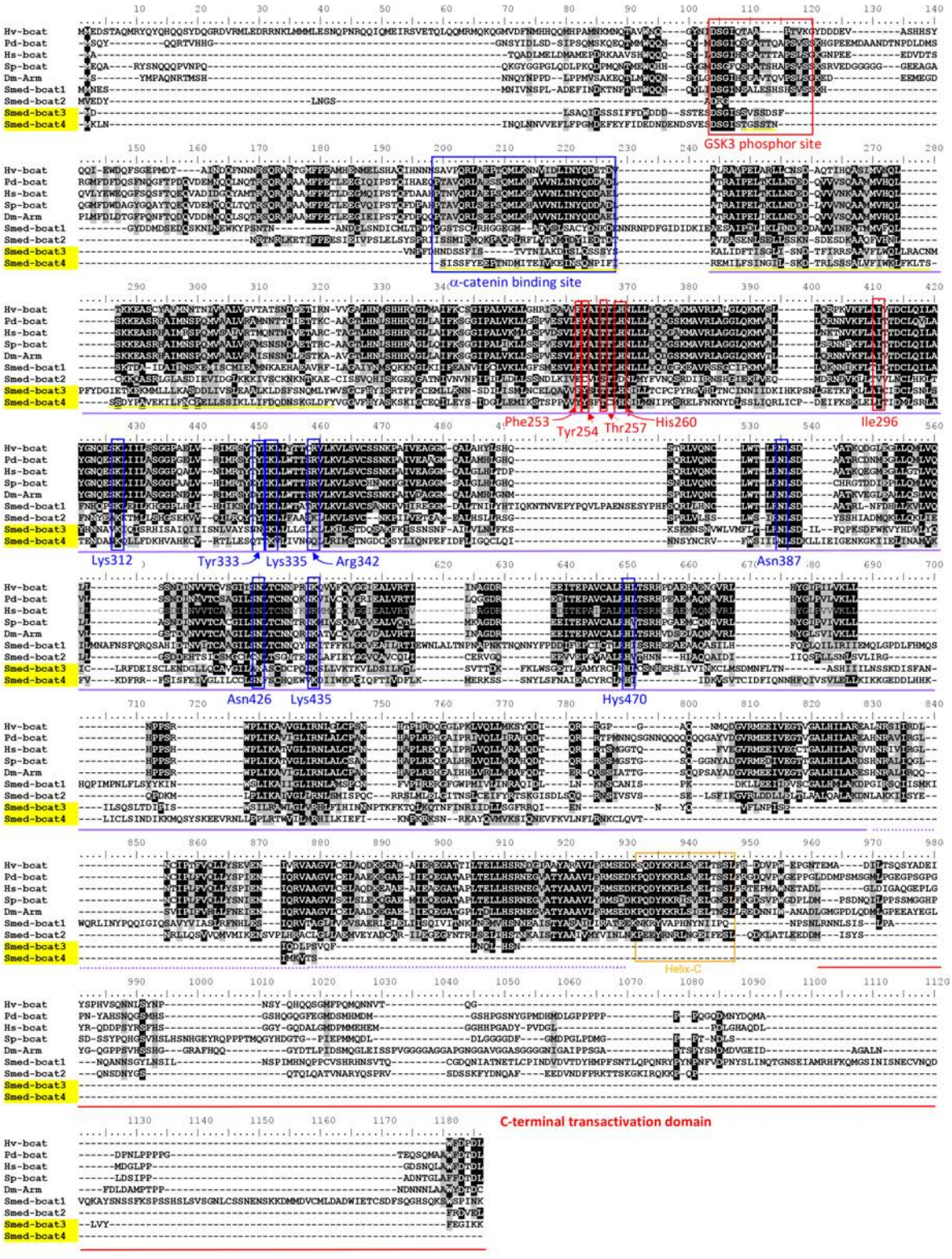


Fig 6. Plakoglobin and Neural Arm inhibit Wnt/β-catenin activity 'in vitro'.

(A) Upper panel, schematic representation of Plakoglobin or β-catenin proteins, showing the protein interacting domains and the degree of homology of the N-terminal, central Arm repeat and C-terminal region between both proteins. Lower panel, activation of TOPflash reporter signal by β-catenin (S37A) and Plakoglobin in HEK293T cells. Plakoglobin showed limited capacity to activate reporter signal, and inhibited β-catenin (S37A) activity in a dose-dependent manner. (B) Upper panel, schematic representation of the exon composition of Arm and Neural Arm (in which exon 6 is skipped). Lower panel, activation of TOPflash reporter signal by Armadillo and Neural Arm in HEK293T cells. Neural Arm showed limited capacity to activate reporter signal and inhibited Arm activity in a dose dependent manner.

Supplementary Figure 1

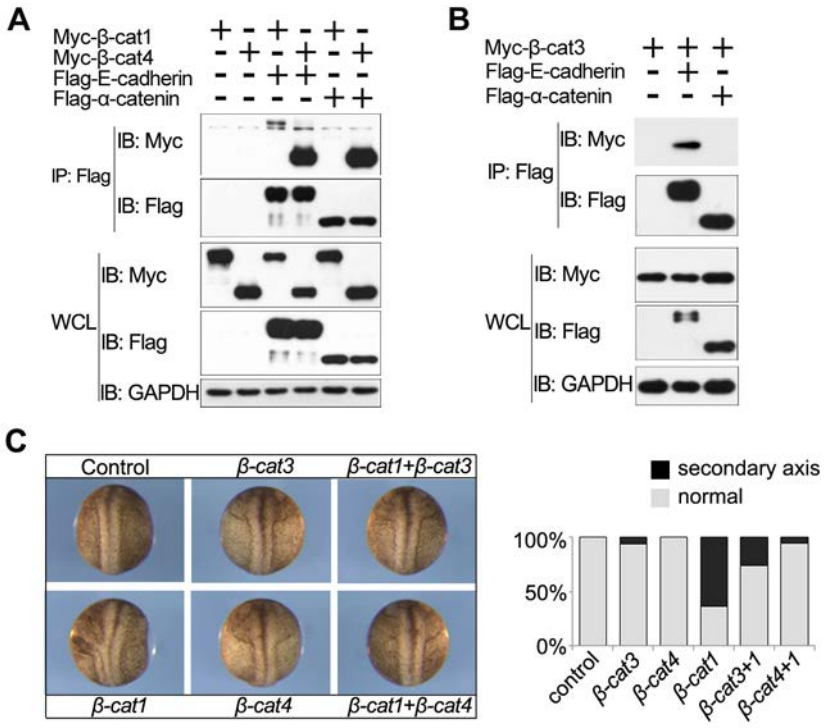


S1 Fig. Protein sequence analysis of β-catenin homologs from different species.

Protein regions and amino acids required for Wnt signaling are indicated in red. β-cat3 and 4 conserve the GSK3 phosphorylation sites in the N terminus, the hydrophobic pocket formed by Phe 253 and some of the surrounding residues, essential for Axin and TCF binding [1]. The red line underlies the C-terminal transactivation domain, which is necessary and sufficient for signaling through TCF factors [2, 3]. The C-terminal transactivation domain is lost in β-cat3 and 4 proteins. Amino acids required for cell-cell adhesion but also involved in TCF interaction, which are

conserved in β-cat3 and 4 proteins, are labeled in blue: the armadillo repeats 4-9 in human β-catenin constitute a core interacting platform for cadherin and TCF binding, in which Lys312 and Lys435 form the critical salt bridges [4, 5]. The α-catenin binding sites [6, 7] appear conserved in β-cat4 but not in β-cat3. Armadillo repeats are underlined with a violet line. The conserved two last repeats, which are not present in β-cat3 and 4 proteins, are underlined by a dashed line. The Helix-C (orange square), a helix-α structure found C-terminal to the last Armadillo repeat and required for transcriptional co-activation [8], is also not conserved in β-cat3 and 4 proteins. The amino acids used to generate the anti β-cat4 antibody are underlined in yellow. Accession numbers of the sequences analyzed are: Hv-bcat, AAQ02885.1; Pd-bcat, ABQ85061.1; Hs-bcat, NP_001895.1; Sp-bcat, NP_001027543.1; Dm-Arm, NP_476666.1; Smed-bcat1, ABW79875.1; Smed-bcat2, ABW79874.1. The accession numbers of the new planarian β-catenins are: β-cat3, KY196224; and β-cat4, KY196225. Abbreviations: *Hv, Hidra vulgaris; Pd, P. dumerilii; Hs, H. spiens; Sp, Strongilocentrotus purpuratus; Dm, Drosophila melanogaster; Smed, S. mediterranea*.

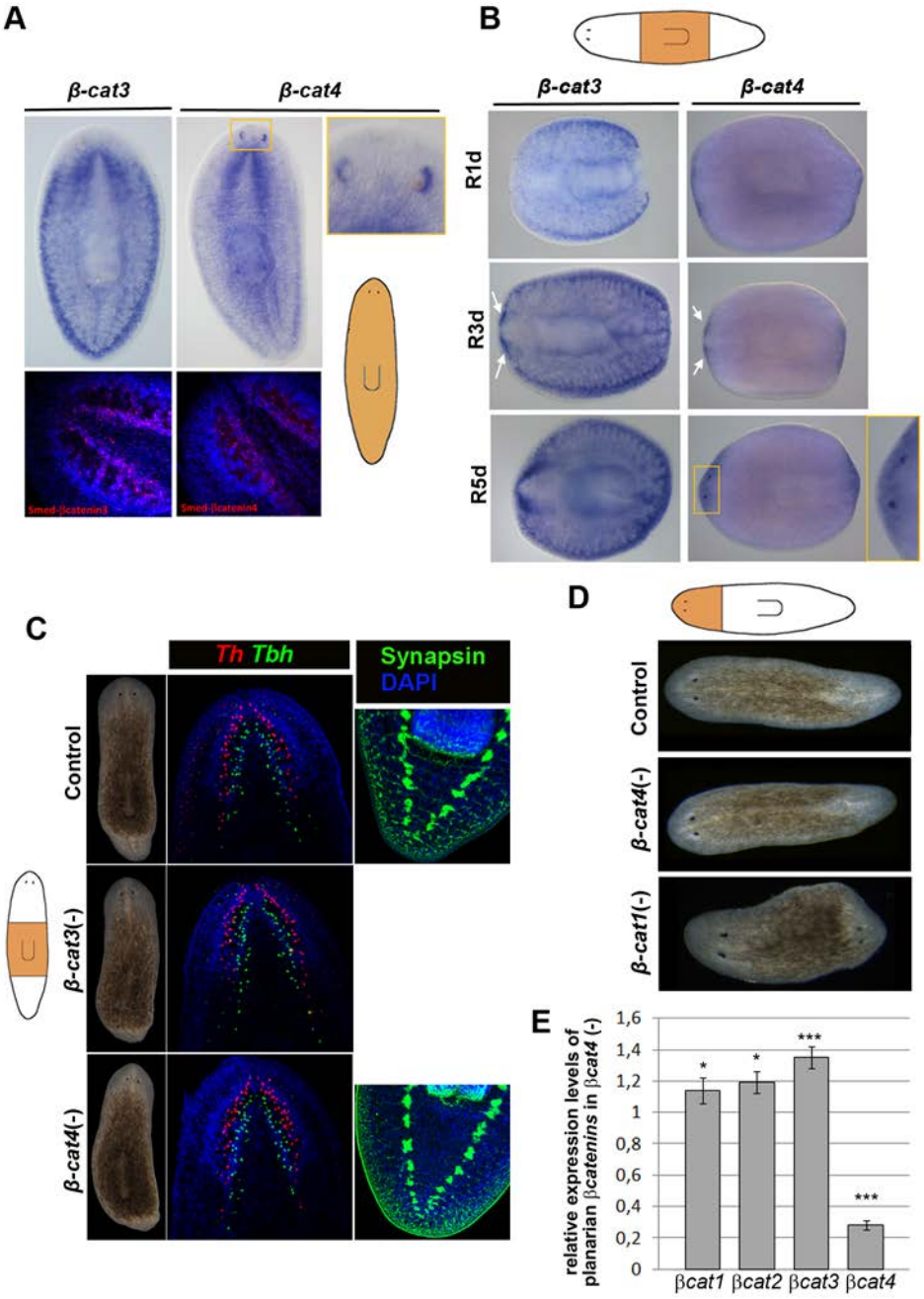
Supplementary Figure 2



S2 Fig. β-cat3 and 4 inhibit β-cat1-dependent Wnt signaling in vitro.

(A-B) Co-immunoprecipitation assays in 293T cells showed the ability of β-cat4 to interact with E-cadherin and α-catenin, while β-cat3 could only interact with E-cadherin. β-cat1 was used as a control since it is reported its interaction with E-cadherin [8]. (C) Axis duplication assays in *Xenopus* embryos. *β-cat1* mRNA injection induces a secondary axis in *Xenopus* embryos, as reported [8, 9], whereas no effects are observed after *β-cat3* or *4* mRNA injection. However co-injection of *β-cat3/4* together with β-cat1 rescued the "double axis" phenotype. The percentages of embryos with the indicated phenotypes are shown (n = 50).

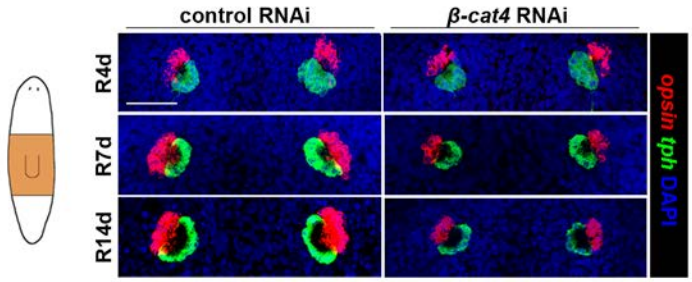
Supplementary Figure 3



S3 Fig. β-cat3 and 4 expression pattern and their RNAi phenotype.

(A) Both *β-cat3* and *β-cat4* were mainly expressed in the CNS after WISH and FISH (red). *β-cat4* was also expressed in photoreceptors (see magnification in the orange box). Analyzed planarians correspond to intact animals. (B) *β-cat3* and *β-cat4* expression pattern during regeneration of trunk fragments, which must regenerate the head and the tail. *β-cat3* and *β-cat4* were expressed in the newly formed brain (arrows in R3d) and *β-cat4* is also expressed in the regenerating eyes (see magnification in the orange box). (C) Phenotype of control and *β-cat3* and 4 RNAi animals after dsRNA injection and induced regeneration. No polarity defects were observed in *β-cat3* or *β-cat4* RNAi animals. No obvious affection in the brain was neither observed through analysis of brain markers, 3C11 (green), *Smed-th (tyrosine hydroxylase)* (red), and *Smed-tbh (tryptophan hydroxylase*) (green) [11]. Planarians shown were trunk fragments at 12 days of regeneration. (D) *β-cat1 RNAi* caused anteriorization of regenerating head fragments, as expected [10, 12, 13], while *β-cat4* RNAi planarians show a normal regenerated posterior region, indicating that *β-cat4* RNAi does not cause antero-posterior defects. (E) Relative expression levels of *β-cat1/2/3/4* after *β-cat4* RNAi by qRT-PCR. Values represent the means of three biological replicates. Error bars represent standard deviation. Data were analyzed by Student's t-test. *p<0.05; **p<0.01; ***p<0.001. Scale bars= 250 μm (A, B, C, D) and 50 μm (magnification in A and B).

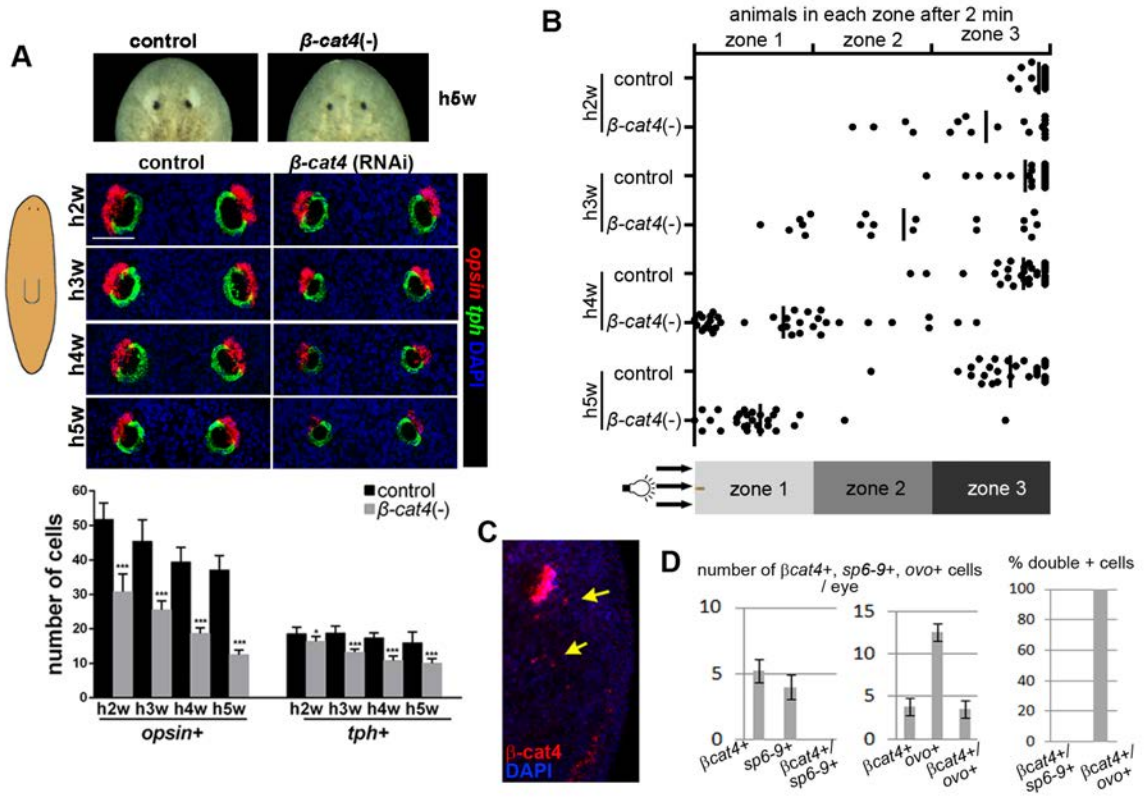
Supplementary Figure 4



S4 Fig. *β-cat4 (RNAi)* produces a decrease of photoreceptor cells during planarian regeneration.

Double FISH of *opsin* (red) and *tph* (green) in control and *β-cat4 (RNAi)* animals, at 4, 7, and 14 days of regeneration. Scale bar=50 μm

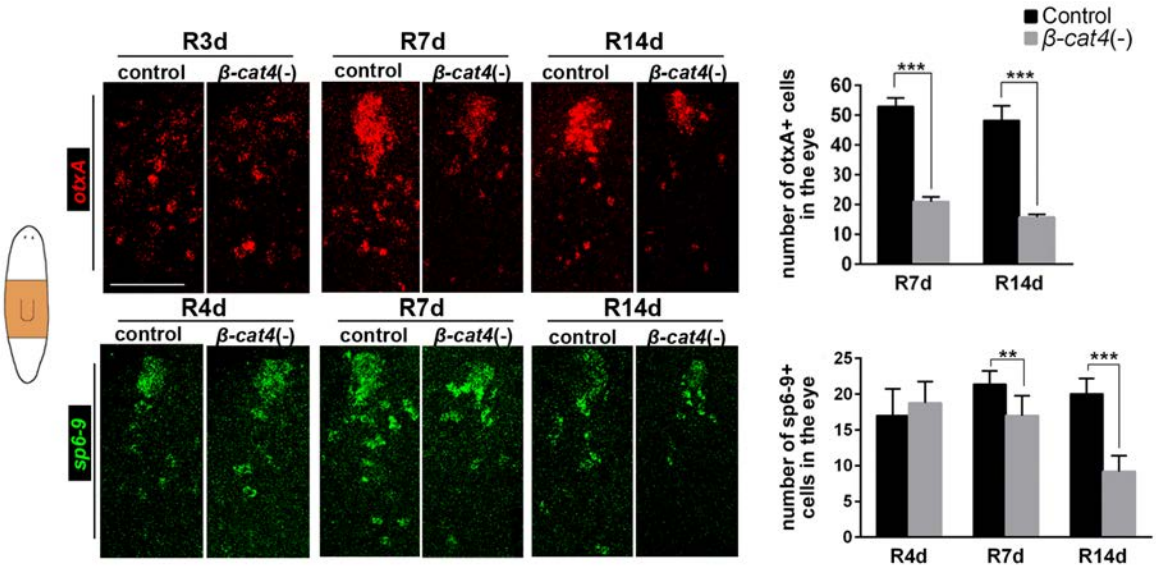
Supplementary Figure 5



S5 Fig. *β-cat4 (RNAi)* produces a decrease of photoreceptor cells during planarian homeostasis.

(A) *β-cat4 (RNAi)* planarians after 5 weeks injection show smaller eyes with small photoreceptor area. FISH of *opsin* (red) and *tph* (green) in control and *β-cat4 (RNAi)* intact animals along the 5 weeks of *β-cat4* dsRNA injection and its quantification, showing a decrease mainly in photoreceptor cells. *opsin*+ cells in control h2w, 51.80±4.71 (SD; n= 10 eyes); *β-cat4 (RNAi*) h2w, 30.75±5.20 (SD; n= 8 eyes); control h3w, 45.40±6.26 (SD; n= 10 eyes); *β-cat4 (RNAi)* h3w, 25.50±2.62 (SD; n= 8 eyes); control h4w, 39.40±4.25 (SD; n= 10 eyes); *β-cat4 (RNAi)* h4w, 18.70±1.64 (SD; n= 10 eyes); control h5w, 37.08±4.14 (SD; n= 10 eyes); *β-cat4 (RNAi*) h5w, 12.50±1.35 (SD; n= 10 eyes). tph+ cells in control h2w, 18.60±1.90 (SD; n= 10 eyes); *β-cat4 (RNAi)* h2w, 16.38±1.41 (SD; n= 8 eyes); control h3w, 18.80±2.00 (SD; n= 10 eyes); *β-cat4 (RNAi*) h3w, 13.25±0.89 (SD; n= 8 eyes); control h4w, 17.40±1.43 (SD; n= 10 eyes); *β-cat4 (RNAi)* h4w, 10.80±1.32 (SD; n= 10 eyes); control h5w, 16.00±3.10 (SD; n= 12 eyes); *β-cat4 (RNAi*) h5w, 10.00±1.33 (SD; n= 10 eyes). *p<0.05, ***p<0.001 (t test). (B) Phototaxis assay of *β-cat4 (RNAi)* intact animals. Graphical representation of the percentage of control and *β-cat4 (RNAi)* planarians found in the different regions in 2 minutes. The scheme of the container with the different zones is shown. β-*cat4 (RNAi)* animals became more insensitive to photophoby along the experiment. (C) FISH of *β-cat4* (red) in intact animals. Yellow arrows indicate isolated *β-cat4*+ cells in the trail posterior to the eyes, corresponding to eye precursor cells. (D) Quantification of *β-cat4*+, *sp6-9*+ and *ovo*+ cells in the eye of 7 days regenerating animals (n=6 eyes). Scale bars=50 μm

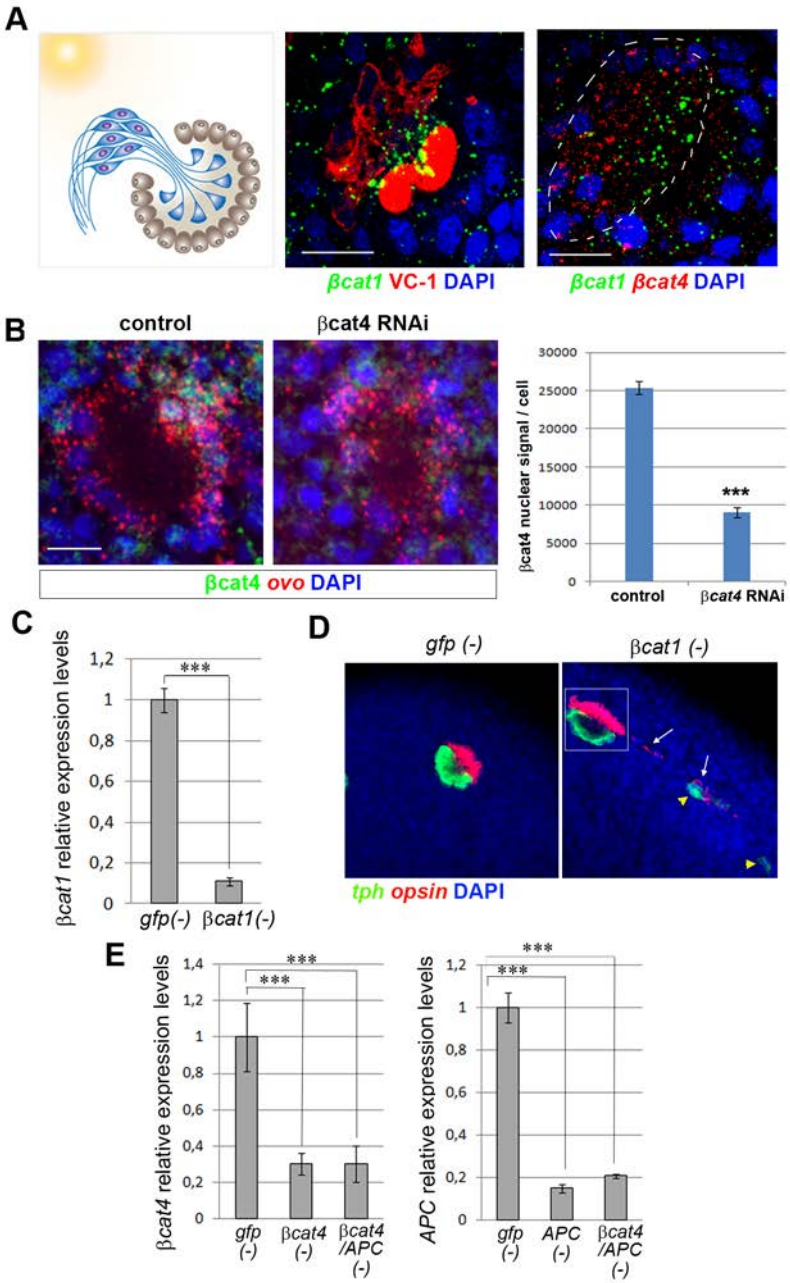
Supplementary Figure 6



S6 Fig. *β-cat4* expression in eye progenitors and eye cells quantification during regeneration.

FISH of *otxA* (red) and *sp6-9* (green) in control and *β-cat4 (RNAi*) animals at indicated regeneration time points and its quantification in the eye structure. otxA+ cells in control R7d, 52.9±2.96 (SD; n= 10 eyes); *β-cat4 (RNAi)* R7d, 21.00±1.58 (SD; n= 9 eyes); control R14d, 48.25±4.95 (SD; n= 8 eyes); *β-cat4 (RNAi)* R14d, 15.80±0.92 (SD; n= 10 eyes). The *sp6-9*+ cells number for control R4d, 17.00±3.74 (SD; n= 6 eyes); *β-cat4 (RNAi)* R4d, 18.75±3.01 (SD; n= 8 eyes); control R7d, 21.38±1.85 (SD; n= 8 eyes); *β-cat4 (RNAi)* R7d, 17.00±2.78 (SD; n= 8 eyes); control R14d, 20.00±2.16 (SD; n= 10 eyes); *β-cat4 (RNAi)* R14d, 9.20±2.20 (SD; n= 10 eyes). **p<0.01, ***p<0.001 (t test). Scale bar=50 μm.

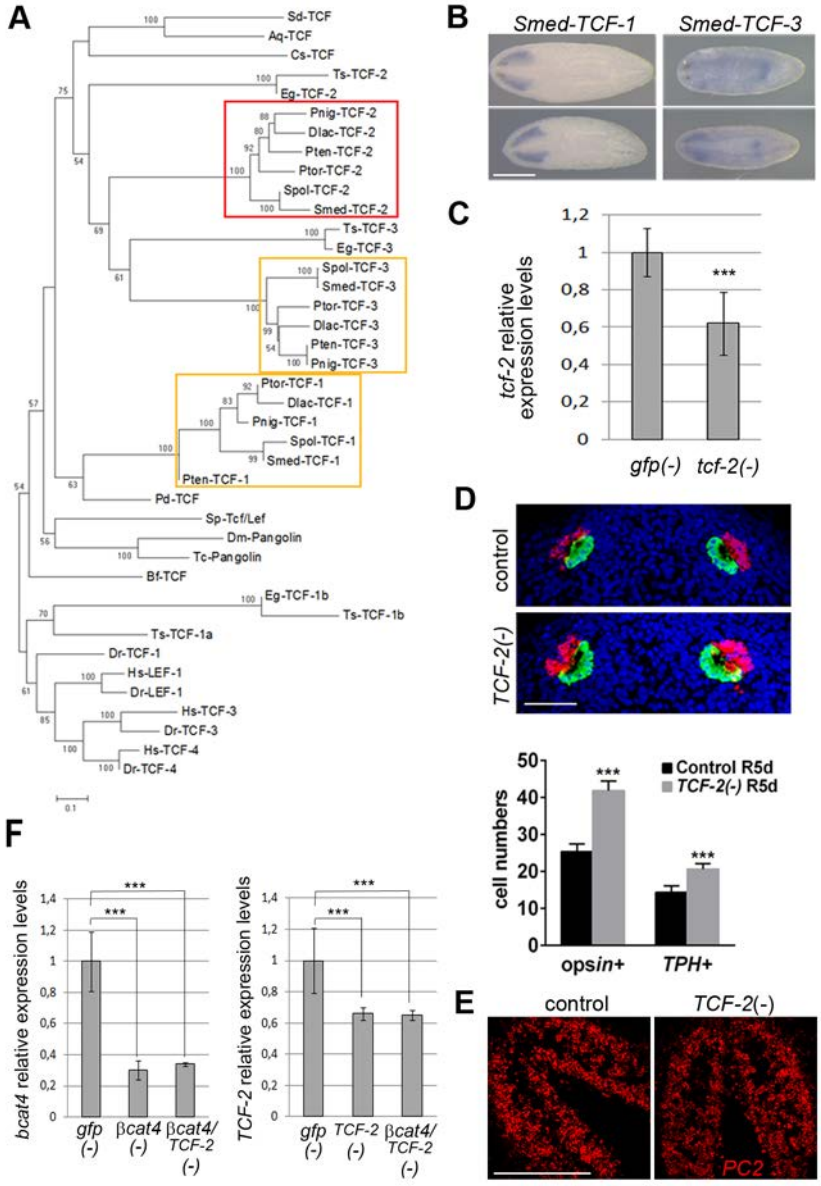
Supplementary Figure 7



S7 Fig. β-cat4 and β-cat1 are expressed in the nucleus of photoreceptor cells.

(A) Left, scheme of a planarian eye showing the eye photoreceptors (in blue) and the pigment cells (in brown). middle, FISH of *β-cat1* (green) combined with immunostaining with anti-VC-1 (red), which labels rhabdomeres of photoreceptor cells (red), demonstrates the localization of *β-cat1* mRNA (green) in the photoreceptor area. Double FISH of *β-cat4* (red) and *β-cat1* (green) demonstrates their localization in the photoreceptor area (dashed line) (B) Immunostaining of control and *β-cat4* RNAi planarians with the anti- β-cat4 antibody (green) to demonstrate its specificity. Quantification of β-cat4 signal per cell shows that it decreases significantly in *β-cat4* RNAi animals compared to controls. ***p<0.001 (t test). n=140 cells of 5 different animals per condition. β-cat4 signal was measured to obtain the raw integrated density (RID) for each individual nuclei. (C) Relative expression level of *β-cat1* after *β-cat1* RNAi by qRT-PCR. Values represent the means of three biological replicates. (D) FISH of *opsin* (red) and *tph* (green) in control and *β-cat1 (RNAi)*. *β-cat1 (RNAi)* planarians show larger eyes and the appearance of ectopic photoreceptor (white arrow) and pigment cells (yellow arrowhead). Images correspond to 12 days regenerating animals. Nuclei are stained with DAPI. The area corresponding to the eye structure (white square) was the one used for the quantification in Fig 3B. (E) Relative expression level of *β-cat4* and *APC* after their RNAi by qRT-PCR. In (D) and (E) values represent the means of three biological replicates. Error bars represent standard deviation. Data were analyzed by Student's t-test. *p<0.05; **p<0.01. Scale bars=20 μm.

Supplementary Figure 8

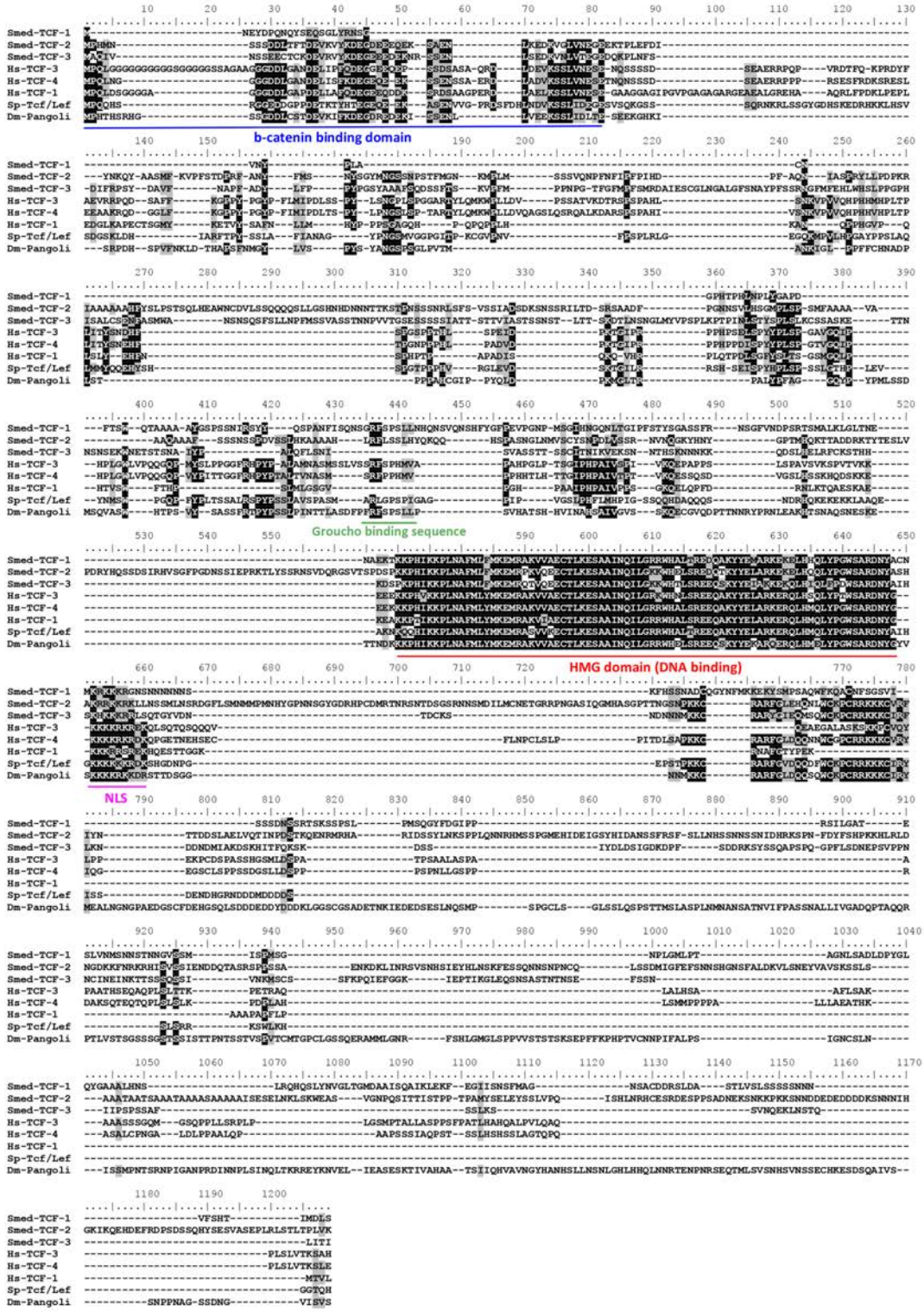


S8 Fig. TCF-2 is involved in photoreceptor regeneration.

(A) Phylogenetic analysis of TCF homologs from different species. Confidence values are shown at the main nodes. Accession number of the analyzed sequences: Aq-TCF ADO16566.1; NBf-TCF AAZ77711.1; Cs-TCF BAB68354.1; Dm-Pangolin P91943.1; Dr-LEF-1 NP_571501.1; Dr-TCF-1 NP_001012389.1; Dr-TCF-3 Q9YHE8.1; Dr-TCF-4 NP_571334.1; Eg-TCF-1b EUB55217.1; Eg-TCF-2 EUB60264.1; Eg-TCF3 CDS20600.1; Hs-LEF-1 NP_057353.1; Hs-TCF-1 AAH48769.1; Hs-TCF-3 NP_112573.1; Hs-TCF-4 NP_110383.2; Pd-TCF ANS60442.1; Sd-TCF CAH04889.1; Sp-Tcf/Lef AAD45010.1; Tc-Pangolin XP_008191151.1; Ts-TCF-1b OCK35857.1; Ts-TCF-2 OCK32932.1; Ts-TCF-3 OCK34187.1. TCF sequences from planarian species were found in Planmine [14]. Accession numbers of Smed-TCF1, Smed-TCF2 and Smed-TCF3 are: KY196226, KY196227, KY196228. Abbreviations: Aq, *Amphimedon queenslandica*; Bf, B*ranchiostoma floridae*; Cs, *Ciona savignyi*; Dlac, *Dendrocelum lacteum*; Dm, *Drosophila melanogaster*; Dr, *Danio rerio;* Eg, *Echinococcus granulosus;* Hs, *Homo sapiens*; Pd, *Platynereis dumerilii*; Pnig, *Polycelis nigra*; Pten, *Polycelis tenuis*; Ptor, *Polycelis torva*; Sd, *Suberites domuncula*; Smed, *Schmidtea mediterranea*; Sp, *Strongylocentrotus purpuratus*; Spol, S*chmidtea polychroa*; Tc, *Tribolium castaneum*; Ts, *Taenia saginata*. (B) Expression pattern of *TCF-1* and *TCF-3*. Both of them are predominantly expressed in the CNS. (C) Relative expression level of *TCF-2* after RNAi by qRT-PCR. (D) Double FISH of *opsin* (red) and *tph* (green) in control and *TCF-2 (RNAi)* animals at 5 days of regeneration with the corresponding quantification *opsin*+ and *tph*+ cells. *opsin*+ cells in control R5d, 25.38±2.07 (SD; n= 8 eyes); *TCF-2 (RNAi)* R5d, 41.88±2.59 (SD; n= 8 eyes). tph+ cells number for control R5d, 14.38±1.77 (SD; n= 8 eyes); *TCF-2 (RNAi*) R5d, 20.63±1.51 (SD; n= 8 eyes). ***p<0.001 (t test). (E) FISH of *pc2 (prohormone convertase)* 2) (red)

[15] in control and *TCF-2 (RNAi)* at 7 days of regeneration. No difference is observed between control and *TCF-2 (RNAi)* brains. (F) Relative expression levels of *TCF-2* and *β-cat4* after their RNAi by qRT-PCR. In (C) and (F) values represent the means of three biological replicates. Error bars represent standard deviation. Scale bar= 250 μm (B), 50 μm (C) and 200 μm (D).

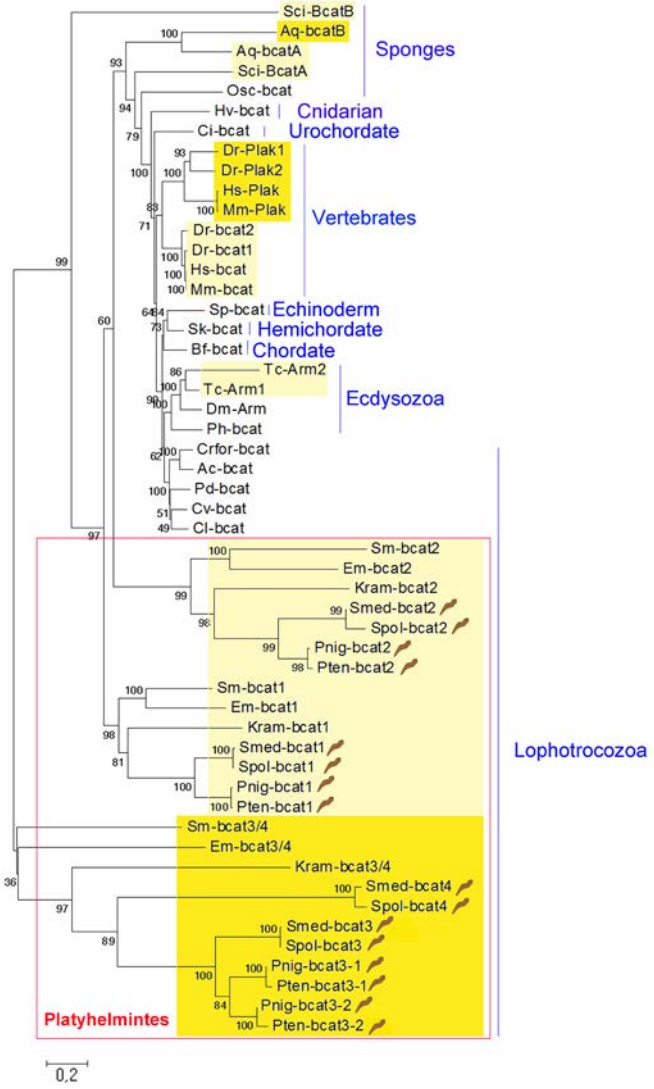
Supplementary Figure 9



S9 Fig. Sequence alignments of TCFs proteins from different species.

Alignment of the three *S. mediterranea* TCF proteins (Smed-TCF-1/3). The β-catenin binding domain [16], underlined in blue, is not conserved in TCF-1. The Groucho binding sequence [17], underlined in green, is not conserved in TCF-2. The HMG (High Mobility Group domain) [18], in red, and the NLS (Nuclear Localization Signal), are conserved in all TCFs. Accession numbers and abbreviations are found in Figure S8 legend.

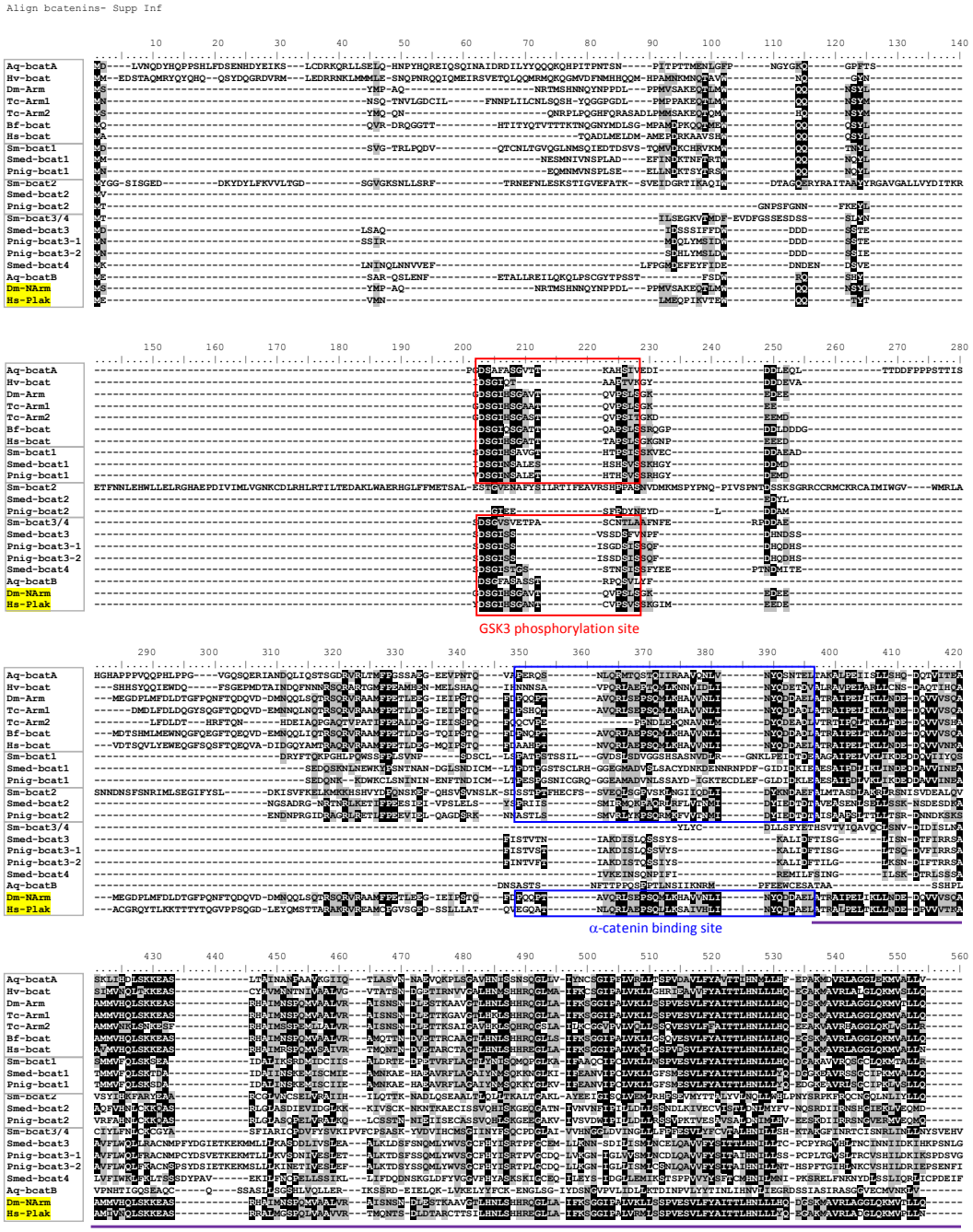
Supplementary Figure 10

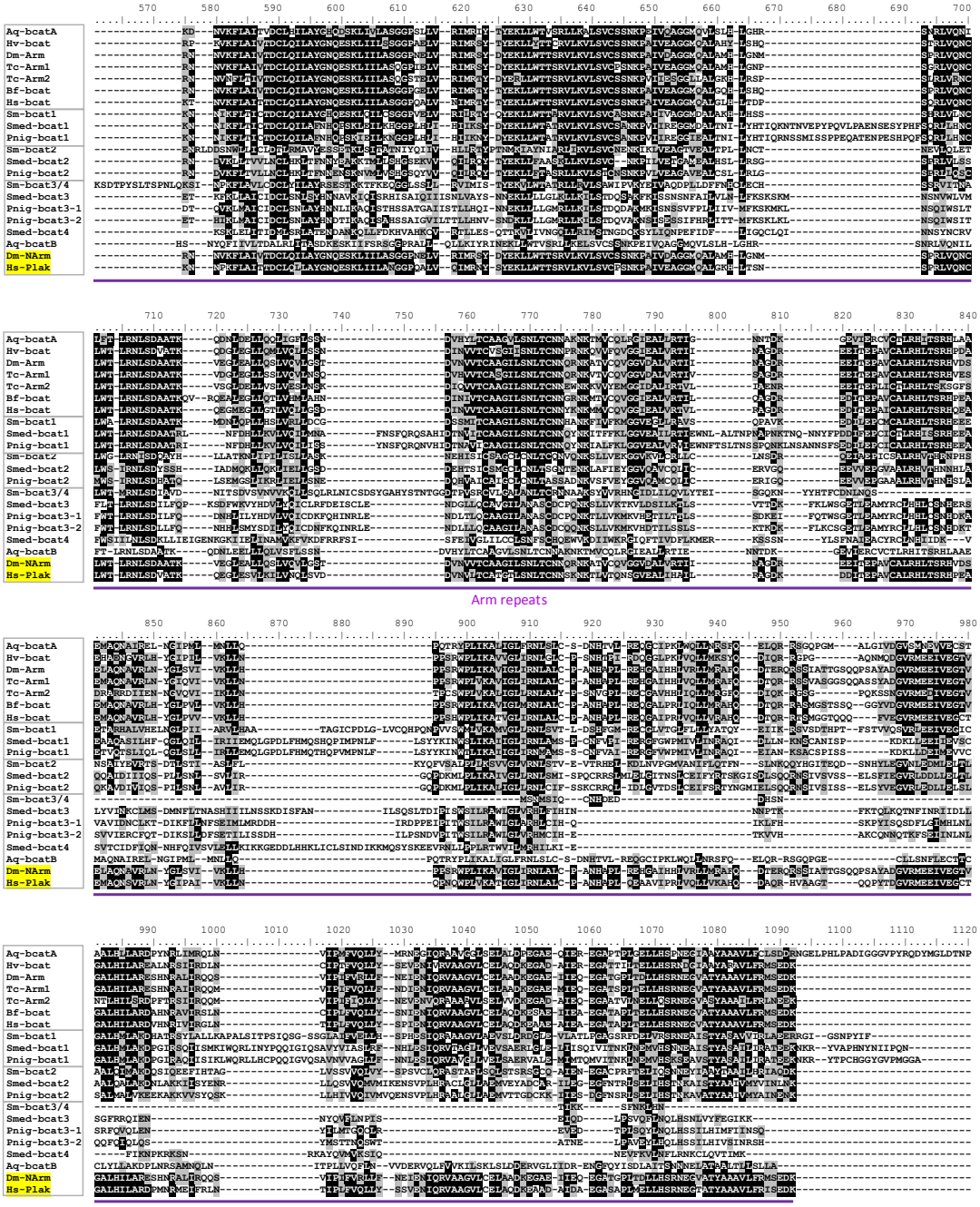


S10 Fig. Phylogenetic analysis of β-catenin proteins

β-catenin sequences from planarian species were identified in the Planmine database [14]. Platyhelminth species are squared in red. Planarian sequences are indicated with a planarian drawing. β-catenin proteins from species that show more than one β-catenin in the genome are labeled in yellow. From those, the ones that show a shorter C-terminal domain are marked in darker yellow. Accession number of the analyzed sequences: Ac-bcat NP_001191600.1; Aq-bcatA ADO16578.1; Aq-bcatB ADO16577.1; Bf-bcat XP_002588232.1; Ci-bcat BAA92185.1; Cl-bcat ABY21456.1; Crfor-bcat ADI48180.1; Cv-bcatAAL49497.1; Dm-Arm NP_476666.1; Dr-bcat1 NP_571134.2; Dr-bcat2 NP_001001889.1; Dr-Plak1 AAH58305.1, Dr-Plak2 XP_002665522.2; Hs-bcat NP_001895.1; Hs-Plak AAA64895.1; Hv-bcat AAQ02885.1; Mm-bcat NP_031640.1 NP_034723.1; Mm-Plak ; Osc-bcat AEC12440.1; Pd-bcat ABQ85061.1; Ph-bcat; Sci-BcatB ; Sci-BcatB; Sk-bcat NP_001158477.1; Sm-bcat1 XP_018651394.1; Sm-bcat2 XP_018646608.1, Sm-bcat3/4 XP_018649674.1; Smed-bcat1 ABW79875.1; Smed-bcat2 ABW79874.1; Sp-bcat NP_001027543.1; Tc-Arm1 EFA10737.1; Tc-Arm2 NP_001164124.1. β-catenin sequences from *Echinococcus multilocularis* and *Kronborgia cf. amphipodicola* were found in the available databases [19, 20]. β-catenin sequences from planarian species were found in the Planmine [14]. Accession numbers of the new planarian β-catenins are: β-cat3, KY196224; and β-cat4, KY196225. Abbreviations: Ac, *Aplysia californica*; Aq, *Amphimedon queenslandica*; Bf, *Branchiostoma floridae*; Ci, *Ciona intestinalis*; Cl, *Cerebratulus lacteus*; Crfor, *Crepidula fornicata*; Cv, *Chaetopterus variopedatus*; Dm, *Drosophila melanogaster;* Dr, *Danio rerio*; Em, *Echinococcus multilocularis*; Hs, *Homo sapiens;* Hv, *Hydra vulgaris*; Kram, *Kronborgia amphipodicola*; Mm, *Mus musculus*; Osc, *Oscarella carmela*; Pd, P*latynereis dumerilii*; Ph, *Parhyale hawaiensis*; Pnig, *Polycelis nigra*; Pten, *Polycelis tenuis;* Sci, *Sycon ciliatum*; Sk, *Saccoglossus kowalevskii;* Sm, *Schistosoma mansoni*; Smed, *Schmidtea mediterranea*; Sp, *Strongylocentrotus purpuratus;* Spol, *Schmidtea polychroa*; Tc, *Tribolium castaneum*.

Supplementary Figure 11




Aq-bcatA
Hv-bcat
Dm-Arm
Tc-Arm1
Tc-Arm2
Bf-bcat
Hs-bcat
Sm-bcat1
Smed-bcat1
Pnig-bcat1
Sm-bcat2
Smed-bcat2
Pnig-bcat2
Sm-bcat3/4
Smed-bcat3
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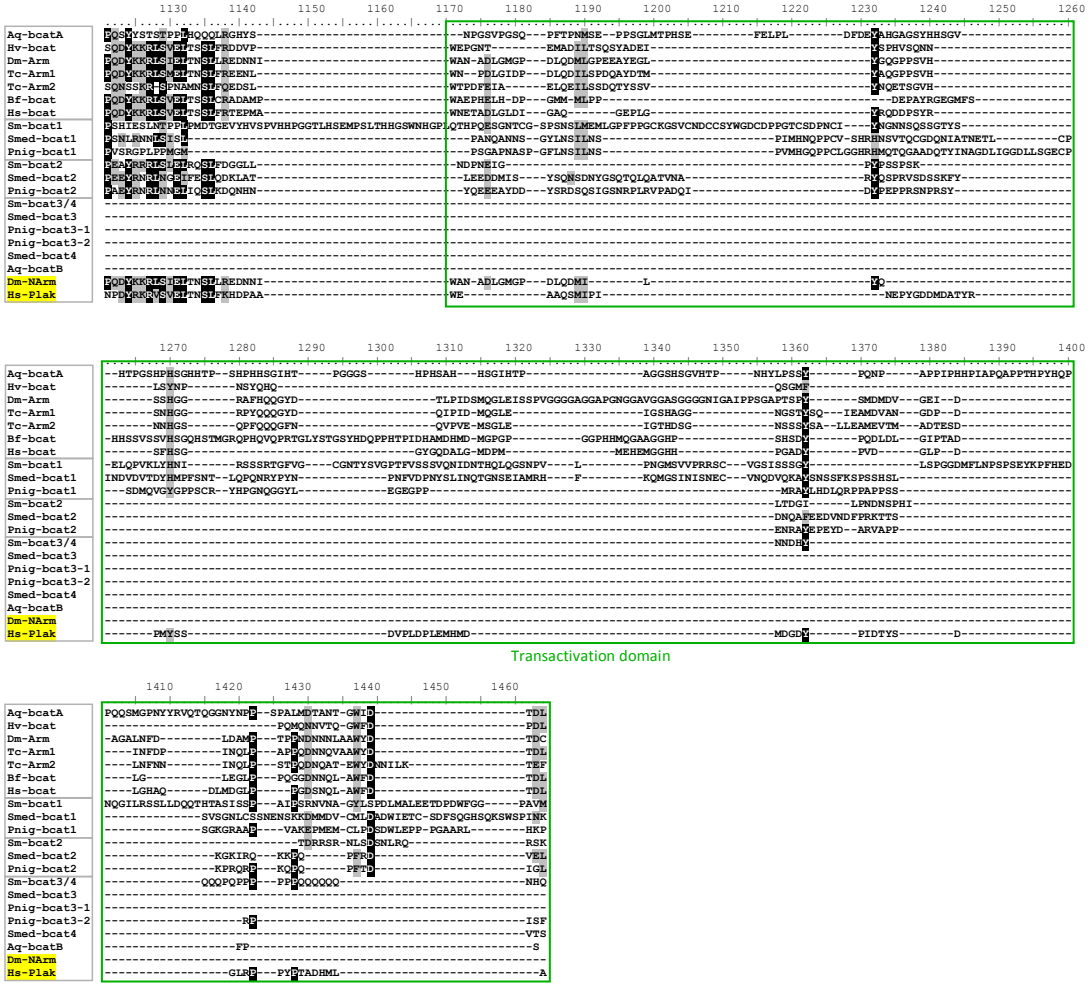
Dm-NArm
Hs-Plak
Arm repeats



S11 Fig. Alignment of β-catenin protein sequences which show a shorter C-terminal transactivation domain.

The functional domains are indicated. Arm splicing isoform (Neural Arm, accession number AAB58731.1) and Plakoglobin, highlighted in yellow, conserve all functional domains but show a shorter C-terminal transactivation domain. Accession numbers and abbreviations are indicated in Fig S10.

Supplementary Table 1

gene	Primer sequences	
	for probe	for dsRNA
β-catenin1	Iglesias et al. 2008	Iglesias et al. 2008
β-catenin3	5'-ATGGATTTAAGTGCTCAAATCGATTCGTCATC-3'	5'-TGCTCTATTGGGTGAGCGGATGTTTTCA-3'
	5'-CATTAATCAAGAGGTAGAAAATGAAGTTTGTTTG-3'	5'-ACTCCCCGATACGGACAGGGACAGGT-3'
β-catenin4	5'-TTTGCTGGCTTTGATGAAACTAAATATAAATC-3'	5'-AAGCATGTCGCTCACAAATGCGTTCG-3'
	5'-TCATGAAGTTACCTTCATAATGGTTACCTG-3'	5'-TTCTCCGATTTCAATCAGCAGCTTATCGG-3'
TCF-1	5'-TCACCATTGAAGCCCACTCC-3'	
	5'-TAGGTGGTACCGAAGGCTCA-3'	
TCF-2	5'-ATGGCATGAGTTGTCTCGGG-3'	5'-TGCGCCATGCTCGAATAGAT-3'
	5'-ACTGGCGGTTTGATCGTCAT-3'	5'-ACTGGCGGTTTGATCGTCAT-3'
TCF-3	5'-GCGTTGACTCGAGAAGACCA-3'	
	5'-GGTCAGCCGATAGATTCCCG-3'	
APC-1		Iglesias et al. 2010

gene	Primer sequences
	For Real time PCR
β-catenin1	5'-CAAGAGCCCATCATCTCA-3'
	5'-CGTCCAACATACATACGTCC-3'
β-catenin2	5'-GGCACTTCGTCATGTTACC-3'
	5'-CACGAATCAGCACCGATA-3'
β-catenin3	5'-TCGACCACAATGACAGTTCT-3'
	5'-TGTGAAATCGATGAGTGCTT-3'
β-catenin4	5'-TACCTCCTCTTCGCACTTG-3'
	5'-ACTTGGTAGGCTTTTCGATTT-3'
APC-1	5'-CAAACGGATGGCGTTAC-3'
	5'-ATGAGGAACTACTGCGGG-3'
TCF-2	5'-CATGGCAAAATCAAACAGG-3'
	5'-CGATAAACGAAGGGGTTCT-3'
URA4	5'-TTCACGTTGTCGATCTAGCC-3'
	5'-CGAATATCCTCTGCCAGTGC-3'

S Table 1. Primers used for dsRNA and ssRNA synthesis and for qPCR analysis.

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Artículo 4:

βcatenin-1 dynamics during the embryonic development of the planaria Schmidtea polychroa.

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Manuscrito en preparación

Resumen en castellano:

Dinamica de la βcatenina-1 durante el desarrollo embrionario de la planaria *Schmidtea polychroa*.

El rol de la vía de señalización Wnt canónica ha sido extensamente estudiado durante la embriogénesis y la homeostasis del adulto en multitud de metazoos. En los últimos años , las planarias se han convertido en un sistema modelo importante para estudiar la vía Wnt canónica durante su desarrollo en el adulto. Durante la regeneración y la homeostasis de las planarias, la vía Wnt canónica especifica la identidad posterior y participa en la organogénesis. Sin embargo, el rol de esta vía de señalización durante la embriogénesis de planaria no está claro. En este estudio, hemos caracterizado la dinámica de distribución de la proteína βcatenina-1 durante la embriogénesis de la planaria. Mostramos que la βcatenina-1 se nuclearíza en los primeros estadios en algunas células vitelinas y en las primeras estructuras endomesodérmicas. Además, mostramos que durante la metamorfosis la βcatenina-1 se nuclearíza de forma masiva en diferentes tipos celulares. Finalmente, a partir de estadio 5 en adelante, la distribución βcatenina-1 es muy similar a la que encontramos en adultos, siendo nuclearizada en diferentes órganos. En definitiva, nuestros resultados sugieren que la βcatenina-1 podría ejercer un papel señalizador durante los primeros estadios del desarrollo, durante la especificación de las capas germinales, al igual que en otros organismos, y que a partir de la metamorfosis los procesos celulares y moleculares observados son comparables a los descritos en planarias adultas. El estudio descriptivo de la localización subcelular de la βcatenina-1 durante el desarrollo embrionario de las planarias aporta nuevos datos para comprender mejor las diferentes etapas de este proceso.

βcatenin-1 dynamics during the embryonic development of the planaria Schmidtea polychroa.

Abstract

The role of canonical Wnt signaling during embryogenesis and adult homeostasis is very well known in a multitude of metazoa. In the last years, planarians have become an important model system to study canonical Wnt signaling in adult planarian development. During planarian regeneration and homeostasis, canonical Wnt signaling specifies posterior identity and participates in the organogenesis. However, the role of this major pathway during planarian embryonic development remains unclear. Here, we characterized the βcatenin-1 protein dynamics during planarian embryogenesis. We show that βcatenin-1 is nuclearized in yolk cells in the first endomesodermal structures. Moreover, during metamorphosis βcatenin-1 is massively nuclearized en different cell types. Finally, from stage 5 onwards we show a similar distribution of βcatenin-1 protein as in adult planarians, being nuclearized in different organs. Our results suggest that βcatenina-1 could have a signaling role during the specification of the germ layers at early embryonic stages, as described in most organisms, and that the cellular and molecular mechanisms observed during metamorphosis are comparable to ones described in adult planarians. The descriptive analysis of βcatenina-1 localization during planarians embryogenesis has provided clues for a better understanding of the different stages of planarian embryogenesis.

Introduction

During embryonic development the canonical Wnt pathway, which key intracellular component is the transcription factor βcatenin, controls multiple events mainly including cell fate (Clevers and Nusse, 2012). The activation of the pathway through the binding of the Wnt ligand with their receptor and co-receptor frizzled and lrp6 respectively, triggers the inhibition of the βcatenin destruction complex, allowing the nuclearization and functional activation of βcatenin (Macdonald and He, 2012; Stamos and Weis, 2013). During early embryogenesis βcatenin nulcearization specifies the site of gastrulation and the formation of the endomesoderm (Byrum and Wikramanayake, 2013; Darras et al., 2011; Henry et al., 2008; Wikramanayake et al., 2003). βcatenin exerts also an evolutionary conserved role in body patterning, promoting and specifying the posterior fates of most animals studied (Petersen and Reddien, 2009). During organogenesis βcatenin controls the cell fate of multiple tissues and cell lineages (Ciani and Salinas, 2005). The canonical wnt signaling is also essential during adult homeostasis, for instance for proper cell renewal in the gut system (Beumer and Clevers, 2016). Thus, βcatenin malfunction leads colon cancer, among others (Clevers, 2006; Grigoryan et al., 2008).

During the last years, adult planarians have become an important animal model to stu-

dy and elucidate how Wnt/βcatenin dependent signaling controls different homeostatic and regenerative processes (Almuedo-Castillo et al., 2012; Gurley et al., 2008; Gurley et al., 2010; Iglesias et al., 2008; Petersen and Reddien, 2008; Sureda-Gómez et al., 2015; Sureda-Gómez et al., 2016). It has been demonstrated that canonical Wnt signaling specifies posterior identity in adult planarians (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008). Moreover, it was suggested that a nuclear βcatenin-1 gradient establishes the different identities along the anteroposterior (AP) axis (Sureda-Gómez et al., 2016). Furthermore, recent studies indicate that βcatenin dependent signaling participates during organogenesis of different tissues such as the nervous system (Hill and Petersen, 2015; Owen et al., 2015; Sureda-Gómez et al., 2016), or the visual system (Owen et al., 2015)(Su et al., unpublished). While in adult planarians our knowledge about the role of the canonical Wnt signaling is broad and is an active area of research, little is known about the role of this major signaling pathway during planarian embryogenesis that is the time at which the body axis and different cell types are specified.

Planarian embryogenesis shows significant differences with respect to the typical spiralian development, since micromeres and macromeres cannot be distinguished (Thomas, 1986). Planarians lay cocoons, which are polyembryonic eggs, containing 1-5 embryos. Inside the cocoon, the blastomeres, the embryonic cells of planarians, of each embryo display a so called "blastomere anarchy"(Stevens, 1904). They remain detached from each other into the mass of yolk cells (Baguñà and Boyer, 1990). Then, some yolk cells together with blastomeres fuse and form a syncytium. The syncytium will give raise to a larva, with an embryonic ciliated epidermis and a pharynx, which remains inside the cocoon. This larva eats the surrounding yolk cells and after 4 days it suffers a metamorphosis, in which the transient structures are substituted by the definitive structures of the adult planaria (Cardona et al., 2005).

The embryonic development of planarians is relatively well documented thanks to studies from the past decade, especially the process of organogenesis that takes place during metamorphosis (Martín-Durán and Romero, 2011; Martín-Durán et al., 2010; Martín-Durán et al., 2012b; Monjo and Romero, 2014). However, which signaling pathways are involved in the processes of organogenesis and especially at the early embryonic stages, remain unknown. Here, we aimed to elucidate the role of the canonical Wnt signaling pathway during planarian embryonic development through the study of the subcellular localization of βcatenin-1.

Taking advantage of a specific antibody against βcatenin-1 previously generated (Sureda-Gómez et al., 2016), we analyzed the subcellular localization of βcatenin-1 protein during the different embryonic stages of the planarian Schmidtea polychroa. The inmunohistochemical results show highly dynamic distribution changes of βcatenin-1 trough embryonic development. The first nuclearization of βcatenin-1 occurs in few yolk cells, suggesting a maternal role of the protein. At this early stage some blastomeres also begin to nuclearize βcatenin-1. Later, βcatenin-1 is highly nuclearized in the cells that form the primordium of the transient embryonic pharynx and gut, which could suggest its evolutionary conserved role during endomesodermal specification. During metamorphosis and the formation of the definitive organs βcatenin-1 is massively nuclearized in embryonic cells, which suggests its conserved role in cell fate specification.

At metamorphosis, the pattern of βcatenin-1 expression resembles the one of adult planarians, being localized more in the ventral region than in the dorsal and especially in the developing adult organs. Although our approach is mainly descriptive, it contributes significantly to the understanding of the molecular and cellular mechanism of planarian embryonic development.

Results

1. βcatenin-1 is a maternal protein first nuclearized in yolk cells and then in a subset of blastomeres

In order to study the localization of the βcatenin-1 protein through the different developmental stages, we followed the stages proposed by Martin-Duran and collaborators (Martín-Durán et al., 2010) (SFig. 1). Stage 1 corresponds to the process that extends from laying the cocoon, which contains 1 to 5 zygotes per egg in a mass of yolk cells, to the formation of the early larva with the embryonic gut and pharynx primordium and the epidermis. During early stage 1, before syncytium formation, blastomeres, which remain isolated and detached from each other, do not show βcatenin-1 protein in the cytoplasm or in the nucleus (Fig. 1A). However, few yolk cells near to the blastomeres present βcatenin-1 in the nucleus (Fig. 1A). Since these yolk cells are deposited by the mother, thus they have a maternal origin (Baguñà and Boyer, 1990), this result indicates that βcatenin-1 could act as a maternal protein. βcatenin-1 in the yolk cells could have a role in signaling to the blastomeres.

After the formation of the syncytium, the first βcatenin-1 signal in blastomeres was visualized. However it was not nuclear but cytoplasmatic (Fig. 1A and SFig. 2A). Importantly, in some embryos few elongated blastomeres, showed βcatenin-1 protein in the nucleus (Fig. 1A). The elongated shape of the blastomeres with nuclear βcatenin-1, compared to the rounded shape of blastomeres with cytoplasmatic βcatenin-1, suggests that the first one correspond to migrating cells, and that nuclearization of βcatenin-1 promotes the earliest asymmetry in planarian embryos. This hypothesis would agree with the evolutionary conserved role of βcatenin signaling in specifying the site of gastrulation. At this stage, yolk nucleus positive for βcatenin-1 inside the syncytium are still detected (Fig. 1A).

2. βcatenin-1 is highly nuclearized in the embryonic gut and pharynx

During late stage 1, few blastomeres migrate towards the periphery to give raise to the larva, mainly composed by the embryonic epidermis and to the embryonic pharynx (Cardona et al., 2005). During this process it is though that migrating blastomeres first form the primordium of the embryonic pharynx, and, simultaneously, the transient embryonic gut appears beneath it as an overture where the embryo will store the ingested yolk (Martín-Durán and Romero, 2011). Interestingly, at this stage, the blastomeres that conform the pharynx primordium and the transient gut present βcatenin-1 in the nucleus (Fig. 1B and SFig. 2B). These data suggest that βcatenin-1 could specify the first endomesodermal structures (pharynx and gut) in planarians, as described in most metazoans (Petersen and Reddien, 2009).

In addition, we found few blastomeres in the periphery of each larva showing nuclear

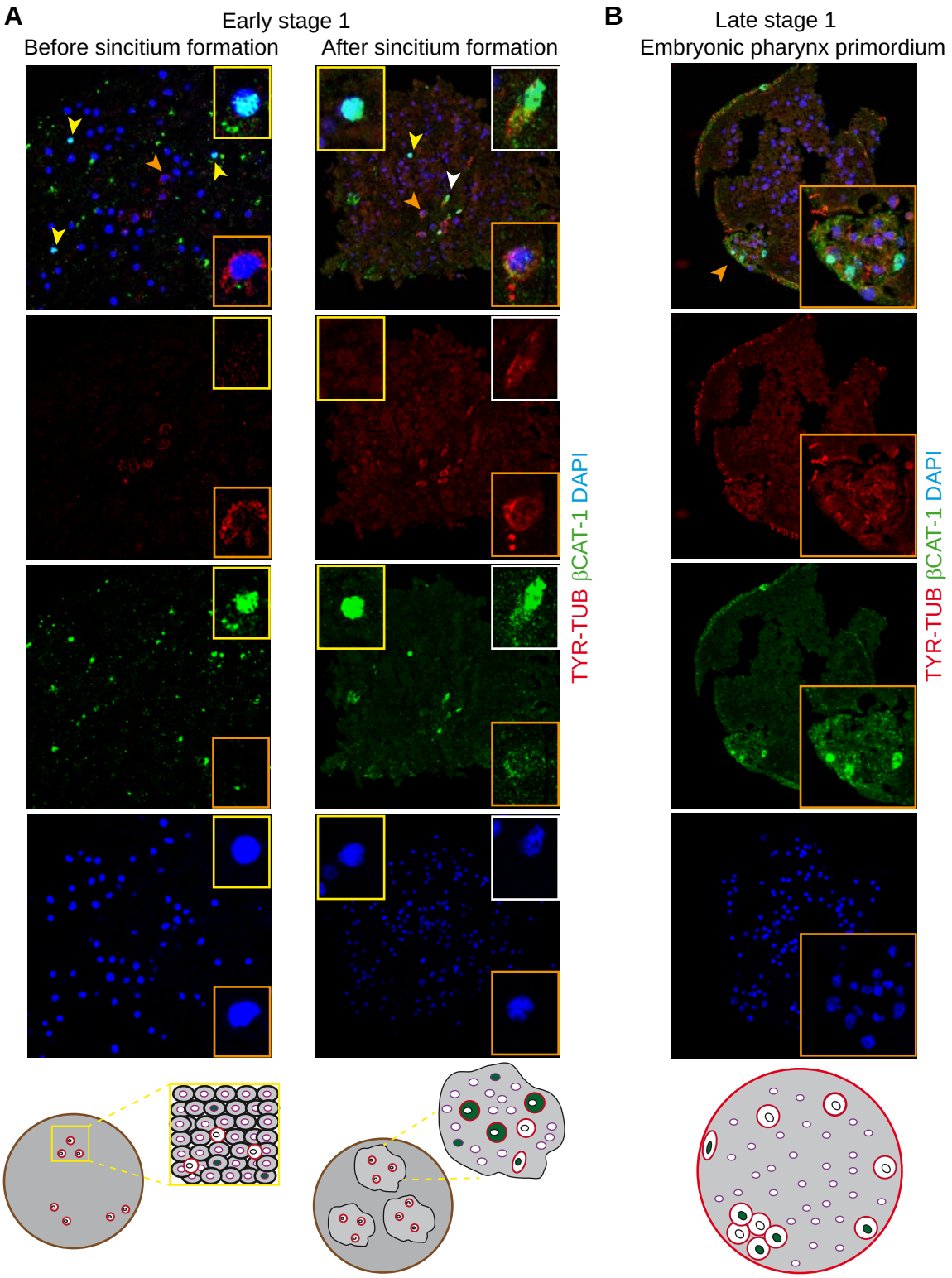

Figure 1. βcatenin-1 is nuclearized in yolk cells and in the pharynx primordium. (A-B) Z-projection of confocal stacks of an stage 1 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). Yellow arrowhead indicates yolk cell shown at higher magnification in insets. Orange arrowhead indicates blastomeres shown at higher magnification in insets. White arrowhead indicates migrating blastomere shown at higher magnification in insets. Draws summarize the images. Green color indicates presence of βcatenin-1.

βcatenin-1 (Fig. 1B). Its location, just underneath the epidermal layer, and nuclear shape (rounded) suggest that they could be precursors of epidermal cells.

At stage 2, the embryo has completely formed the embryonic pharynx and the embryonic epidermis. At this stage nuclear βcatenin-1 continues to be found in pharynx cells and in the presumptive progenitors of the epidermis, but never in the differentiated epidermal cells (Fig. 2A). We still detect few blastomeres located in the transient gut positive to βcatenin-1 in the nucleus (Fig. 2A).

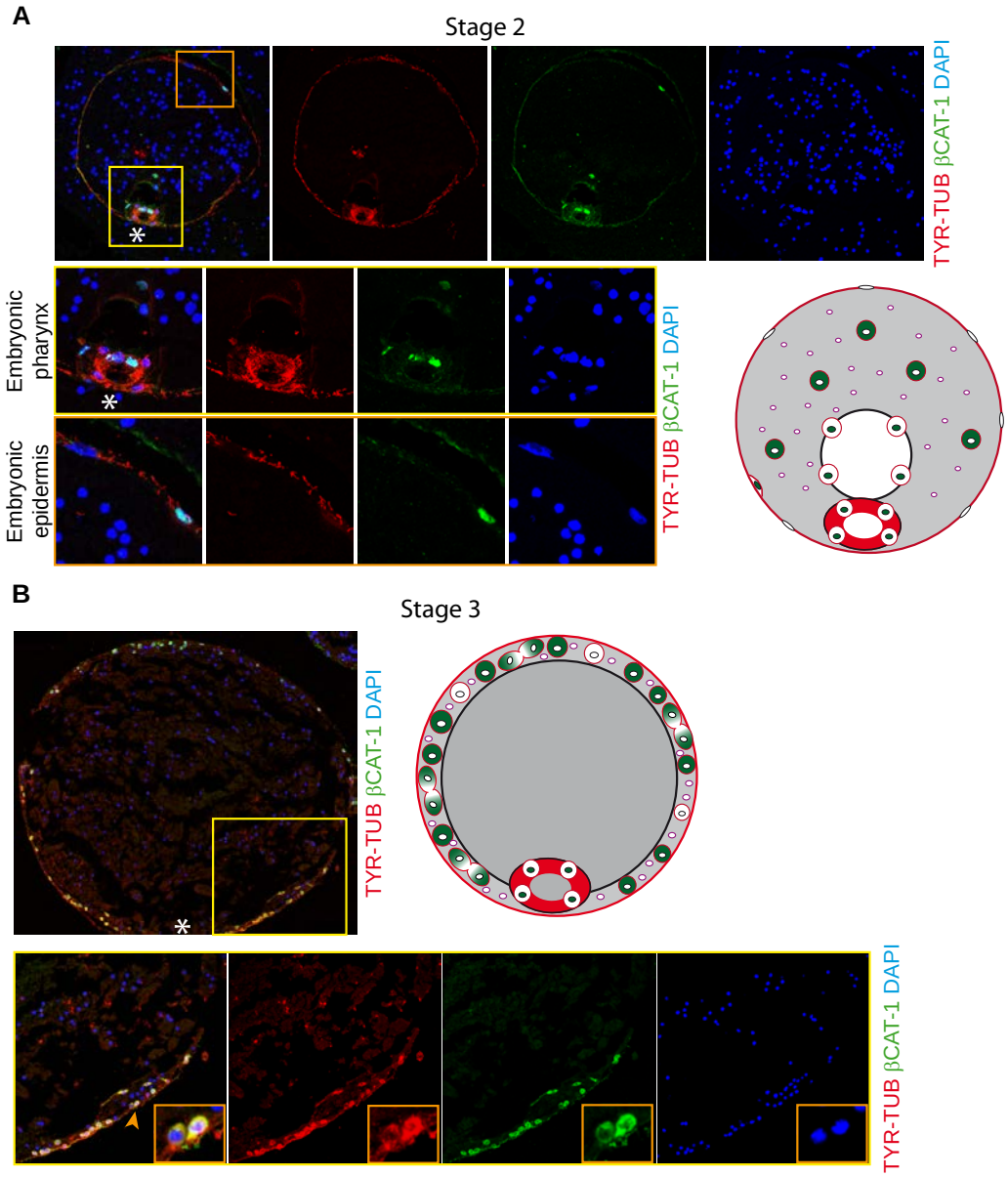

Figure 2. βcatenin-1 is nuclearized in the embryonic pharynx and remains cytoplasmatic in the rest of blastomeres. A-B) Z-projection of confocal stacks of an stage 2 (A) and stage 3 (B) embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). Yellow and orange squares indicate higher magnification in insets. Orange arrowhead indicates blastomeres shown at higher magnification in insets. White asterisk indicates pharynx location. Draws summarize the images. Green color indicates presence of βcatenin-1.

3. During the growth of the larva blastomeres show cytoplasmatic βcatenin-1 and share it equally during division.

As development proceeds, during stage 3 the larva starts feeding the surrounding yolk cells. The ingestion of yolk displaces blastomeres towards the periphery of the larva, giving raise to the germ band, a multilayer of blastomeres and yolk nucleus (Cardona et al., 2005; Martín-Durán et al., 2010). During this stage blastomeres start to divide in the germ band to increase the size of the larva. We found that with exception of the embryonic pharynx cells, which still present nuclear βcatenin-1 (SFig. 2), the vast majority of the blastomeres in the germ band show βcatenin-1 in the cytoplasm (Fig. 2B). Interestingly, during mitosis blastomeres share cytoplasmatic βcatenin-1 equally. This result suggests that during stage 3, which mainly consists in the increase in size of the larva with no differentiation of specific cell types, the mitosis of blastomeres are symmetric.

4. During early metamorphosis βcatenin-1 is massively nuclearized, coinciding with the specification of the adult cell types.

After the ingestion of the surrounding yolk cells, the larva suffers a metamorphosis which consists in the disappearance of the embryonic tissues and the differentiation of the definitive or adult tissues and organs. This process starts at stage 4 and ends after the embryo hatches the egg and appears the juvenile, showing all the adult traits but the sexual organs (Cardona et al., 2005; Martín-Durán et al., 2010).

At early stage 4, we found some blastomeres migrating from the germ band towards the inside of the larva. Interestingly those migrating blastomeres showed nuclear βcatenin-1 (SFig. 3A), being the first blastomeres of the germ band that nuclearize it. Afterwards, at late stage 4, when the first definitive structures are formed (Cardona et al., 2005) a massive nuclearization of βcatenin-1 was observed in the embryos. Interestingly, in the transition from stage 4 to 5, several cell types, according to the subcellular localization of βcatenin-1 could be distinguished: blastomeres with no expression of βcatenin-1, blastomeres with cytoplasmatic βcatenin-1 and blastomeres showing nuclear βcatenin-1 (Fig. 3A). An important observation was that during this stage both symmetric and asymmetric divisions in terms of βcatenin-1 distribution were visualized (Fig. 3A). This result could suggest that the appearance of asymmetric divisions is linked to the process of cell specification, since at this stage the different cell types that will give rise to the adults are specified (Cardona et al., 2005; Martín-Durán and Romero, 2011; Monjo and Romero, 2014).

A further observation during this stage was that blastomeres were distributed asymmetrically in the embryo, thus most of the blastomeres were accumulated in one half of the embryo, which was the one where the embryonic pharynx was located. According to Monjo and Romero this side of the embryo would correspond to the ventral, were the nervous system is developed (Monjo and Romero, 2014). Interestingly, nuclear βcatenin-1 was mainly found in the blastomeres of this side of the embryo (Fig. 3B and SFig. 3B). This result agrees with the ventral enrichment of βcatenin-1 found in adult planarians (Sureda-Gómez et al., 2016), and could be related with the process of neurogenesis (Monjo and Romero, 2014).

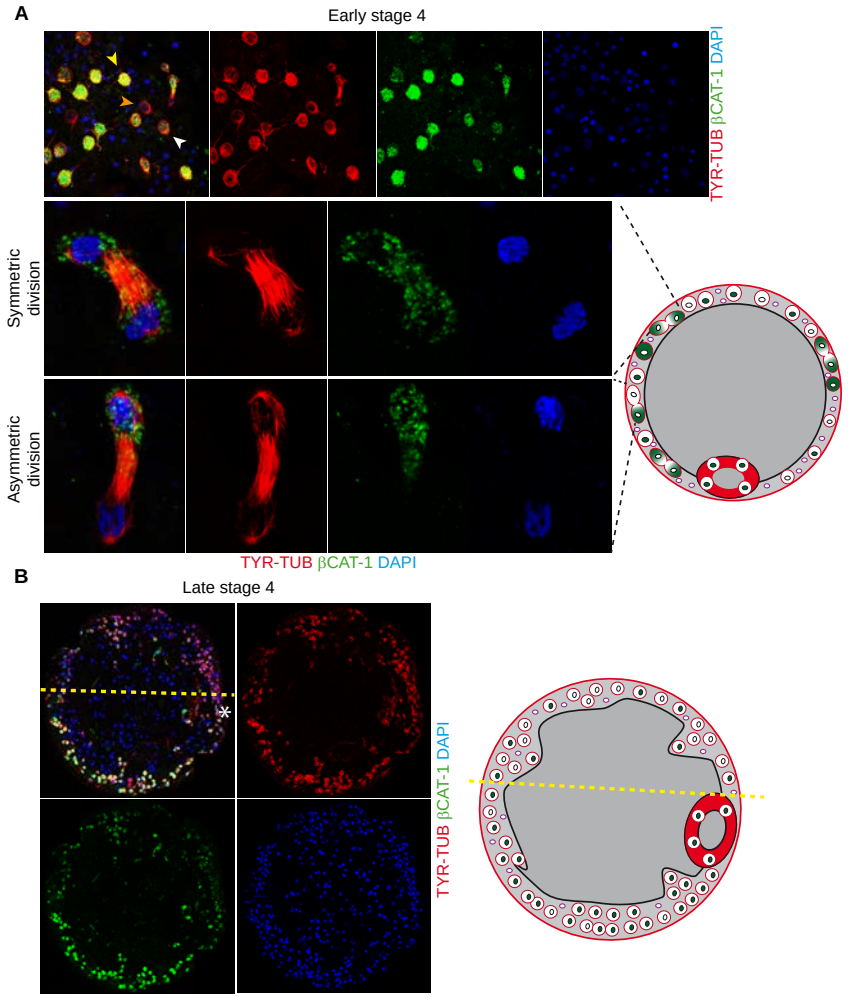

Figure 3. βcatenin is massively nuclearized in blastomeres and show asymmetric distribution. A) Z-projection of confocal stacks of an early stage 4 embryo from an immunohistochemistry on whole mount (A) and a late stage 4 embryo from an immunohistochemistry on paraffin sections (B) against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). Yellow arrowhead indicates blastomeres with nuclear βcatenin-1. White arrowhead indicates blastomeres with cytoplasmatic βcatenin-1. Orange arrowhead indicates blastomeres negative for βcatenin-1. Yellow dashed line indicates the presumptive dorsoventral border. White asterisk indicates pharynx location. Draws summarize the images.

5. During late metamorphosis βcatenin-1 is localized in the nucleus of many cells which end up restricted to specific organs, showing a similar pattern than in adult planarians.

From stage 5 onwards the larva will develop the definitive tissues that appear in the juvenile. At this stage the processes of polarity determination and organogenesis can be compared with the ones described in the adult (Martín-Durán et al., 2010; Martín-Durán et al., 2012a). Thus, at this stage the first markers of axial polarity and differentiated cell types are detected (Martín-Durán et al., 2010). With respect to axial polarity, it has been reported that in adult planarians a nuclear gradient of βcatenin-1 from the prepharynx region to the tip of the tail is the responsible to specify the AP axial identities (Sureda-Gómez et al., 2016). In our immunostainings, at stage 5 βcatenin-1 shows a pattern very similar to the one observed at stage 4 (Fig. 4A), and we could not detect any apparent AP gradient. However, it is also reported that in adult planarians βcatenin-1 is involved in organogenesis, and it is found nuclearized in organs as the pharynx and the CNS (Sureda-Gómez et al., 2016). The massive nuclearization of βcatenin-1 observed at this

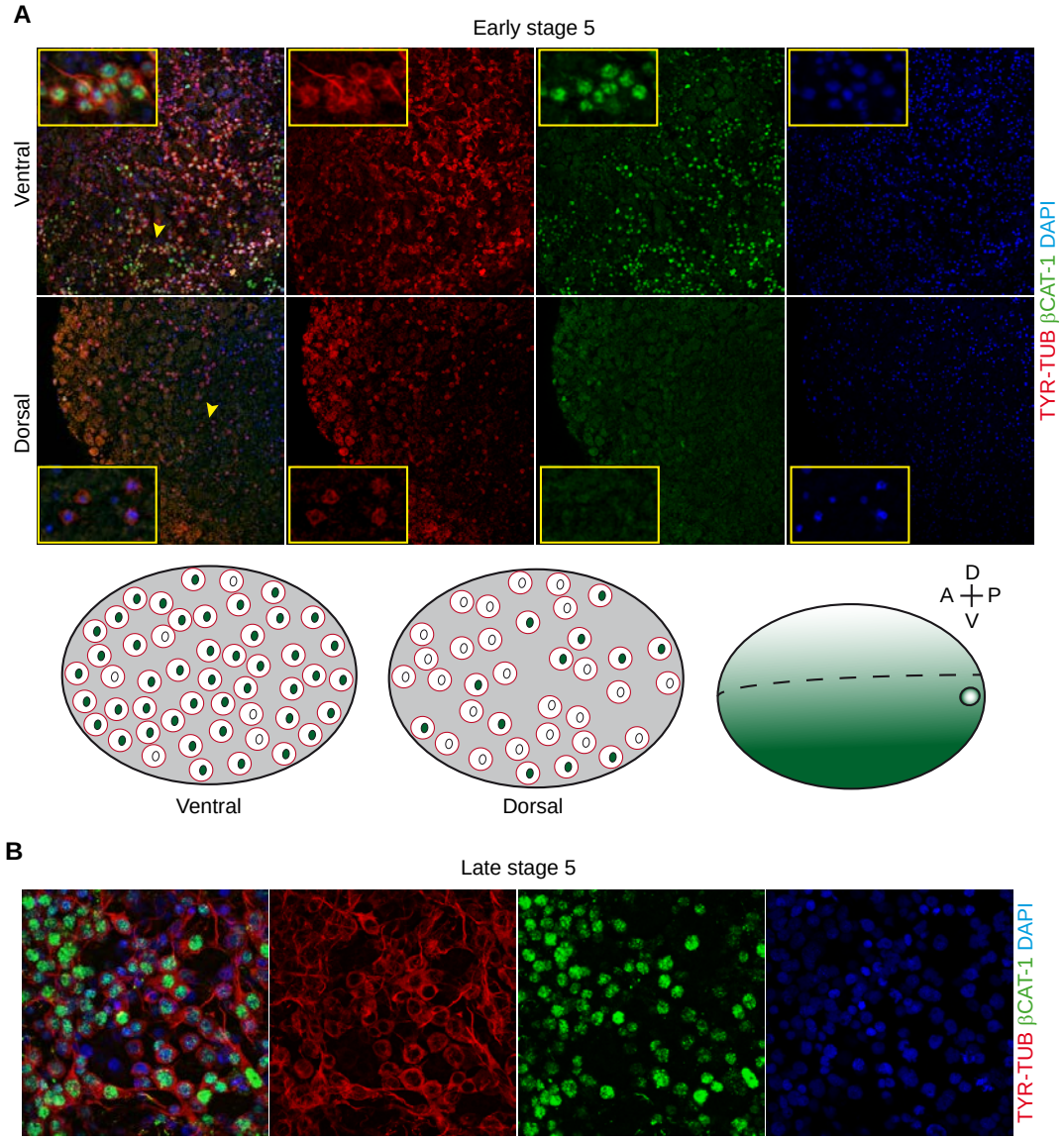

Figure 4. βcatenin-1 is higly nuclearized and preferentially located in the ventral region. A-B) Z-projection of confocal stacks of an early and late stage 5 embryos from an immunohistochemistry on whole mount against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). Yellow arrowhead indicates areas shown at higher magnification in insets. Draws summarize the images.

stage 5 agrees with a role of βcatenin-1 in cell type specification and organogenesis. In fact, its accumulation in the ventral side (Fig. 4A) suggests its role during the embryonic development of the CNS, in agreement with its described requirement during regeneration of the CNS in adult planarians (Sureda-Gómez et al., 2016). For that reason, although any apparent AP gradient of βcatenin-1 could be observed, βcatenin-1 could have also a role in AP axial specification in planarian embryos, since the gradient could be hidden by the large amount of βcatenin-1 positive cells found through the embryo at this stage.

At late stage 5 the cephalic glanglia, the ventral nerve cords and the muscular system became apparent through the labeling with specific markers (Monjo and Romero, 2014). During this stage we found a variety of embryonic cells with different size and

shape presenting different amount of nuclear βcatenin-1 (Fig. 4B), as described in adult planarians (Sureda-Gómez et al., 2016), which suggest that βcatenin-1 is found in different cell lineages. Interestingly, from late stage 5 onwards, cells showing cytoplasmatic βcatenin-1 are not found, a situation also found in adult planarians (Sureda-Gómez et al., 2016). Taking into account that cytoplasmatic βcatenin-1 is mainly found in early embryos, this data suggests that the ability to maintain cytoplasmatic βcatenin-1 is restricted to early blastomeres (embryonic cells) and is not found in adult cells.

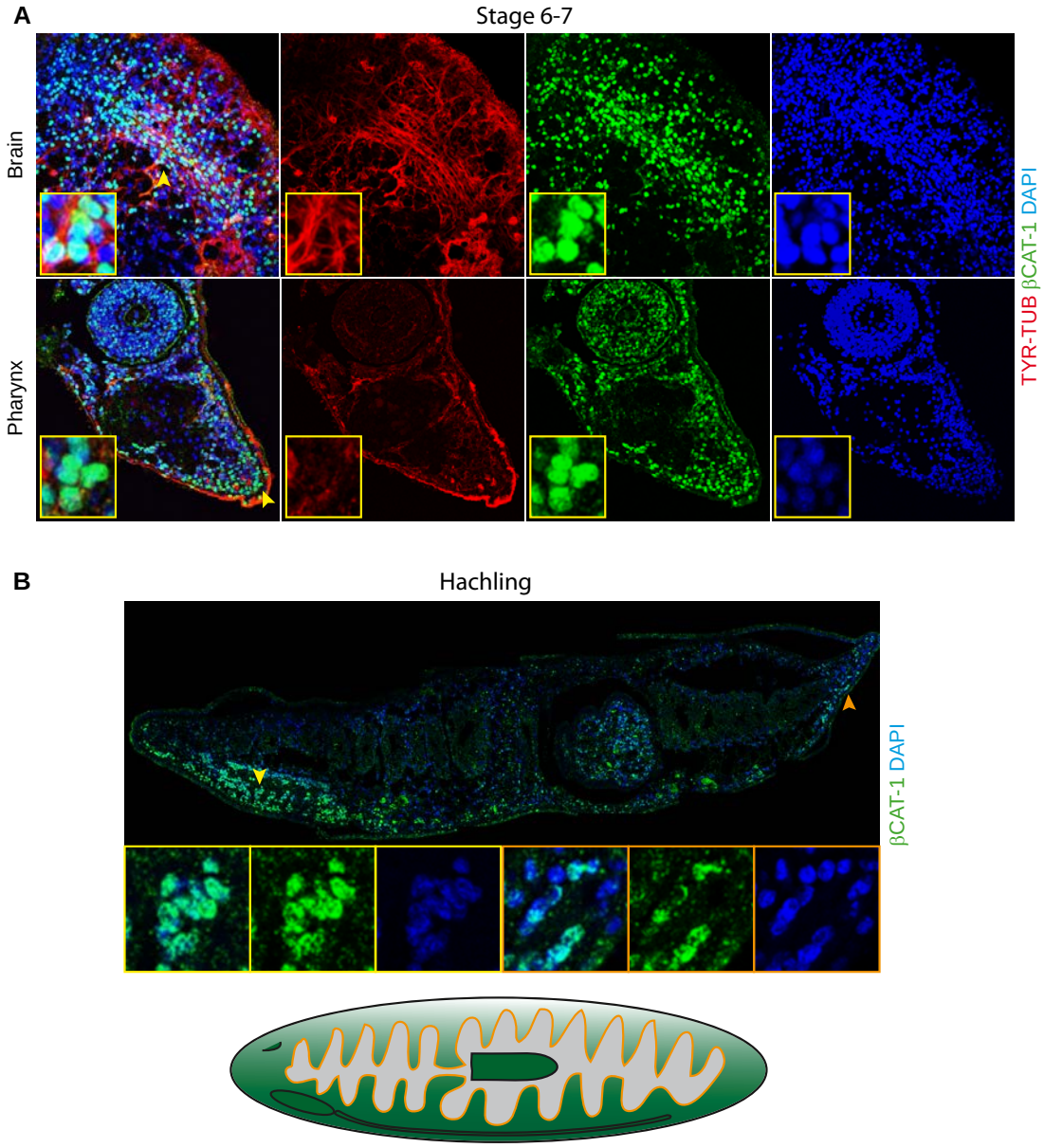

Figure 5. βcatenin-1 is found in the nucleus of diferent organs. A) Z-projection of confocal stacks of a late stage 4 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red). Nuclei are labeled with DAPI (blue). B) Reconstructed image corresponding to Z-projection of confocal stacks of a late stage 4 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green). Nuclei are labeled with DAPI (blue). Yellow and orange arrowheads indicate areas shown at higher magnification in insets. Draws summarize the images.

From stage 5 onwards, embryos consolidate the definitive adult organs, as the brain, nerve cords, eyes and pharynx (Martín-Durán and Romero, 2011; Martín-Durán et al., 2010; Martín-Durán et al., 2012b; Monjo and Romero, 2014). At these stages we found nuclear βcatenin-1 in different developing organs as brain or pharynx, as described in adult planarians (Fig. 5A and SFig. 4).

After fifteen days, the embryo will hatch the capsule showing the juvenile traits (Cardona et al., 2005; Martín-Durán et al., 2010). Inmunohistochemistry against βcatenin-1 in hatchling planarians shows the same pattern of nuclear βcatenin-1 compared with adult planarians (Fig. 5B) (Sureda-Gómez et al., 2016). Nuclear βcatenin-1 is detected in the definitive brain, pharynx and digestive system. The accumulation of βcatenin-1 nuclear positive cells remains in the ventral side. However, at this stage the accumulation of cells with nuclear βcatenin-1 in the posterior parenchyma is not observed (Fig. 5B), as described in adults.

Discusion

βcatenin-1 and its role during planarian early embryonic development

Canonical or βcatenin-dependent Wnt signaling exerts multiple functions during early development in all metazoan, but little is known about the role of βcatenin in embryonic yolk cells. In Zebrafish, it has been demonstrated a role of βcatenin in the yolk sincitial layer (YSL) (Kelly et al., 2000), which is involved in the formation of the zebrafish early organizer, the shield organizer that acts as the Nieuwkoop center in *Xenopus* (Joubin and Stern, 2001). Interestingly in both cases, in Zebrafish and in *Xenopus*, βcatenin acts as a maternal protein and is necessary for the organizer activity. In planarians, some authors have shown that yolk cells are transcriptionally active (Cardona et al., 2005; Le Moigne, 1966). Moreover, Martin-Duran and collaborators have shown high levels of *βcatenin-1* expresion during early stage 1 by qPCR, but they did not report the expression pattern at this stage (Martín-Durán et al., 2010). We found a specific nuclearization of βcatenin-1 in few yolk cells surrounding the blastomeres before and after the syncytium formation (Fig. 1). These results suggest that yolk cells with nuclear βcatenin-1 could act as inductors of the syncytium formation and/or the progression of the early cleavage of blastomeres. From cellular and morphological studies, a role of nuclei yolk in signaling during larva formation providing maternal determinants was already proposed (Cardona et al., 2005; Le Moigne, 1966). Whether these yolk cells and yolk nuclei positive for βcatenin-1 could act as an organizer center and confer axial identities in planarians requires further experiments.

In most bilaterians, βcatenin is nuclearized in blastoporal cells and specifies the endomesoderm, for example in sea urchin (Logan et al., 1999), *P. dumerelys* (Schneider and Bowerman, 2007), *C. elegans* (Korswagen et al., 2000) or mouse (Huelsken et al., 2000). In planarians, we found nuclear βcatenin-1 in the primordium of the embryonic pharynx and gut, the first endomesodermal structures (Fig. 1; Fig. 2). These results agrees with previously studies of *βcatenin-1* expresion (Martín-Durán et al., 2010) and strongly suggest that βcatenin-1 has a conserved role in specifying the endomesoderm

during planarian embryogenesis. To clarify this possibility it would be necessary to analyze whether the first blastomeres that nuclearize βcatenin-1 and establish the first asymmetric population (Fig. 1) are the ones that give raise the pharynx primordium (endomesoderm).

It is important to mention that we observed blastomeres under the embryonic epidermis that also presented nuclear βcatenin-1, which probably do not contribute to endomesdermal tissues (Fig. 2A). Their location just underneath the epidermis and its elongated shape suggest that those blastomeres could be the epidermal progenitors. Since we never found differentiated epidermal cells with nuclear βcatenin-1, it could be that βcatenin-1 is not involved in terminal differentiation of epidermal cells but in determination of the epidermal fate. Alternatively, it could be that nuclear βcatenin-1 confers migration properties, which would agree with the finding of the first elongated blastomers showing also nuclear βcatenin-1 (Fig. 1) (Gonzalez and Medici, 2014).

An interesting observation in early embryos was the finding that the blastomeres in the germ band localized βcatenin-1 in the cytoplasm and that they distributed it equally to both daughter cells (Fig. 3). In contrast, at later stages (Stage 4) the situation was completely different, since dividing cells were not so abundant and, although in most cases βcatenin-1 was distributed symmetrically, in some cases we could observe asymmetric distribution of cytoplasmatic βcatenin-1 (Fig. 3). It was already described that during the first embryonic stages blastomeres were continuously proliferating (Cardona et al., 2005; Martín-Durán et al., 2010). However, there was any evidence of symmetric or asymmetric mitosis, since until stage 5 blastomeres show the same morphology. In fact, it was assumed that the purpose of the active mitotic activity in early embryos was to expand the undifferentiated population (Cardona et al., 2005; Martín-Durán et al., 2010). However, our data suggests that although at stage 3 it is true that only symmetric divisions in terms of βcatenin-1 are found, probably related with the rapid increase in size of the larva, at stage 4 asymmetric divisions are observed, coinciding with the time when the genes involved in cell fate determination start to be expressed (Martín-Durán and Romero, 2011; Monjo and Romero, 2014). These results suggest that the mitotic activity at stage 4 is not only involved in expanding the undifferentiated population as proposed (Cardona et al., 2005; Martín-Durán et al., 2010), but also to originate differentiated cells as the metamorphosis starts. Furthermore, they suggest that βcatenin-1 could be involved in specification of different cell fates. Future experiments of in situ co-localization with progeny and stem cell markers will clarify the specific role of βcatenin-1 in cell fate determination during early metamorphosis.

The establishment of the anteroposterior and dorsoventral axis during S. polychroa embryogenesis.

The presence of micromeres on one side of the embryo defines the vegetal pole in spiralians (Hyman, 1951), which is the formerly posterior pole in bilateria (Imai et al., 2000; Logan et al., 1999; Wikramanayake et al., 1998). However, in *S. polychroa* the blastomeres are the same size, and micromeres or macromeres cannot be identified (Cardona et al., 2005). Furthermore, no anterior or posterior pole can be distinguished in the early embryo based on the morphology of the cells. Morphologically, the fist asymmetry observed is during the formation of the larva, with the appearance of the embryonic

pharynx at one pole, which confers a radial symmetry. This symmetry correlates with the expression pattern of elements of the Wnt pathway, which in the adult specify the AP axis. Thus, in the early larva (stages 1-4), polarity genes such as *sFRP-1* and *wnt1*, which in adults are located in the most anterior and posterior tips respectively (Gurley et al., 2010), do not display any AP distribution but are found in discrete blastomeres all around the larva, with the exception of the pharynx (Martín-Durán et al., 2010). It is important that although the expression of the Wnt elements is not polarized at this stage, βcatenin-1 is specifically nuclearyzed in the pharynx and its primordia. Taking into account that in most metazoans the site of gastrulation and endomesoderm formation defines the posterior pole of the adult, our data suggests that the pole where the embryonic pharynx is found could be the prospective posterior of the late larva and the adult planarian. This hypothesis would agree with the evolutionary conserved role of βcatenin-1 in specifying posterior fates in different developmental stages in most metazoans (Petersen and Reddien, 2009). In fact, after metamorphosis, the embryonic pharynx is assumed to present a posterior-ventral location (Cardona et al., 2005; Monjo and Romero, 2014).

At late metamorphosis, stage 5-6, the AP and DV determinants (elements of the Wnt and BMP signaling pathway, respectively) start to appear polarized as in the adults (Martín-Durán et al., 2010). However, during metamorphosis βcatenin-1 do not shows any AP apparent gradient, as described in the adults (Sureda-Gómez et al., 2016), but it is massively nuclearized in the whole embryo. In fact, during metamorphosis βcatenin-1 is predominantly found in the ventral part with respect to the dorsal (Fig. 3 and Fig. 4). Although this result could suggest that during metamorphosis βcatenin-1 has a role in DV patterning and not in AP patterning, we hypothesize that the accumulation of βcatenin-1 in the ventral part is associated with the differentiation of the nervous system at this stage (Monjo and Romero, 2014) and not directly with polarity.

In other spiralians such as *Platynereis*, and in the nematode *C. elegans*, it has been described that the AP axial identities are specified by βcatenin but not in terms of a gradient of activity but through βcatenin asymmetric distribution during cell division. Thus, the posterior territories are specified through the nuclearization of βcatenin in the posterior syster cell (Rocheleau et al., 1999; Schneider and Bowerman, 2007). In our study we observed asymmetric cell divisions in terms of βcatenin-1 during stage 4, when the larva starts to polarize. However, in our model, those asymmetric divisions distribute cytoplasmatic and not nuclear βcatenin-1. Whether the planarian blastomeres nuclearize βcatenin-1 after division it is unknown and a more precise study is needed to elucidate the meaning of those asymmetric divisions.

In general, our study provides new data to understand different aspects of planarian embryogenesis, but further analysis should be performed to clarify the role of the βcatenin-dependent Wnt signaling in axial patterning. For instances, it is not known if any Wnt triggers βcatenin-1 nuclearization during early embryogenesis, or if the posterior Wnts involved in AP axial patterning in adult planarians (Sureda-Gómez et al., 2016) also trigger βcatenin-1 nuclearization in the establishment of the definitive body axis during metamorphosis.

Embryogenesis, regeneration and homeostasis

Any process of regeneration can be seen as a recapitulation of the process of embryogenesis. In planarians, the study of the function of several signaling pathways during adult regeneration and homeostasis, supports this notion, since they exert similar functions than the ones reported during embryogenesis of many metazoans For instances, the Wnt and the BMP pathway specify the AP and DV axis in most metazoans during embryogenesis and also in adult planarians (Almuedo-Castillo et al., 2012; Molina et al., 2011; Niehrs, 2010).

But, is planarian regeneration a recapitulation of planarian embryogenesis? Are the molecular mechanisms re-used in the different situations? Studies on the specification of the eye (photoreceptor and pigment cells) suggest that in fact the genes involved in the specification of the different eye cell types during regeneration are also responsible for its speciation during embryogenesis, at stage 6, that is when eyes first appear (Martín-Durán et al., 2012b). And the same is observed with other organs system as nervous system (Monjo and Romero, 2014) and digestive system (Martín-Durán and Romero, 2011).

With respect to the role of βcatenin-1, recent results demonstrate multiple roles for βcatenin-1 in adult planarians. Thus, it is not only involved in axial patterning but also in organogenesis during brain or pharynx regeneration or during maturation of the sexual organs (Sureda-Gómez et al., 2016). Our data supports that during embryogenesis βcatenin-1 exerts an important role in organogenesis, since it is expressed in the primordia of the brain and pharynx (Fig 5) (Sureda-Gómez et al., 2016), during late metamorphosis. Interestingly, βcatenin-1 is also nuclearyzed in the embryonic pharynx, at stage 2, (Fig. 2), suggesting that even in this early stage βcatenin-1 could have a role in organogenesis.

A more difficult question is if during embryogenesis βcatenin-1 has a role in posterior specification, as broadly documented that it has in adult animals (Petersen and Reddien, 2009). In adult planarians, βcatenin-1 is essential to specify posterior identity, and its levels are related with the different axial identities (Gurley et al., 2008; Iglesias et al., 2008; Petersen and Reddien, 2008; Sureda-Gómez et al., 2016). Thus, during homeostasis βcatenin-1 protein presents a nuclear gradient from the prepharingeal region to the tail. And during regeneration it is preferentially located in posterior facing wounds (Sureda-Gómez et al., 2016). However, during metamorphosis and onwards, when in fact the polarity determinants of adult planarians are already polarized in embryos (Martín-Durán et al., 2010) we do not detect βcatenin-1 preferentially located in the posterior part of the animal, but in the ventral side. This result could be explained by the active role of βcatenin-1 in organogenesis at this stage, which hides its possible enrichment in posterior.

An interesting question is whether βcatenin-1 exerts a polarizing role at stage 1, during the organization of the larva. However, since the larva does not show a bilateral symmetry, this process would not be considered a recapitulation of any adult event.

Materials and methods

Animals and eggs

A sexual population of *S. polychroa* (Sant Celoni, Barcelona) was maintained in the lab as previously described (Martín-Durán et al., 2008). Egg capsules were collected and fixed as previously described (Cardona et al., 2005).

Immunohistochemistry

Embryos were dissected as previously described (Cardona et al., 2005). Paraffin embedding and sectioning were conducted as previously described (Cardona et al., 2005). Inmunohistochemistry of whole mount samples and paraffin sections were conducted as previously described (Sureda-Gómez et al., 2016). Primary antibodies were used at the following dilutions: anti-Bcat1, 1:200 (Sureda-Gómez et al., 2016); anti α-tubulin AA4.3, 1:20 (Developmental Studies Hybridoma Bank); anti-FMFRamide, 1:500 (DiaSorin, 2009) . Secondary antibodies were used at the following dilutions: anti-rabbit HRP-conjugated antibody, 1:500 (Pierce); anti-mouse Alexa Fluor 568-conjugated antibody, 1:1000 (Molecular Probes). Nuclei were counterstained with DAPI, 1:5000 (Sigma). Embryos were mounted and stored in 70% glycerol.

Image analisys

Immunostaining images were obtained under a Leica TCS-SP2 confocal laser-scanning microscope (Leica Lasertchnik, Heidelberg, BW, Germany) adapted for an inverted microscope and a Leica TCS-SP5 confocal laser-scanning microscope (Leica Lasertchnik, Heidelberg, BW, Germany). Images were processed using Fiji software (Schindelin et al., 2012), Adobe Photoshop and Illustrator.

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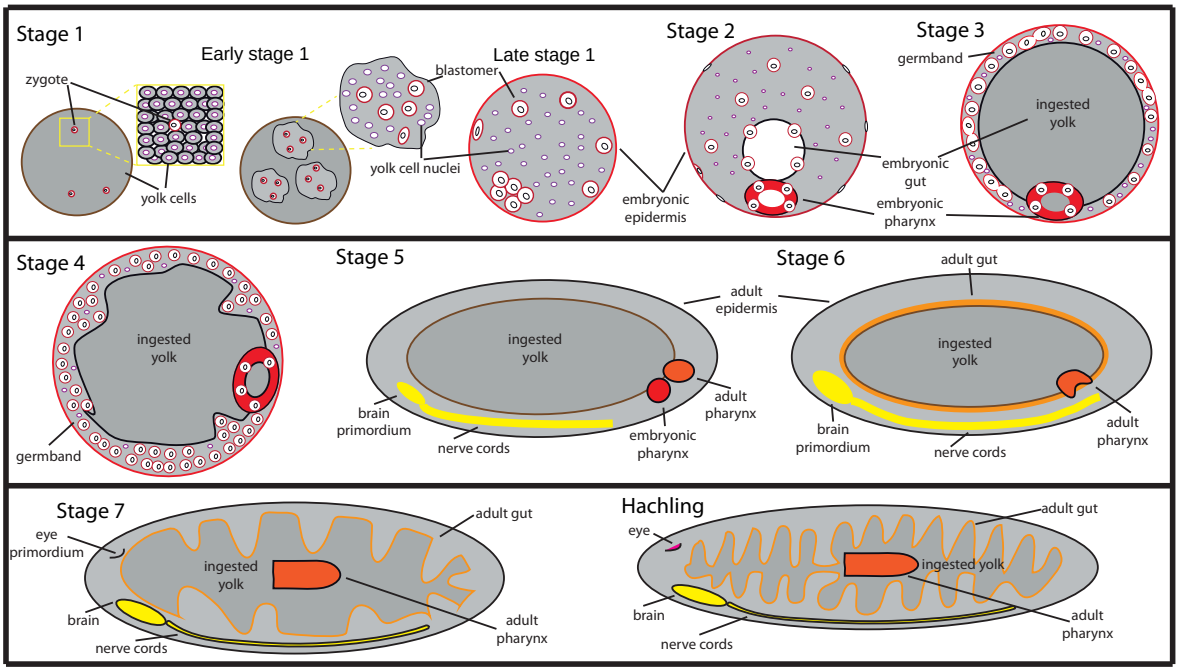
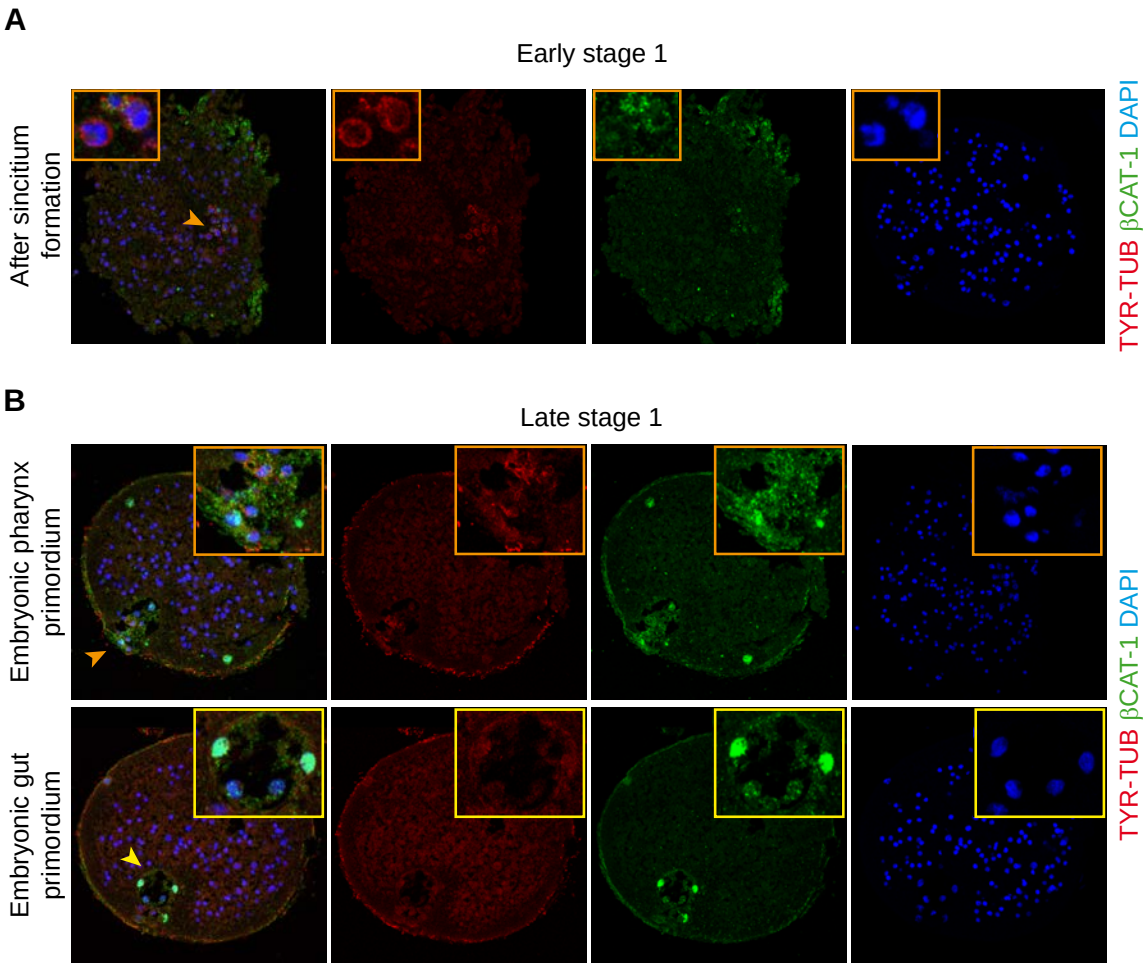
Supplementary information

Supplementary Figure 1



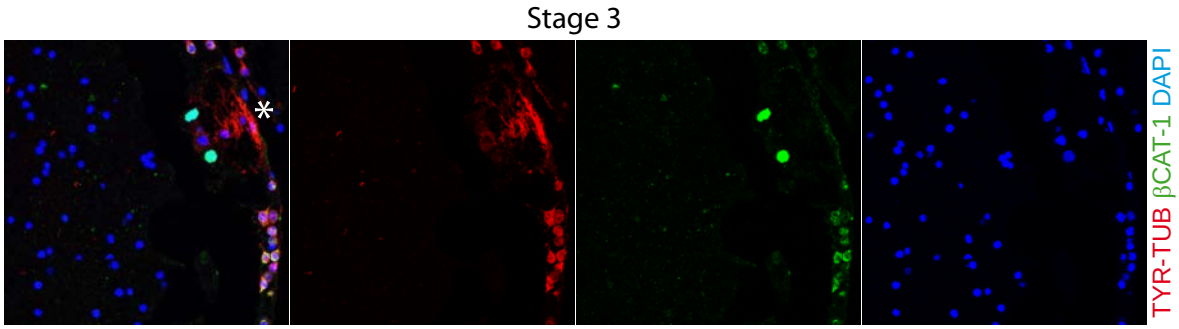
Supplementary figure 1. Picture scheme of the Schmidtea polychroa embryonic development of. Draw pictures represent the different developmental stages and show the embryonic tissues and structures.

Supplementary Figure 2



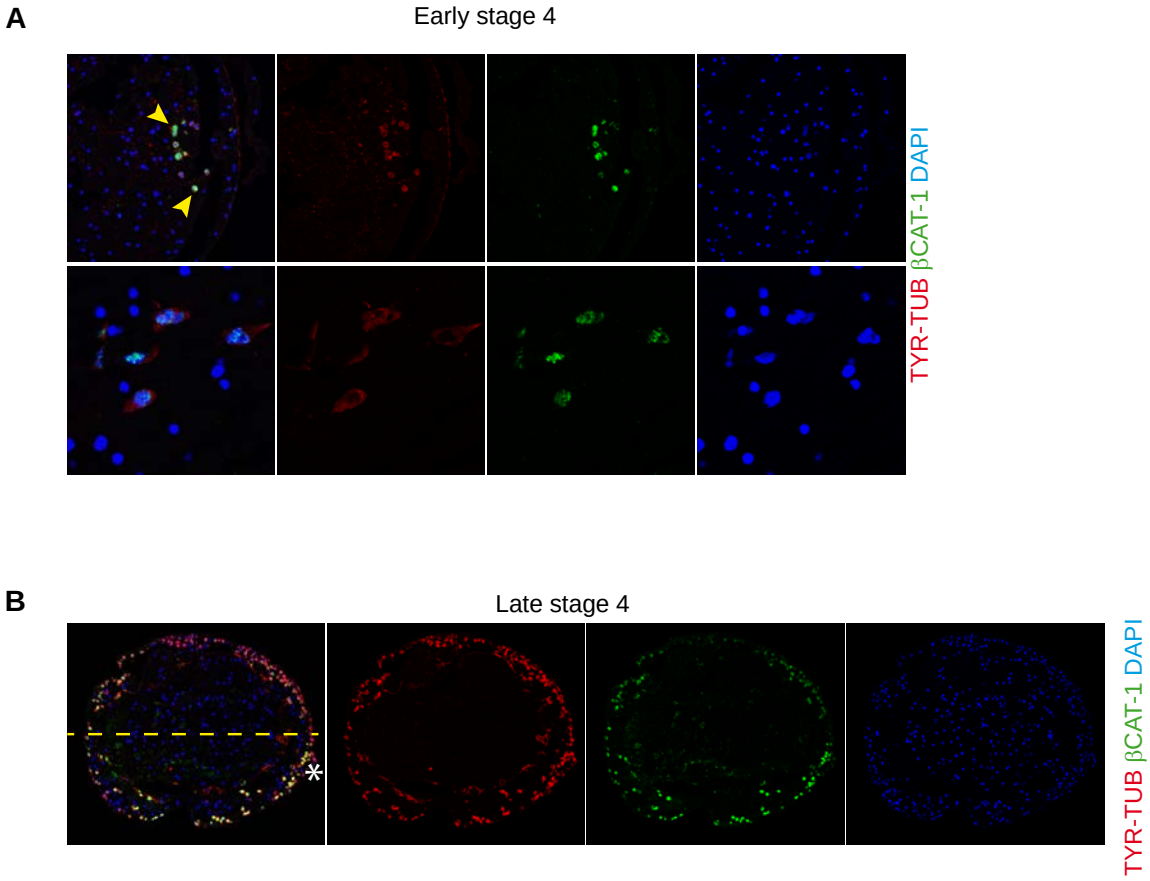
Supplementary figure 2. βcatenin-1 location at stage 1. (A-B) Z-projection of confocal stacks of an stage 1 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). Yellow and orange arrowheads indicate areas shown at higher magnification in insets.

Supplementary Figure 3



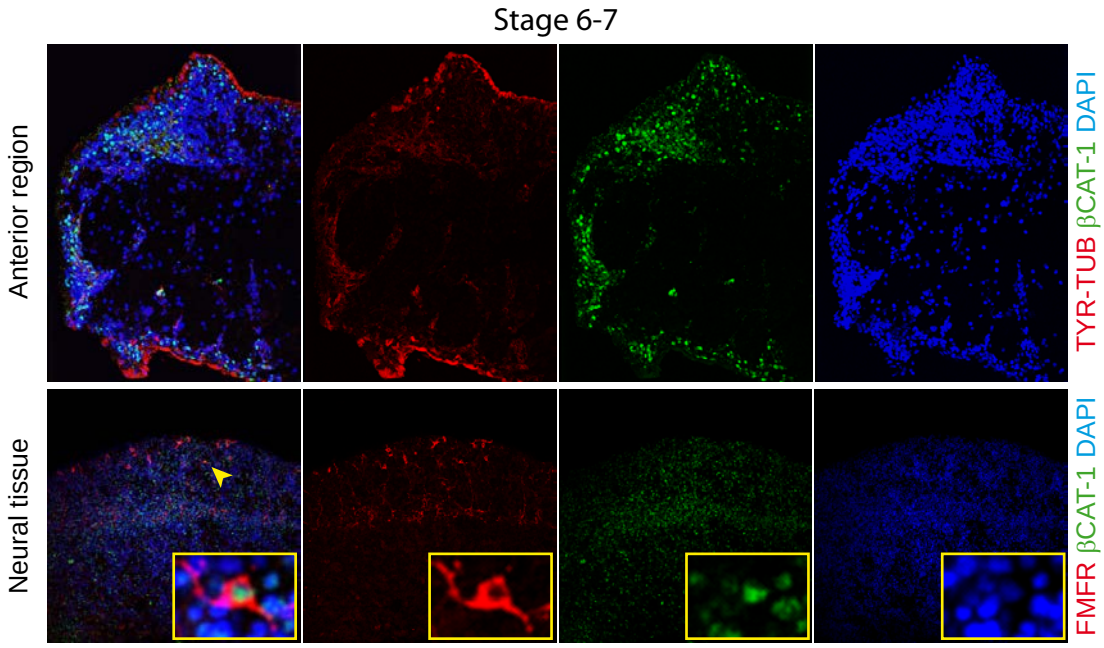
Supplementary figure 3. βcatenin is only nuclearized in the embryonic pharynx. Z-projection of confocal stacks of an stage 3 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). White asterisk indicates pharynx location.

Supplementary Figure 4



Supplementary figure 4. βcatenin-1 is nuclearized in migrating blastomers and is preferentially accumulate in the ventral region. (A-B) Z-projections of confocal stacks of an stage 4 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). (A) Top images show blastomeres migrating towards the middle of the larva. Bottom images show magnification of migrating blastomeres. Yellow arrowheads indicate the migrating blastomeres. (B) Dashed line indicate the presumtive dorsoventral border. White asterisk indicates pharynx location.

Supplementary Figure 5



Supplementary figure 5. βcatenin-1 is found in neural tissues. (Top images) Z-projection of confocal stacks of an stage 6-7 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and tubulin (red) which labels blastomeres. Nuclei are labeled with DAPI (blue). (Bottom images) Z-projection of confocal stacks of an stage 6-7 embryo from an immunohistochemistry on paraffin sections against βcatenin-1 (green) and FMFR-amide (red) which labels neural cells. Nuclei are labeled with DAPI (blue). Yellow arrowhead indicates areas shown at higher magnification in insets.

DISCUSIÓN

1. La vía Wnt y su papel en el establecimiento del eje anteroposterior en planarias

Desde Morgan y Child hasta nuestros días, una de las cuestiones más estudiadas en planarias es por qué se regenera una cabeza y una cola en el lugar que les corresponde, es decir, cómo se re-establece el eje anteroposterior (AP) durante la regeneración (Child, 1911; Child, 1941; Morgan, 1904a; Morgan, 1904b; Morgan, 1905). En los últimos años, diferentes estudios han demostrado que la vía Wnt canónica es la responsable de re-establecer el eje AP, durante la regeneración y también de mantenerlo durante la homeostasis de las planarias (Adell et al., 2009; Gurley et al., 2008; Iglesias et al., 2008; Iglesias et al., 2011; Petersen y Reddien, 2008; Petersen y Reddien, 2009a; Petersen y Reddien, 2011). En base a estos estudios y a la observación de la localización asimétrica de algunos elementos de la vía Wnt canónica (Adell et al., 2009; Gurley et al., 2010; Petersen y Reddien, 2008; Petersen y Reddien, 2009a; Petersen y Reddien, 2011), se ha propuesto que un gradiente de actividad de βcatenina-1 a lo largo del eje AP, menor en anterior y mayor en posterior, puede establecer las diferentes identidades cabeza, tronco y cola (Adell et al., 2010). Sin embargo, antes de los estudios realizados durante esta tesis, no había ninguna prueba experimental directa de la existencia del gradiente de actividad de βcatenina-1. Además, no se había profundizado en el estudio de la función de los ligandos Wnt.

En esta tesis doctoral hemos demostrado que existe un gradiente de actividad βcatenina-1 mediante el uso de un anticuerpo específico contra esta proteína (Sureda-Gómez et al., 2016). Además, hemos progresado en la compresión de la función de los ligandos Wnts expresados en posterior (Sureda-Gómez et al., 2015). Por otra parte, hemos demostrado que la βcatenina-1 también tiene un papel principal en la regeneración anterior (Sureda-Gómez et al., 2016), que era una función no descrita hasta el momento.

1.1 El gradiente de actividad βcatenina-1 durante la homeostasis.

La hipótesis propuesta por Adell y colaboradores de que un gradiente de actividad βcatenina-1 patronea el eje AP parte en esencia de dos observaciones (Figura 41 A) (Adell et al., 2010):

1. La gradación de los fenotipos obtenidos después del silenciamiento de *βcatenina-1*. Esta gradación depende de la dosis y el tiempo de inhibición (Gurley et al., 2008; Iglesias et al., 2008; Petersen y Reddien, 2008).

2. La localización en la parte posterior de los *wnts* y en la parte anterior de sus inhibidores (*notum*, *sFRP1*) (Adell et al., 2009; Gurley et al., 2010; Petersen y Reddien, 2008; Petersen y Reddien, 2009a; Petersen y Reddien, 2011).

Utilizando un anticuerpo específico contra βcatenina-1, hemos demostrado que existe un gradiente respecto a la nuclearización de esta proteína, que se relacionaría con su actividad, ya que en el núcleo es donde ejerce su función. Sin embargo, hemos observado que el punto más bajo no se encuentra en el extremo anterior (cabeza), como se pensaba, sino que aparece en la prefaringe (Figura 41 B). Este gradiente AP más corto

concuerda con la expresión de los *wnts* posteriores, los cuales se expresan desde la cola hasta la prefaringe (Gurley et al., 2010; Sureda-Gómez et al., 2015). Por otra parte, los genes *teashirt* y *axinaB*, descritos como targets de βcatenina-1, también se expresan en forma de gradiente desde la región prefaringe hacia posterior (Iglesias et al., 2011; Owen et al., 2015; Reuter et al., 2015). Estos datos son consistentes con nuestros resultados del gradiente de βcatenina-1 que excluye la zona de la cabeza.

En la región anterior, en la cabeza, el escenario es más complejo. Detectamos un aumento de la proteína βcatenina-1, y su inhibición produce defectos en el cerebro, los ojos y la pérdida de la expresión de notum, regulador negativo de la vía Wnt que especifica identidad anterior (Sureda-Gómez et al., 2016). Estos datos sugieren que la βcatenina-1 de la región anterior no participa directamente en la especificación del eje AP. Posibles funciones de esta actividad local de βcatenina-1 en anterior podrían ser en el control del polo anterior de la planaria (apartado 1.4 de la discusión) y en la organogénesis del cerebro (apartado 2.1 de la discusión), tal como se ha descrito en otros animales (Ciani y Salinas, 2005) (Figura 41 B).

Aunque hemos demostrado que existe un gradiente de proteína βcatenina-1, quedan cuestiones no resueltas. Por ejemplo, ¿qué células presentan βcatenina-1 nuclear y participan en el gradiente? Nuestros datos indican que al menos los neoblastos (receptores

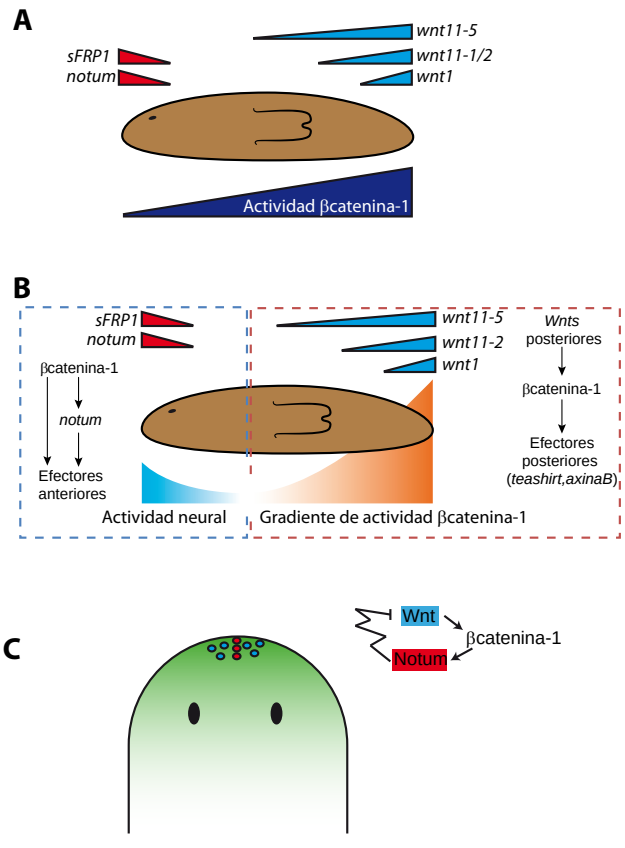


Figura 41. Modelo pasado y nuevo sobre la función de la βcatenina-1 en el eje AP. (A) Modelo pasado de la dinámica y el establecimiento del gradiente de actividad de βcatenina-1 propuesto por Adell et al., 2010. La expresión en forma de gradiente en la región posterior de los wnts posteriores (wnt1, wnt11-1, wnt11-2, wnt11-5) establece un gradiente de actividad βcatenina-1 a lo largo del eje AP. La expresión localizada en la región anterior de notum y sFRP1 inhibe la actividad de los Wnts y por consiguiente de la actividad βcatenina-1 decae. (B) Nuevo modelo de la dinámica y el establecimiento del gradiente de actividad de βcatenina-1. La expresión en forma de gradiente en la región posterior de los wnts posteriores (wnt1, wnt11-2, wnt11-5) establece un gradiente de actividad βcatenina-1 desde la prefaringe hasta la cola. βcatenina-1 activa a diferentes efectores para el correcto establecimiento de la región central y posterior (cuadro rojo). En anterior se observa un incremento de la actividad de βcatenina-1 relacionada con una posible actividad neural y el control del polo anterior (activación de notum). (C) Modelo de "local self-enhancement and long-range inhibitor" para establecer la actividad de βcatenina en la región anterior. Un activador, Wnt (caja azul) activa βcatenina-1 y esta a su vez al inhibidor del activador, Notum (caja roja). Notum inhibe a largo alcance al activador, provocando que decaiga la actividad de βcatenina a medida que se aleja de la fuente de secreción del activador.

de las señales de diferenciación y decisión del destino celular) y las células musculares (que secretan los determinantes, como los Wnts) pueden participar en el gradiente (Sureda-Gómez et al., 2016). Se debería comprobar si otros tipos celulares también participan o no del gradiente.

Otra pregunta que surge de la demostración del gradiente es si los inhibidores (Notum y sFRP1) y los activadores (Wnts posteriores) son los responsables de establecerlo y mantenerlo. En cuanto a los inhibidores, existen evidencias de que sFRP1 está relacionado con el sistema nervioso y no con la polaridad anterior (Fraguas et al., 2014). Por otro lado, *notum* se expresa en dos dominios: en la punta anterior (o polo anterior) y en el cerebro. Se ha demostrado que la fracción de las células que expresan *notum* en el cerebro están implicadas en el control del tamaño de este órgano, y no en el establecimiento de la identidad anterior (Hill y Petersen, 2015). Sin embargo, la fracción que se encuentra en el polo anterior no se ha relacionado con ninguna función durante la homeostasis. En mi opinión, una posible función de Notum en el polo anterior puede ser controlar la actividad local de βcatenina-1 en anterior, a través de la inhibición de un hipotético Wnt. Sin embargo, ¿cómo se explica que la concentración más alta de βcatenina-1 se encuentre donde se expresa su inhibidor? Una posibilidad es que estemos ante un modelo de "local self-enhancement and long-range inhibitor", propuesto por Gierer y Meinhard (Gierer y Meinhardt, 1972). En este caso, Notum es la señal inhibidora y un Wnt la activadora. Cerca de la fuente el activador tiene más potencia (βcatenina-1 alta) y más lejos es el inhibidor el que ejerce su función (βcatenina-1 baja) (Figura 41 C).

En cuanto a los activadores, ¿son los Wnts los que generan y/o mantienen este gradiente de actividad βcatenina-1? Para que sea más sencilla la discusión sobre el papel que tienen los Wnts, primero discutiré los resultados obtenidos durante el proceso de regeneración, ya que es donde han sido más extensamente estudiados.

1.2 Los Wnts patronean las estructuras posteriores durante la regeneración

Diferentes estudios basados en el silenciamiento de los *wnts* posteriores durante la regeneración sugieren que pueden activar a la βcatenina-1. El caso más claro es el de Wnt1 (Adell et al., 2009; Petersen y Reddien, 2009a). Su silenciamiento fenocopia el producido por el ARNi de *βcatenina-1*. Además se expresa en posterior en animales intactos, y en animales regenerantes se expresa de forma temprana en blastemas anteriores y posteriores, para despues localizarse de forma tardía en posterior (especialmente a partir de 48 horas). Estos datos sugieren que Wnt1 especifica la identidad posterior a través de activar a la βcatenina-1. Por contra, antes de esta tesis, no estaba clara la función que ejercen el resto de Wnts y por tanto, se desconocía si podian activar a la βcatenina-1 (Adell et al., 2009; Gurley et al., 2010).

Nuestros resultados indican que, excepto Wnt11-1, el resto de Wnts posteriores pueden ejercer su función (o parte de ella) a través de βcatenina-1. Además demostramos que ejercen funciones independientes entre ellos, patroneando diferentes estructuras posteriores (Sureda-Gómez et al., 2015). Se asemejaría a un código Hox, como se ha propuesto en Hydra (Guder et al., 2006). Así, Wnt11-5 se encargaría de delimitar la región

del tronco, mientras que Wnt1 y Wnt11-2 se encargaría de especificar y patronear la cola.

Los datos obtenidos sobre Wnt1 demuestran que efectivamente su función durante la regeneración es promover la nuclearización de la βcatenina-1 en el blastema posterior, y no en el anterior, y, por tanto establecer específicamente la identidad posterior (Figura 42) (Sureda-Gómez et al., 2016).

Con respecto al *wnt11-2*, su inhibición produce el fenotipo "sin cola", además de una duplicación de la boca y una reducción de la identidad posterior (Figura 42 A). Esto indica que tiene una función en la formación del patrón de la cola. Gurley y colaboradores interpretan el fenotipo producido por la inhibición del *wnt11-2* como un problema en la elongación de la línea media posterior. Sin embargo, no determinan si la identidad posterior está afectada (Gurley et al., 2010). Nuestros datos indican que Wnt11-2 participa en el establecimiento de la identidad posterior, probablemente a través de activar a βcatenina-1 (Figura 42 B). Una evidencia es que la inhibición conjunta de *wnt1* y *wnt11-2* incrementa el fenotipo "dos cabezas" (Figura 42 A) (Sureda-Gómez et al., 2015). Asimismo, la inhibición de *βcatenina-1* a bajas dosis también produce un fenotipo "sin cola" (Almuedo-Castillo et al., 2011). Por otra parte, puede ser que Wnt11-2 también se encargue de posicionar las células que secretan Wnt1 en la línea media posterior. Desconocemos si la afectación de la línea media posterior es la causa o la consecuencia de que la identidad esté afectada. Por otra parte, otros genes que su inhibición produce un defecto similar al silenciamiento de *wnt11-2* son *pitx* e *islet*. La inhibición de ambos produce el fenotipo "sin cola" y la pérdida de la expresión de los *wnts* posteriores (Currie y Pearson, 2013; Hayashi et al., 2011; März et al., 2013). En este caso März y colaboradores proponen que Pitx e Islet controlan la identidad de la cola y la línea media. Sin embargo consideran ambos procesos independientes entre ellos. Es decir, la afectación de la línea media no tiene porque ser la causa de la perdida de la identidad posterior y viceversa (März et al., 2013).

En el caso del *wnt11-5*, su inhibición produce una duplicación de la región central (dos faringes en tándem) sin afectar a la región posterior (Figura 42 A). Estos datos sugieren que su función es especificar/modelar la zona del tronco. A posteriori, otros dos trabajos han propuesto que la función de Wnt11-5 es delimitar la región del tronco (Lander y Petersen, 2016; Scimone et al., 2016). Mientras que la función de especificar la región central parece clara, existe controversia sobre si Wnt11-5 ejerce su función a través de βcatenina-1. Lander y Petersen han demostrado que la expresión de *axinaB* solo se ve alterada tras silenciar *βcatenina-1* y no tras inhibir *wnt11-5*. Así pues, defienden que la función de Wnt11-5 no es activar a βcatenina-1. Por contra, nuestros datos apuntan a que sí puede ejercer su función (o parte de ella) a través de activar a la βcatenina-1. La inhibición de *wnt11-5* junto con *wnt1* incrementa la penetrancia del fenotipo "dos cabezas", y esto sugiere que una de las funciones del Wnt11-5 debe ser activar a βcatenina-1 (Figura 42 B) (Petersen y Reddien, 2009a; Sureda-Gómez et al., 2015). Además, la inhibición a bajas dosis de *βcatenina-1* provoca la duplicación de la faringe (Almuedo-Castillo et al., 2011), del mismo modo que el ARNi de *wnt11-5*.

Mientras que existe controversia sobre si el Wnt11-2 y el Wnt11-5 pueden activar a la βcatenina-1, el estudio del *wnt11-1* deja pocas dudas sobre su función no canónica.

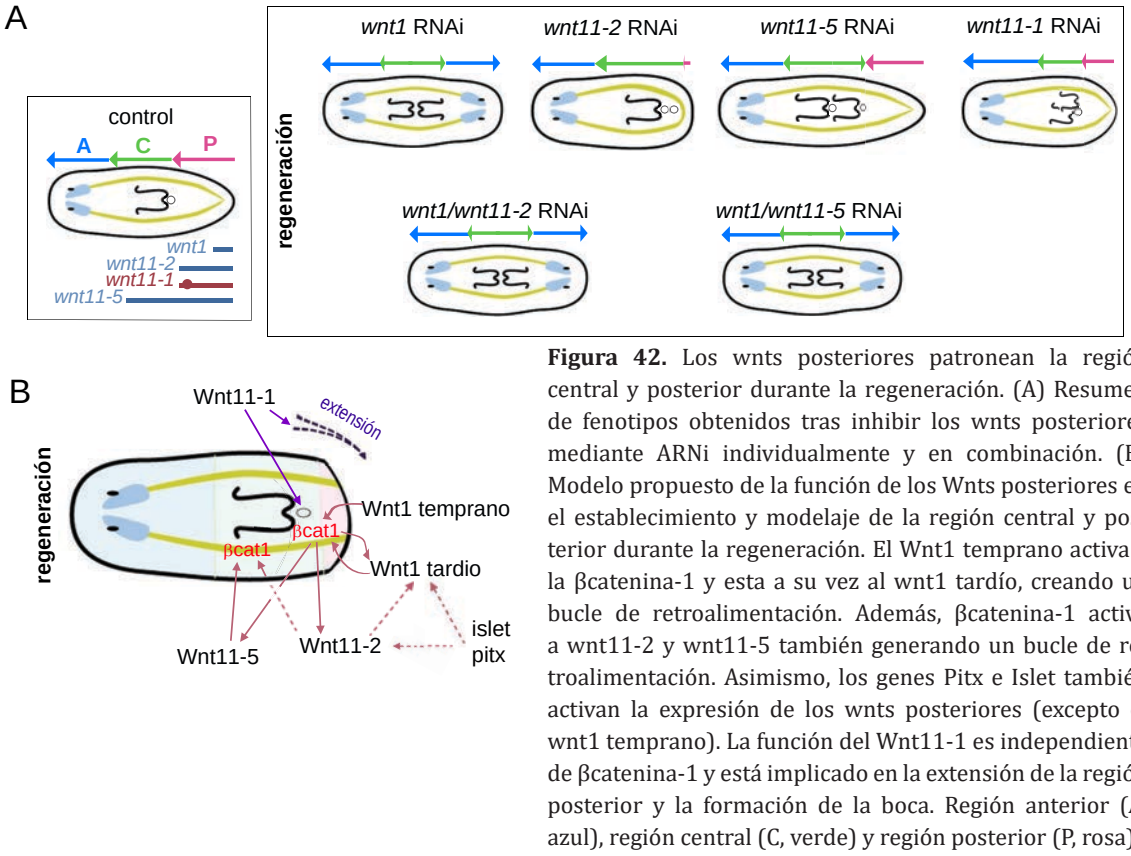


Figura 42. Los wnts posteriores patronean la región central y posterior durante la regeneración. (A) Resumen de fenotipos obtenidos tras inhibir los wnts posteriores mediante ARNi individualmente y en combinación. (B) Modelo propuesto de la función de los Wnts posteriores en el establecimiento y modelaje de la región central y posterior durante la regeneración. El Wnt1 temprano activa a la βcatenina-1 y esta a su vez al wnt1 tardío, creando un bucle de retroalimentación. Además, βcatenina-1 activa a wnt11-2 y wnt11-5 también generando un bucle de retroalimentación. Asimismo, los genes Pitx e Islet también activan la expresión de los wnts posteriores (excepto el wnt1 temprano). La función del Wnt11-1 es independiente de βcatenina-1 y está implicado en la extensión de la región posterior y la formación de la boca. Región anterior (A, azul), región central (C, verde) y región posterior (P, rosa).

Nuestros resultados muestran que la inhibición del *wnt11-1* produce una cola normal pero más corta comparada con animales control. Además, la polaridad posterior no se ve alterada y la inhibición conjunta de *wnt11-1* y *wnt1* no incrementa el fenotipo "dos cabezas"(Figura 42 A). Estos datos sugieren que su función es elongar la cola de la planaria sin activar a βcatenina-1 (Figura 42 B) (Sureda-Gómez et al., 2015). Este problema de extensión observado en las planarias tras inhibir *wnt11-1* se asemeja a los producidos en otros organismos al inhibir la vía no canónica. Por ejemplo, al inhibir la familia del *wnt11* en embriones de rana durante el desarrollo, los movimientos convergentes de extensión no se producen (Heisenberg et al., 2000; Wallingford et al., 2002). Otro dato a favor de su función no canónica es que tras inhibir *wnt11-1* en planarias en ocasiones la faringe se duplica, pero sin provocar una transformación homeótica, como en el caso del *wnt5* (Gurley et al., 2010) o *dishevelled* (Almuedo-Castillo et al., 2011). En el futuro se debería comprobar mediante el uso del anticuerpo contra la βcatenina-1 si los Wnt11 modulan la actividad de βcatenina-1 durante la regeneración.

1.3 ¿Son los Wnts los responsables de mantener el gradiente de βcatenina-1 durante la homeostasis?

Durante la regeneración, Wnt1 y posiblemente Wnt11-2 y Wnt11-5 regulan la nuclearización de la βcatenina-1, no así Wnt11-1. Si Wnt1, Wnt11-2 y Wnt11-5 son los que controlan el gradiente de βcatenina-1, esperaríamos que su inhibición afectara a la polaridad durante la homeostasis, del mismo modo que al silenciar *βcatenina-1*. Y en parte es lo que observamos. La inhibición de forma individual de *wnt11-2* y *wnt11-5* produce

defectos en la parte posterior o central, pero nunca una aparición de una cabeza en posterior. Aunque, de hecho, lo más sorprendente es que inhibir *wnt1* no causa ningún defecto aparente.

En cambio, la inhibición conjunta de todos ellos (incluido *wnt11-1*, ya que no tenemos datos sin inhibirlo también) produce una pérdida de la identidad posterior e incluso de la identidad media (tronco), ya que no se aprecia la faringe en algunos de estos animales (Figura 43 A) (Sureda-Gómez et al., 2015). Esta pérdida de la faringe también se observa tras inhibir la *βcatenina-1* (Iglesias et al., 2008). Este resultado sugiere que los Wnts posteriores sí que pueden establecer el gradiente de βcatenina-1 durate la homeostasis (Figura 43 B). Sin embargo, cabe destacar que al inhibir los Wnts posteriores el fenotipo anteriorizado es menos evidente que al inhibir la *βcatenina-1*. Una posible explicación de que no aparezca una cabeza en posterior tras inhibir los Wnts posteriores podría ser que la inhibición de los ligandos produce una inhibición menor de βcatenina-1 que su inhibición directa, ya que la inhibición de elementos secretables presenta más dificultad que la de factores de transcripción (observación personal). Además, la inhibición de varios genes conjuntamente es menos eficiente que por separado.

Otra posibilidad es que la inhibición conjunta de la vía canónica (*wnt1*, *wnt11-2* y *wnt11-5*) y la no canónica (*wnt11-1*) pueda interferir en la transformación de la parte posterior. Sin embargo, en mi opinión esta opción es poco probable, ya que inhibir *evi/wntless* o *dishevelled*, elementos comunes a la vía canónica y no canónica, produce planarias con dos cabezas y con otros defectos descritos como no canónicos (Adell et al., 2009; Almuedo-Castillo et al., 2011), indicando que la vía canónica y no canónica no interfieren a este nivel.

Quizás la explicación más sencilla es que un mecanismo muy robusto controla la nuclearización de βcatenina-1 durante la homeostasis. Por encima de βcatenina-1 pueden existir otras vías o proteínas además de los Wnts, por ejemplo otros agonistas no descritos en planarias, la vía del Calcio (Nogi et al., 2009; Zhang et al., 2011) o la vía Hippo (Lin y Pearson, 2014). Lo que si podemos descartar es que las otras βcateninas (la -2, -3 y -4) influyan en este proceso de especificar identidad posterior, ya que la inhibición de todas ellas no produce ningún fenotipo relacionado con polaridad (Chai et al., 2010) (Su, Sureda-Gómez et al., en revisión). En el futuro se debería estudiar si la inhibición de los *wnts* posteriores modula el gradiente de actividad de βcatenina-1 utilizando el anticuerpo contra la βcatenina-1.

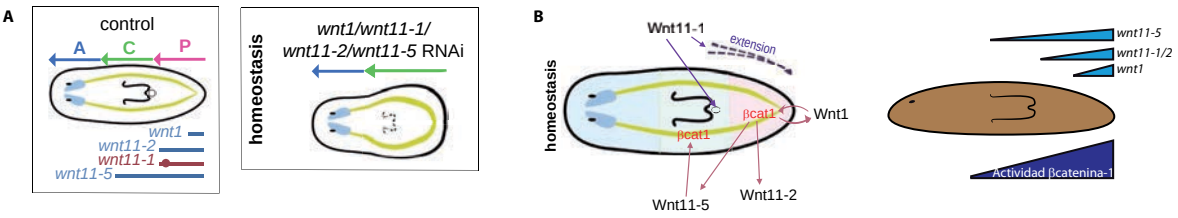


Figura 43. Los wnts posteriores patronean la región central y posterior durante la homeostasis. (A) Resumen del fenotipo obtenido tras inhibir todos los wnts posteriores conjuntamente mediante ARNi. (B) Modelo propuesto de la función de los Wnts posteriores en el establecimiento y modelaje de la región central y posterior durante la homeostasis. El Wnt1, Wnt11-2 y Wnt11-5 activan a la βcatenina-1 en la región posterior y central. Esta actividad de βcatenina-1 establece la identidad central y posterior. El Wnt11-1 ejerce su función de extensión de la región posterior y en la boca de forma independiente a βcatenina-1.

1.3.1 La generación del gradiente de actividad de βcatenina-1: posibles mecanismos.

Nuestros resultados indican que el gradiente de Wnts posteriores establece el gradiente de actividad βcatenina-1. Sin embargo, no se ha demostrado si los Wnts generan dicho gradiente de actividad mediante su secreción al medio. Existen diferentes propuestas sobre cómo se secretan y viajan los Wnts en otros organismos, por ejemplo en vesículas, libres, mediante citonemas, etc. (Langton et al., 2016; Mikels y Nusse, 2006).

En planarias, las células musculares son las que expresan los *wnts* (Witchley et al., 2013), y la musculatura se encuentra cubriendo todo el cuerpo del animal. Una posibilidad es que los Wnts no se secreten, sino que las células musculares proyecten citonemas. Otra posibilidad es que se puedan secretar de forma libre o en vesículas, ya que las células musculares en otros organismos pueden secretar moléculas al medio (Pedersen y Febbraio, 2008).

Si consideramos que los Wnts se secretan al medio, otra cuestión es su rango de acción, es decir, si pueden viajar lejos de su fuente de secreción. Diferentes estudios apuntan hacia la idea de que los Wnts no pueden viajar distancias largas y que tienen un corto alcance (Baena-Lopez et al., 2009; Farin et al., 2016). Además, en *Drosophila* se ha demostrado que la inhibición de la secreción de los Wnts mediante su anclaje a la membrana no produce ningún defecto en el patrón del ala (Alexandre et al., 2014). Estos autores sugieren que las células que estuvieron en contacto con la fuente mantienen su actividad aunque no continúen recibiendo el estimulo de los Wnts, es decir, pueden recordar su influencia por mecanismos epigenéticos. Del mismo modo, en planarias puede estar actuando un mecanismo de corto alcance basado en modificaciones epigenéticas. Este mecanismo generaría el gradiente de actividad βcatenina-1 durante la regeneración y la homeostasis.

En el caso de la regeneración, durante las primeras horas (6-24 horas) *wnt1* se expresa en el postblastema y blastema. Esta señal de Wnt1 es la que instruiria a las células adyacentes (corto alcance). A medida que el blastema crece, las células se alejan de la fuente de Wnt1. Igual que en el ala de *Drosophila*, estas células que se alejan podrían mantener activado el programa de transcripción de βcatenina-1 gracias a modificaciones epigenéticas. De esta manera se podría crear un gradiente de actividad de βcatenina-1 sin necesidad de que exista un gradiente de Wnts de largo alcance (Figura 44).

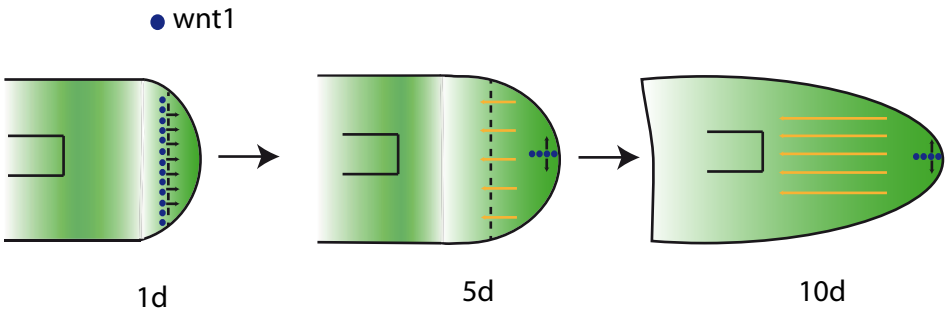


Figura 44. Modelo propuesto de la generación del gradiente de βcatenina-1 durante la regeneración. El Wnt1 temprano que se localiza en la región postblastema activa a βcatenina-1 en el blastema de día 1. A medida que el animal regenera la región posterior las células migran hacia anterior alejándose de la fuente de Wnt1 lo que provoca que la actividad de βcatenina-1 se diluye hacia anterior pero se mantenga por mecanismos epigenéticos.

Esta posibilidad concuerda con la idea de que el re-establecimiento del gradiente de actividad βcatenina-1 a lo largo del eje AP durante la regeneración se da de forma gradual gracias al crecimiento del blastema. Así, el crecimiento del blastema inducirá la 'dilución' de la actividad βcatenina-1 en el tejido nuevo. Finalmente este nuevo tejido se integrará con el pre-existente (Figura 44). El re-establecimiento progresivo del gradiente de βcatenina-1 también concuerda con la dinámica de aparición de los *wnts* durante la regeneración, de forma gradual y escalada (Gurley et al., 2010; Sureda-Gómez et al., 2015).

En el caso de la homeostasis se puede aplicar el mismo mecanismo que en la regeneración, ya que las células migran (Salo y Baguñà, 1985) y que el animal crece más por la región posterior (comunicación personal del Dr. Emili Salo). La fuente principal de los Wnts es la punta de la cola. Las células de la cola reciben los Wnts y a medida que crece el animal, las células se alejan de la fuente pero mantienen su estado activo mediante modificaciones epigenéticas.

Finalmente y ligado con el párrafo anterior, ¿pueden los Wnts posteriores controlar el crecimiento de la planaria además de controlar la identidad posterior? La posibilidad de que los Wnts (o otras moléculas que forman gradientes) puedan controlar el crecimiento ya se ha propuesto en otros organismos (Giraldez y Cohen, 2003). Por ejemplo, en las alas de *Drosophila* en las que los Wnts están anclados son más pequeñas, aunque el patrón es correcto (Alexandre et al., 2014). En vertebrados, la inhibición de Hh en la extremidad también se relaciona con el crecimiento y expansión de los dedos (Lopez-Rios, 2016). En planarias no se ha estudiado si los Wnts afectan o no al crecimiento de la cola, aunque hemos observado que la inhibición de *wnt1*, *wnt11-1* y *wnt11-2* durante la regeneración produce una cola redonda y más pequeña. En el futuro se debería analizar la función de los Wnts de planarias no solo desde el punto de vista de patrón sino del crecimiento.

1.4 Los polos anterior y posterior

Diferentes autores han propuesto que las planarias poseen un polo anterior y uno posterior que pueden actuar como organizadores durante la regeneración y la homeostasis (Almuedo-Castillo et al., 2012; Meinhardt, 2009; Vogg et al., 2016):

- El polo posterior: se ha sugerido que tanto Wnt1 como βcatenina-1 son dos de los elementos que especifican el polo posterior. La inhibición del *wnt1* de primera fase y *βcatenina-1* durante la regeneración produce la desaparición de la identidad posterior y se establece una nueva identidad anterior en su lugar. Además, la inhibición del *wnt1* de segunda fase también produce una pérdida de la identidad posterior, pero en este caso no se substituye por una nueva. Basándome en los datos obtenidos en esta tesis, en mi opinión, la primera señal de Wnt1 activa a la βcatenina-1 promoviendo la formación del polo posterior en el blastema (Sureda-Gómez et al., 2016). Este polo también expresa *wnt1* (el de segunda fase), además de βcatenina-1. Otros elementos propuestos del polo posterior son *pitx* e *islet*, los cuales se expresan en el blastema posterior y su inhibición produce fenotipos "sin cola" (Currie y

Pearson, 2013; Hayashi et al., 2011; März et al., 2013). Asimismo se ha demostrado que la segunda fase de expresión de *wnt1* está controlada por Pitx e Islet. Una vez formado el polo secretará las señales que patronean el blastema, como en el caso del organizador de Spemann en el embrión. Estas señales son los Wnts posteriores, que establecerán un bucle de activación de βcatenina-1 (Figura 45) (Sureda-Gómez et al., 2015). Finalmente estas señales modelarán la región posterior, a través de activar otros genes como teashirt (Owen et al., 2015; Reuter et al., 2015) o los Hox.

- El polo anterior: Este polo se caracteriza por expresar diferentes genes como *foxD*, *zic-1*, *notum* y *follistatin* (Figura 46) (Gaviño et al., 2013; Petersen y Reddien, 2011; Roberts-Galbraith y Newmark, 2013; Scimone et al., 2014; Vásquez-Doorman y Petersen, 2014; Vogg et al., 2014). Se ha propuesto que forman parte del polo ya que el silenciamiento de estos genes produce la incorrecta formación de la cabeza durante la regeneración. Asimismo, existe una estrecha relación entre estos genes a nivel transcripcional. Por todo, esto se ha sugerido que la función de estos genes es modelar la región anterior (Vogg et al., 2014; Vogg et al., 2016).

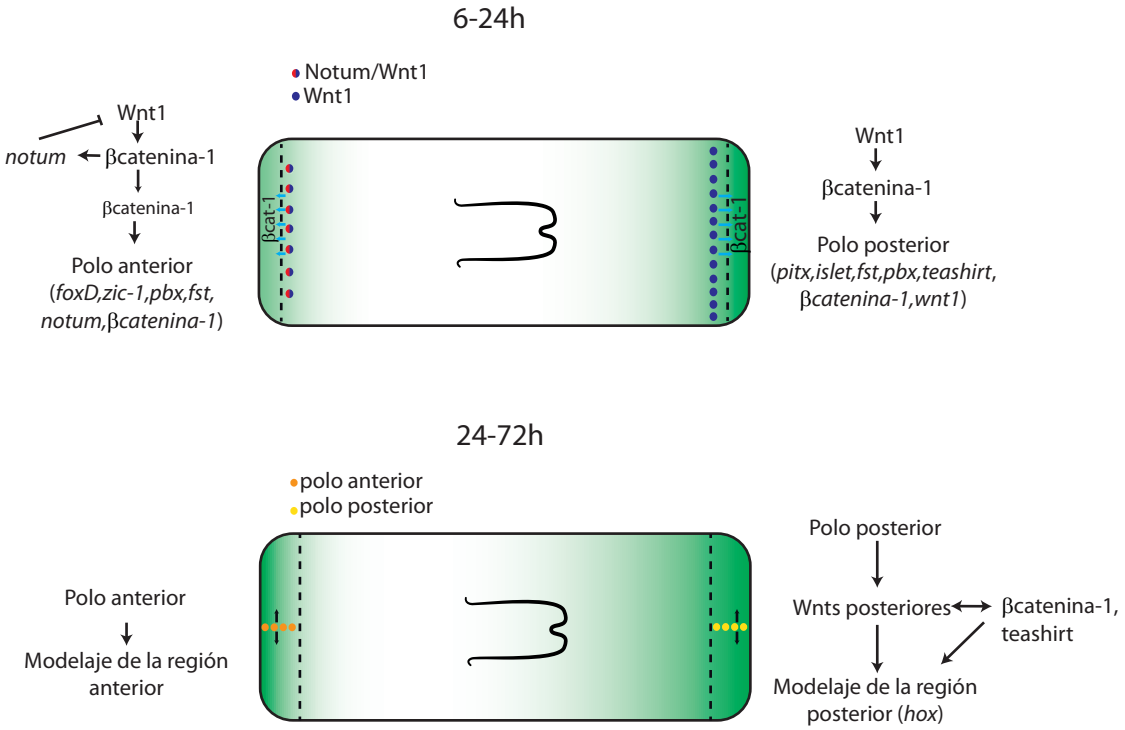


Figura 45. Modelo propuesto del funcionamiento de los polos durante la regeneración. Entre las 6-24 horas post-amputación, el Wnt1 temprano activa a la βcatenina-1 en ambos blastemas, pero de forma preferencial en posterior debido a la presencia de Notum (su inhibidor) en anterior. La actividad baja de la βcatenina-1 de anterior activa la expresión de los genes del polo anterior. La actividad alta de la βcatenina-1 en posterior activa la expresión de los genes del polo posterior. A partir de las 24 horas post-amputación, el polo anterior se encarga de modelar la región anterior. El polo posterior activa la expresión de los *wnts* posteriores, que a su vez activaran a la βcatenina-1 para modelar la región posterior a través de activar otro efectores como los genes *hox* y t*eashirt.*

Algunos de los elementos que encontramos en estos polos no son exclusivos solo de uno de ellos. Por ejemplo, *islet* y *pitx* además de expresarse en el polo posterior también se expresan en el polo anterior durante la regeneración, y su silenciamiento produce defectos en la regeneración anterior (Currie y Pearson, 2013; März et al., 2013). El gen *follistatin* es otro ejemplo, también se expresa en posterior y produce defectos en la

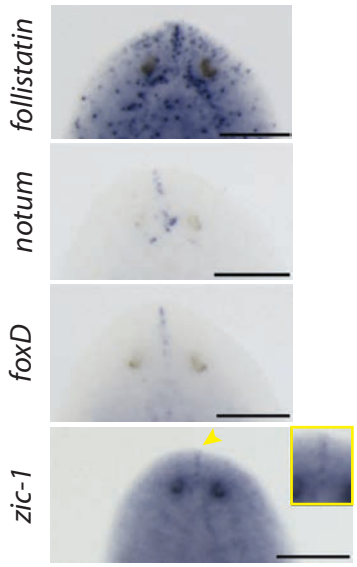


Figura 46. Patrón de expresión de los genes del polo anterior. Hibridación *in situ* de *follistatin*, *notum*, *foxD* y *zic-1*. Flecha amarilla indica ampliación. [Adaptado de Vogg et al., 2014].

cola (Gaviño et al., 2013). Asimismo, el gen *pbx* es común en ambos polos y su silenciamiento bloquea la regeneración, por lo que se ha sugerido que es necesario para la formación de ambos polos (Blassberg et al., 2013; Chen et al., 2013).

Nuestro estudio sobre la localización de la βcatenina-1 sugiere que también puede ser un elemento común de ambos polos/organizadores y no solo del polo posterior, porque:

- encontramos βcatenina-1 en la punta anterior (donde se encuentra el polo anterior),
- la inhibición de *βcatenina-1* produce la pérdida del surco de la línea media anterior donde se encuentra el polo anterior (Sureda-Gómez et al., 2016),
- la inhibición de *βcatenina-1* produce la desaparición de *notum* en el polo anterior (Sureda-Gómez et al., 2016), además de los otros genes del polo como *foxD* o *zic-1* (comunicación personal del Dr. Mathias Vogg). Esto sugiere que βcatenina-1 es un elemento necesario para mantener o establecer el polo anterior (Figura 45).

Este modelo de los polos/organizadores se basa principalmente en datos obtenidos durante la regeneración. Sin embargo, durante la homeostasis no se ha esclarecido si los organizadores continúan activos. Es importante tener en cuenta que el contexto de regeneración y homeostasis es diferente, ya que no es lo mismo regenerar una región del cuerpo que mantenerla. Durante la regeneración, el animal debe organizar el blastema, un nuevo tejido. En cambio durante la homeostasis solo debe mantener lo establecido. Además, existen genes que actúan solo durante la regeneración o que su función varía dependiendo de si el animal regenera o no (Fraguas et al., 2014). Así, la inhibición de los elementos del polo anterior durante la homeostasis no afecta a la polaridad. De igual modo, la inhibición de *wnt1* tampoco parece que produzca ningún tipo de defecto (Sureda-Gómez et al., 2015). Solo la inhibición de *βcatenina-1* o *teashirt* provocan la perdida de la identidad posterior y la aparición de una nueva identidad anterior (Gurley et al., 2008; Iglesias et al., 2008; Owen et al., 2015; Petersen y Reddien, 2008; Reuter et al., 2015). La inhibición de todos los *wnts* posteriores produce una pérdida de la identidad posterior y media, sin transformación anterior. En mi opinión, este fenotipo se puede explicar sin necesidad de mantener un organizador posterior. Cabe recordar que la musculatura puede actuar como un GPS y por tanto la modificación de la información posicional afecta a las diferentes identidades preexistentes (Witchley et al., 2013). Estos datos sugieren que los polos actúan como organizadores durante la regeneración pero su función es transitoria, como en embriones. Sin embargo, cabe remarcar que los genes del polo anterior y posterior continúan expresándose durante la homeostasis.

2. La vía Wnt y su papel durante la organogénesis

La vía Wnt ejerce un papel evolutivamente conservado en el establecimiento del eje AP durante el desarrollo (Petersen y Reddien, 2009b), pero también tiene un función esencial durante la organogénesis de diversas estructuras, como puede ser el sistema nervioso (Ciani y Salinas, 2005) o el sistema visual (Cho y Cepko, 2006). Muchas de las funciones de la βcatenina se han caracterizado durante el desarrollo embrionario, no así durante la regeneración de los órganos.

Las planarias son un organismo ideal para estudiar la regeneración de los diversos órganos (Roberts-Galbraith y Newmark, 2015). Sin embargo, no se había descrito ninguna función de la vía Wnt durante la organogénesis de las planarias, posiblemente debido a que puede pasar desapercibida en los fenotipos anteriorizados tan fuertes que provoca la inhibición de la vía. Nuestros resultados demuestran que la vía Wnt sí tiene un papel importante durante la regeneración y la homeostasis de diferentes órganos. El sistema nervioso y visual de las planarias son dos de los principales sistemas donde hemos demostrado la función de la vía Wnt. Además, en planarias del biotipo sexual hemos demostrado que la βcatenina-1 es necesaria para el desarrollo del sistema reproductor. En este capítulo discutiremos el papel de la vía Wnt dependiente de βcatenina en el proceso de organogénesis durante la regeneración y la homeostasis.

2.1 La vía Wnt en el sistema nervioso

En las planarias se han descrito multitud de genes y vías implicadas en la regeneración y la homeostasis del sistema nervioso (Roberts-Galbraith y Newmark, 2015). Sin embargo, la vía Wnt canónica no se consideraba que tuviera un papel en el sistema nervioso.

En esta tesis, gracias al desarrollo de un anticuerpo contra la βcatenina-1, hemos demostrado que la βcatenina-1 esta nuclearizada en multitud de células del sistema nervioso. Además su silenciamiento produce defectos en la regeneración del cerebro. Estos resultados demuestran que la vía Wnt canónica ejerce una función en el sistema nervioso de planarias, en concreto en el cerebro.

La vía Wnt canónica actúa en el sistema nervioso de multitud de especies (Ciani y Salinas, 2005), por lo que es plausible que en planaria también desempeñe esta función. En ratón, el silenciamiento de la función de la βcatenina produce diferentes defectos en el sistema nervioso, desde pérdidas de estructuras como el cerebro medio o el cerebelo, hasta la afectación de precursores neuronales (Brault et al., 2001; Hari et al., 2002). En pez cebra también se ha sugerido que la βcatenina controla genes esenciales para la correcta formación del cerebro, como el gen *fez* (Zhang et al., 2014). Asimismo, en *Xenopus* se ha demostrado que la actividad de la vía wnt canónica es esencial para la regionalización del cerebro (Lake y Kao, 2003). Por otro lado, el incorrecto control de la vía wnt canónica en humanos puede causar diferentes tumores del sistema nervioso (Chenn, 2008). En invertebrados también se ha demostrado el funcionamiento de la vía wnt dependiente de βcatenina en la formación del sistema nervioso, por ejemplo en *Platynereis* (Demilly et al., 2013), *C. elegans* (Korswagen et al., 2000) o *Drosophila* (Loureiro y Peifer, 1998).

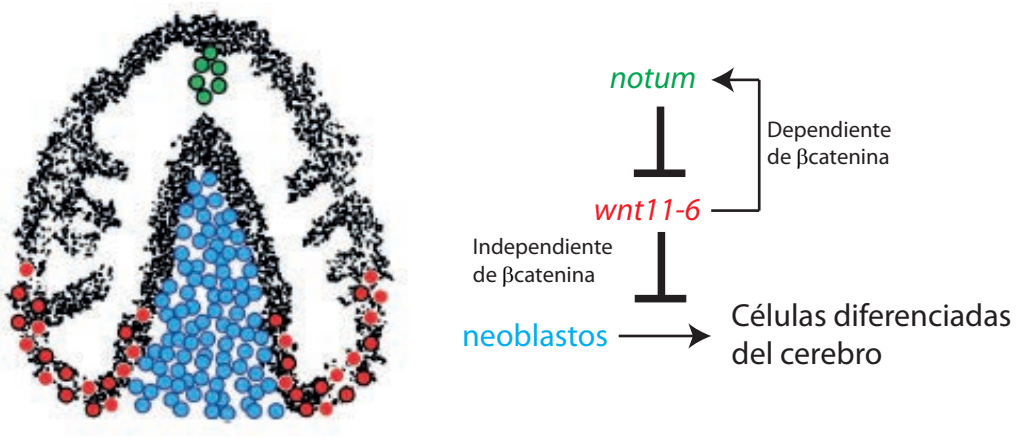


Figura 47. Modelo de la regulación del tamaño del cerebro. El dibujo muestra el cerebro de una planaria, sus diferentes neuronas (gris), las neuronas que expresan *wnt11-6* (rojo), las que expresan *notum* (verde) y los neoblastos (azul) que se encuentran alrededor del cerebro. El esquema indica las diferentes interacciones entre genes. Wnt11-6 inhibe la producción de neuronas de forma independiente a βcatenina y activa la expresión de notum a través de βcatenina-1. Notum inhibe la función de Wnt11-6 y promueve la síntesis de neuronas.

En muchos de estos organismos, la posibilidad de inhibir la βcatenina en una ventana de tiempo en concreto facilitó la identificación de su función en el sistema nervioso. En planarias, aunque diferentes elementos de la vía wnt canónica se expresan en el sistema nervioso, la afectación del eje AP enmascara otros defectos no relacionados con polaridad. Recientemente se ha propuesto que βcatenina-1 es un elemento implicado en el control del tamaño del cerebro en planarias (Hill y Petersen, 2015), aunque de forma no autónoma. Hill y Petersen proponen que un sistema basado en el ligando Wnt11-6 y su posible inhibidor Notum controla el tamaño del cerebro mediante un bucle de retroalimentación negativo (Figura 47). En este sistema Wnt11-6 activa la expresión de notum a través de la βcatenina-1, y la función de notum es promover el crecimiento del cerebro. Sin embargo, Wnt11-6 inhibe la formación del cerebro de manera independiente a βcatenina-1 (no canónico).

Nuestros resultados muestran que, tras inhibir *βcatenina-1*, el cerebro no es solo más pequeño (como en el caso de inhibir notum), sino que también presenta otros defectos en su morfología. En mi opinión, al igual que en otros organismos, la βcatenina-1 puede controlar la diferenciación de diversos tipos neuronales, aunque es necesario profundizar más en el estudio de su función. Por ejemplo, no conocemos qué elemento es el responsable de activar a βcatenina-1 en las neuronas del cerebro. Se puede descartar a Wnt11-6, ya que el fenotipo provocado por su inhibición (elongación del cerebro) es el opuesto de inhibir *βcatenina-1* (cerebro más pequeño). Por otro lado la inhibición conjunta de *wnt11-6* y *βcatenina-1* produce el mismo fenotipo que inhibir *wnt11-6* solo (Hill y Petersen, 2015). En la región anterior, además de *wnt11-6*, también se expresa el *wnt2*. Sin embargo no se ha caracterizado su función. Asimismo, no se puede descartar que otros *wnts* también se expresen en el cerebro. Debido a los límites de sensibilidad de la técnica de in situ puede que no seamos capaces de detectar otros *wnts*, como el *wnt1*. Otra posibilidad es que otro activador no descrito en planarias sea el responsable de activar a la βcatenina en la región anterior, como por ejemplo Norrin (Xu et al., 2004) o las WNK kinasas (Serysheva et al., 2013).

Otra pregunta importante es cuál es el papel de βcatenina-1 en el cerebro y cómo se

puede regular su actividad. En la mayoría de organismos, nuclearizar βcatenina implica activar la transcripción, si bien dentro del núcleo su actividad puede ser modulada negativa o positivamente. Se ha descrito que el factor de transcripción Sox puede unirse a βcatenina para activar un programa de transcripción en concreto o competir con βcatenina por la unión a TCF para inhibir su actividad (Kormish et al., 2010). En los ojos de planarias, hemos demostrado que la βcatenina-4 regula la actividad de βcatenina-1 en el núcleo. Asimismo, tanto la βcatenina-4 como la 3 también se expresan en el cerebro, sugiriendo que pueden tener una función de regulación de la βcatenina-1 en este órgano (Su, Sureda-Gómez y col., en revisión). Además no hay que descartar otros factores de transcripción, como el propio Sox, que también pueden modular la actividad de βcatenina-1 en el sistema nervioso.

Nuestros resultados indican que la βcatenina-1 es necesaria para la regeneración de estructuras anteriores como el cerebro. Sin embargo existen datos que indican que la inhibición de la βcatenina-1 es necesaria para regenerar esas mismas estructuras anteriores. Así, la inhibición de *βcatenina-1* en especies de planarias incapaces de regenerar la cabeza provoca la recuperación de la capacidad regenerativa anterior (Liu et al., 2013; Sikes y Newmark, 2013; Umesono et al., 2013). En realidad, estos datos son compatibles con los aportados hasta ahora. Como se ha visto en el capitulo anterior, la βcatenina-1 debe silenciarse o al menos modularse en blastemas anteriores para definir la identidad anterior. Ahora bien, una vez se ha establecido la identidad, la βcatenina-1 es necesaria para la correcta neurogénesis. Como en muchos otros ejemplos, es esencial el momento y el lugar de su activación. Así, en los experimentos con planarias incapaces de regenerar la cabeza, la inhibición de la *βcatenina-1* no se mantiene en el tiempo, solo es inhibida antes de que se produzca la regeneración para que regeneren la cabeza. Una posible explicación de este hecho es que para activar la regeneración anterior se debe inhibir la *βcatenina-1*, para después recuperar los niveles normales y regenerar los tejidos neurales.

Los estudios de sobreactivación de la vía Wnt canónica en planarias son otro ejemplo que demuestra que la *βcatenina-1* no necesita ser silenciada para poder regenerar tejidos neurales. La inhibición de las *axinas* (elemento del complejo de destrucción de la βcatenina) causa la formación de una cola en la región anterior. Sin embargo también se generan unos primordios de cerebro (Iglesias et al., 2011). En otras palabras, en presencia de una alta concentración de βcatenina-1, pueden aparecen estructuras neurales, aunque no prosperen correctamente.

2.2 La vía Wnt y el sistema visual

El sistema visual de las planarias es uno de los sistemas más estudiados durante la regeneración y la homeostasis (González-Sastre et al., 2012; Lapan y Reddien, 2011; Lapan y Reddien, 2012; Saló et al., 2002). Al igual que con el sistema nervioso, no se había descrito ninguna función de la vía wnt en este sistema.

Nuestros resultados indican que la vía Wnt canónica tiene un papel fundamental en el sistema visual de las planarias. Hemos demostrado la localización de las proteínas

βcatenina-1 y βcatenina-4 en los fotoreceptores. Además la inhibición de ambas causa defectos en el sistema visual. Así, la inhibición de βcatenina-1 produce ojos más grandes, mientras que la inhibición de la βcatenina-4 provoca el fenotipo opuesto, ojos más pequeños. La caracterización de estos fenotipos nos ha permitido demostrar que la función de estas βcateninas es promover (en el caso de la βcatenina-4) o inhibir (en el caso de la βcatenina-1) la especificación/diferenciación de los fotoreceptores.

Por otra parte, hemos identificado un nuevo *TCF* que se expresa también en los fotorecepetores (*TCF-2*). La inhibición de *TCF-2* causa la formación de ojos más grandes, igual que inhibir la *βcatenina-1*. Además, el silenciamiento de forma conjunta de *βcatenina-4* y *TCF-2* provoca la regeneración de ojos más grandes. Este resultado indica que la βcatenina-1 y TCF-2 actúan conjuntamente para activar la transcripción y que la βcatenina-4 actúa como inhibidora de esta actividad. Mediante estudios *in vitro*, hemos demostrado que la βcatenina-4, aunque puede unirse a TCF, no puede activar la transcripción, y esto sugiere que puede actuar como inhibidor por competición. Asimismo, utilizando un ensayo de competición *in vitro* hemos demostrado que la βcatenina-4 inhibe la actividad de la βcatenina-1 por competición a la unión de TCF. En conjunto, estos datos nos llevan a proponer que βcatenina-4 actúa como un inhibidor de la βcatenina-1 por competición a la unión del factor de transcripción TCF-2. Así, pues la modulación de la actividad de βcatenina-1 es indispensable para la correcta especificación de los fotoreceptores (Figura 48).

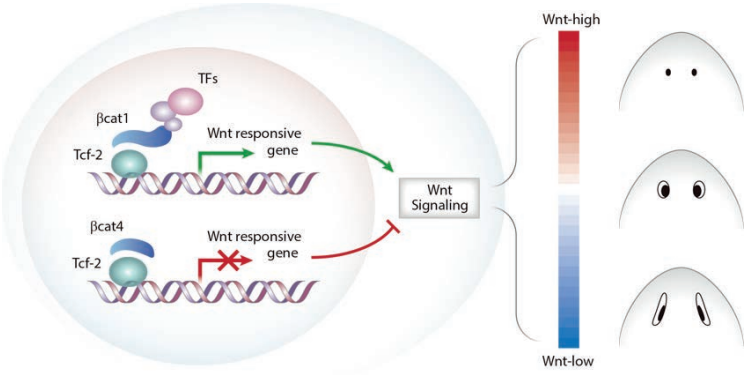


Figura 48. Modelo propuesto de la regulación del tamaño de los ojos. La correcta regulación de la actividad de βcatenina-1 establece el correcto tamaño de los ojos. La βcatenina-1 activa la vía mediante su unión a TCF-2. La βcatenina-4 actúa como inhibidor por competición de la βcatenina-1 por la unión a TCF-2. Esta competición produce la correcta modulación de la actividad de βcatenina-1. La sobreactivación de la vía produce ojos pequeños y la inhibición de la vía ojos grandes.

La función de la vía Wnt canónica en la organogénesis del sistema visual también se ha demostrado en otros organismos (Fuhrmann, 2008). Tanto en el embrión de pollo (Cho y Cepko, 2006), de pez cebra (Meyers et al., 2012) o en *Drosophila* (Legent y Treisman, 2008), la incorrecta regulación de la vía causa defectos en el sistema visual. Sin embargo, la función de la vía wnt en el sistema visual no es siempre a nivel de fotoreceptores. En *Drosophila* se sugiere que la función de Armadillo (βcatenina en *Drosophila*) es organizar la retina (Legent y Treisman, 2008). En cambio en el embrión de pollo la función de βcatenina es determinar el destino de algunos tipos celulares del ojo, como los que forman el iris (Cho y Cepko, 2006). Además, en *Xenopus* se ha demostrado que la activación de la vía Wnt canónica mantiene las células madre de la retina y sus progenitoras en un estado proliferativo, mientras que su inhibición no causa ningún defecto (Denayer et al., 2008). Todos estos datos apoyan que la vía wnt canónica ejerce una función en

el sistema visual a lo largo de la evolución, aunque una función específica a nivel de fotoreceptores no está demostrada en muchos casos.

En planarias la vía wnt canónica está implicada en la especificación de los fotoreceptores, pero desconocemos su función a nivel celular. Una posibilidad es que la vía Wnt canónica organice los fotoreceptores en el ojo, como en *Drosophila* (Figura 49 A). Si bien la inhibición de *βcatenina-1* (ojos grandes) causa la diferenciación ectópica y un incorrecto posicionamiento de las células pigmentarias y fotorecepetoras, el ARNi de *βcatenina-4* (ojos pequeños) no afecta a la organización del ojo. No obstante, cabe remarcar que la diferenciación ectópica no se produce tras inhibir TCF-2, por lo que no se puede descartar que βcatenina-1 pueda ejercer diferentes funciones en este órgano. Otra posibilidad es que la vía wnt canónica controle la proliferación de los progenitores de fotoreceptor, como en el caso de *Xenopus* (Figura 49 B). Esta opción es más plausible, ya que la *βcatenina-4* se encuentra en los progenitores de fotoreceptor (*h2b+/ovo+*) y al inhibirla se observa una reducción en el numero de progenitores. Asimismo, inhibir la *βcatenina-1* da como resultado un aumento de los fotoreceptores, si bien se debería estudiar si aumentan los progenitores, que sería lo esperable. Por último, otra opción es que la vía Wnt canónica no afecte a la proliferación, sino a la diferenciación hacia fotoreceptor (Figura 49 C). En este caso, se debería estudiar el destino alternativo de esas células, si se diferencian en otro tipo celular o si entran en apoptosis.

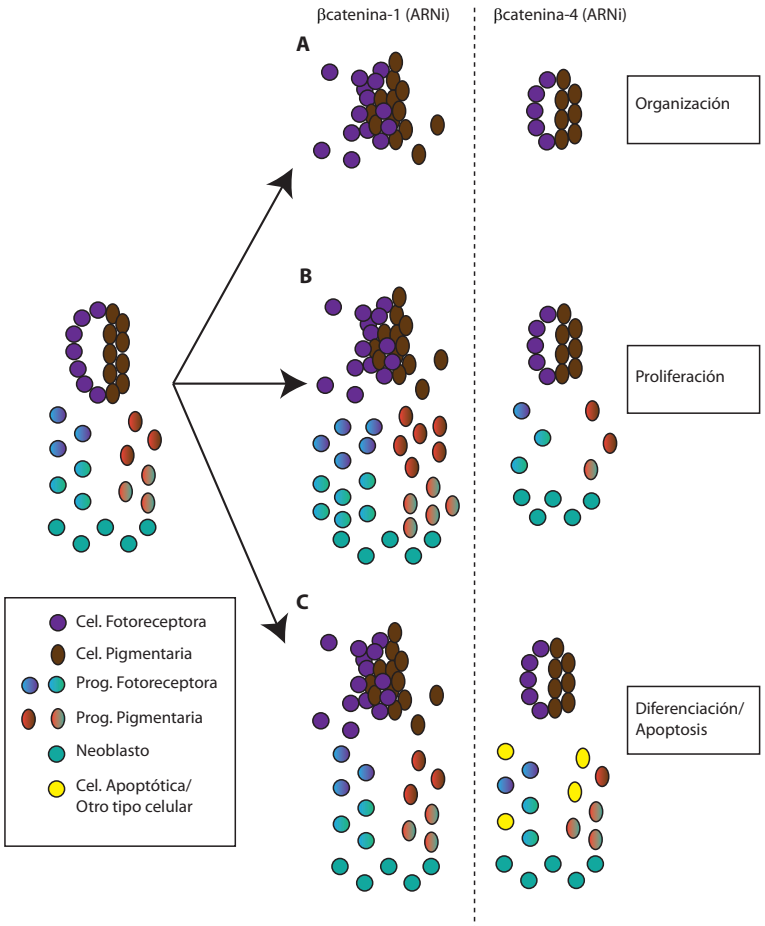


Figura 49. Posible mecanismo de como la vía Wnt canoníca controla el tamaño y forma de los ojos en planaria. El dibujo de la izquierda muestra la situación control. (A) La vía canónica controla la correcta organización del ojo. La inhibición de la vía provoca la desorganización de la estructura. (B) La vía wnt canónica controla la proliferación de los progenitores. La inhibición de la vía canónica provoca el aumento de la proliferación de los progenitores y por consiguiente el tamaño del ojo. La sobreactivación de la vía provoca la disminución de la proliferación de los progenitores y por consiguiente el tamaño del ojo. (C) La vía wnt canónica controla la diferenciación/apoptosis de los progenitores de ojo. La inhibición de la vía provoca la diferenciación de los progenitores de ojo, diferenciándose fuera de la estructura. La sobreactivación de la vía provoca la apoptosis/diferenciación hacia otro tipo celular diferente de ojo y provoca un tamaño más pequeño del ojo.

2.3 La vía Wnt y su papel en el sistema reproductor

El sistema reproductor de las planarias nos permite estudiar un tipo de células madre diferentes a los neoblastos, las células madre germinales. Mediante el anticuerpo contra la βcatenina-1 hemos demostrado que la proteína se encuentra nuclearizada en las presuntas células madre germinales. Asimismo, la inhibición de βcatenina-1 bloquea la aparición de las células germinales durante la maduración del juvenil de planaria. Estos resultados muestran que la vía wnt canónica es necesaria para la determinación o aparición de las células madre germinales, tanto masculinas como femeninas.

La función de la vía wnt en el sistema reproductor no ha sido muy estudiada en otros organismos. En ratón se ha demostrado que la vía wnt canónica no tiene ningún papel en el mantenimiento de las células germinales (Rivas et al., 2014) pero sí en su determinación durante la embriogénesis (Ohinata et al., 2009). En las células madre germinales de *Drosophila* también se ha demostrado que la vía Wnt canónica no ejerce ninguna función en el mantenimiento de las células germinales (Losick et al., 2011). A pesar de la falta de evidencias sobre la función de la vía wnt canónica en células madre germinales, sí que existen en otros tipos de célula madre (Clevers y Nusse, 2012). Por ejemplo la vía wnt canónica es necesaria para el mantenimiento de las células madre intestinales o las del folículo piloso (Clevers et al., 2014).

En planarias, la situación parece la opuesta a la descrita en otros organismos. La βcatenina-1 está implicada en el mantenimiento o determinación de las células germinales en un estado indiferenciado, pero no así en los neoblastos. La variación de la vía wnt no parece alterar la población de neoblastos. Es decir, la inhibición de la βcatenina-1 no produce ningún fenotipo asociado a la perdida de neoblastos, como es la regresión de la cabeza o el "curling" del animal. Sin embargo, no podemos descartar que afecte a alguna subpoblación de neoblastos, por ejemplo a las células progenitoras de los fotoreceptores.

3. La vía Wnt canónica y su papel durante la embriogénesis de planaria

Gran parte de los estudios sobre la vía Wnt canónica se han realizado durante el desarrollo embrionario de multitud de organismos. Sin embargo, en las planarias, el estudio de la vía Wnt se ha centrado exclusivamente en el adulto. Debido a que las planarias presentan un desarrollo embrionario divergente, a que sus embriones son de difícil acceso y que el adulto posee esta impresionante capacidad de regeneración, el estudio de la embriología de planarias ha pasado desapercibido.

A pesar de ello, los diferentes resultados obtenidos en planarias adultas respecto a mecanismos del desarrollo, son comparables a los descritos en embriones de otros organismos. De hecho, se puede decir que las planarias en su estado adulto se comportan molecular y celularmente como embriones ya que: 1) presentan una gran plasticidad, 2) sus células madre muestran una alta pluripotencia y 3) diferentes vías de señalización como la vía Wnt o la vía BMP están continuamente activadas. Pero, ¿las planarias adultas se comportan como sus propios embriones? Dicho de otra forma, ¿es la regeneración y la homeostasis del adulto una recapitulación del desarrollo embrionario a nivel molecular y celular?

En esta tesis doctoral hemos abordado esta cuestión a través del estudio de la vía Wnt canónica. Además, esta estrategia ha aportado nuevos datos sobre la embriogénesis de planaria.

3.1 La βcatenina-1 y su papel durante la fase larvaria

En la introducción ya se ha mencionado que durante la embriogénesis de planaria se pueden distinguir diferentes estadios, del 1 al 8. En este apartado de la discusión me centraré principalmente en los considerados de fase larvaria, es decir, desde el de zigoto (estadio 1) hasta el comienzo de la metamorfosis (estadio 4).

Durante las primeras horas del desarrollo de la planaria, el zigoto se divide dando lugar a los blastómeros, los cuales permanecen aislados entre ellos. Estos blastómeros están rodeados por las células vitelinas, de las cuales algunas se fusionaran entre ellas para englobar a los blastómeros y formar un sincitio. Martin-Duran y colaboradores demostraron mediante PCR cuantitativa que la βcatenina-1 se encuentra muy expresada en estadio 1, pero no mostraron su localización celular (Martín-Durán et al., 2010). Nuestro estudio sobre la localización de la proteína βcatenina-1 durante el estadio 1 temprano muestra que algunas células vitelinas presentan βcatenina-1 en el núcleo. Los blastómeros por contra no presentan βcatenina-1 antes de la formación del sincitio (Figura 50). La presencia de βcatenina-1 en células vitelinas también se ha descrito en el embrión de pez cebra (Kelly et al., 2000). La función de la βcatenina en estas células es inducir la formación del escudo, el organizador del embrión de pez cebra equivalente al centro de Nieuwkoop en rana (Joubin y Stern, 2001). En ambos casos la βcatenina actúa de forma materna en la inducción de la actividad organizadora del embrión. Nuestros datos apuntan a que en el embrión de planaria las células vitelinas con βcatenina-1 nuclear

pueden actuar como inductores de la formación del sincitio y/o la correcta progresión de los blastómeros. Es importante remarcar que ya se había propuesto un rol activo de las células vitelinas durante el estadio 1, basándose en estudios sobre su actividad transcripcional y su morfología celular (Cardona et al., 2005; Le Moigne, 1966).

Después de la formación del sincitio, los blastómeros se agregan entre ellos para formar la epidermis (ectodermo) y, la faringe y la cavidad digestiva (mesoendodermo) que son las estructuras embrionarias de la larva. Las planarias no presentan una blástula como tal, sin embargo sí que presentan las diferentes capas germinales. En muchos organismos bilaterales la formación del mesoendodermo se debe a la nuclearización de la βcatenina en las células del blastoporo (Huelsken et al., 2000; Korswagen et al., 2000; Logan et al., 1999; Schneider y Bowerman, 2007; Wikramanayake et al., 2003). Resultados previos en planarias sugieren que la βcatenina-1 está implicada en la formación del mesoendodermo ya que se expresa en la faringe embrionaria (Martín-Durán et al., 2010). Nuestros datos concuerdan con estos resultados previos, ya que encontramos βcatenina-1 nuclear en los primordios de la faringe y del digestivo. Cabe resaltar que antes de la formación de los primordios de ambas estructuras detectamos algunas cé-

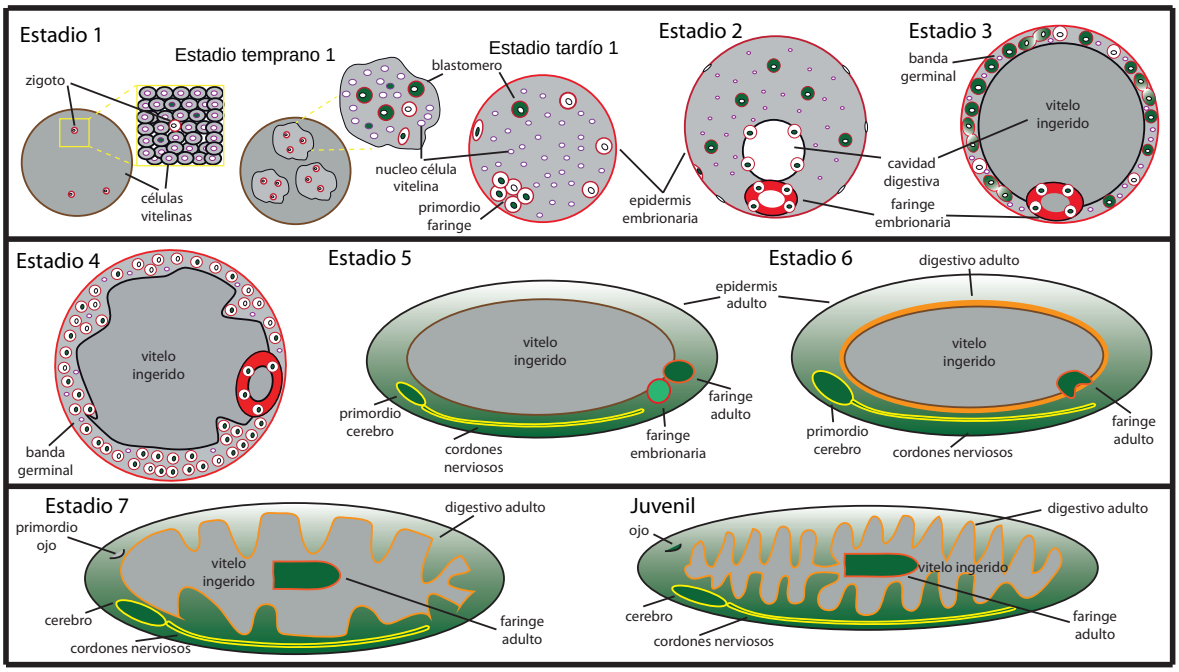


Figura 50. Resumen de la localización de la βcatenina-1 durante la embriogénesis de planaria. (Estadio 1 temprano) La βcatenina-1 (color verde) se encuentra nuclearizada en alguna células vitelinas. En el sincitio encontramos βcatenina-1 nuclear en algunos núcleos de células vitelinas fusionadas y en blastómeros elongados (migrando); algunos blastómeros con forma esférica presentan βcatenina-1 citoplasmática. (Estadio 1 tardío) La βcatenia-1 se nuclearíza en blastómeros del primordio de la faringe y en las presuntivas células progenitoras de la epidermis. También encontramos βcatenina-1 citoplasmática en los blastómeros indiferenciados. (Estadio 2) La βcatenina-1 se nuclearíza en la faringe y el digestivo embrionario. También se nuclearíza en las presuntivas células progenitoras de la epidermis. La gran mayoría de blastómeros indiferenciados presenta βcatenina-1 citoplasmática. (Estadio 3) La βcatenina continua nuclearizada en la faringe y digestivo embrionario. Los blastómeros se dividen de forma simétrica en términos de distribución de βcatenina citoplasmática. (Estadio 4) La βcatenina-1 se nuclearíza en multitud de blastómeros. Los blastómeros con βcatenina-1 nuclear se acumulan en la región ventral. (Estadio 5) Nuclearización masiva de βcatenina-1. Acumulación de blastómeros con βcatenina-1 nuclear en ventral y en los primordios de diferentes órganos como el cerebro y faringe definitiva. (Estadio 6 - Juvenil) La βcatenina-1 se encuentra nuclearizada en diferentes órganos y células parenquimáticas.

lulas, posiblemente migrando, que muestran βcatenina-1 nuclear, siendo los primeros blastómeros en nuclearizarla (Figura 50). En el futuro se debería estudiar si esta primera asimetría poblacional provocada por la nuclearización de βcatenina-1 es la que da lugar al mesoendodermo.

Por otra parte, también hemos observado que algunos blastómeros que probablemente no provienen del mesoendodermo también nuclearizan la βcatenina-1. Su posición justo debajo de la epidermis y su forma alargada sugieren que podrían ser células progenitoras de la epidermis. Sin embargo nunca se observan células epidérmicas que expresen βcatenina-1 (Figura 50). Una posible explicación de la función de βcatenina-1 es que esté implicada en determinación pero no en diferenciación terminal a epidermis. Otra posibilidad es que se nuclearice para inducir la migración, como se propone en otros sistemas dada su función en la transición epitelio-mesénquima (Gonzalez y Medici, 2014). El hecho de que los primeros blastómeros que nuclearizan βcatenina1 también presenten morfología elongada y se encuentren apartados del resto apoyaría esta posibilidad.

Durante los primeros estadios embrionarios de la planaria, los blastómeros de la larva están continuamente proliferando. Se ha propuesto que esta proliferación está asociada al incremento del tamaño de la larva y que por tanto los blastómeros se mantienen en estado indiferenciado (Cardona et al., 2005; Martín-Durán et al., 2010). Gracias al estudio de la distribución de la βcatenina-1, hemos demostrado que durante los primero estadios (1-3) las mitosis son simétricas en términos de distribución de la βcatenina-1. Este resultado apoya la asunción de que los blastómeros se mantienen en el mismo estado indiferenciado. Sin embargo, al comienzo del estadio 4, algunas de las mitosis observadas son asimétricas en cuanto a la distribución de la βcatenina-1. En este mismo estadio muchos genes implicados en diferenciación comienzan a expresarse (Martín-Durán et al., 2010; Monjo y Romero, 2014). Estos datos sugieren que las divisiones asimétricas podrían originar diferentes tipos celulares, coincidiendo con el inicio de la metamorfosis y la aparición de genes implicados en determinación celular.

3.2 El establecimiento de los ejes AP y DV durante la embriogénesis de planaria.

Durante el desarrollo embrionario de la gran mayoría de organismos, el establecimiento de los ejes AP y DV está controlado por la vía Wnt y BMP respectivamente (Niehrs, 2010). En las planarias adultas ambas vías también controlan el establecimiento de los ejes AP y DV durante la homeostasis y la regeneración. Sin embargo en los embriones de planarias continua siendo desconocido como se establecen estos ejes en la larva y después de la metamorfosis. Los embriones de planarias a diferencia de otros organismos con desarrollo en espiral, no presentan micrómeros y macrómeros (Cardona et al., 2005; Thomas, 1986). Cabe recordar que la presencia de los micrómeros en un polo del embrión marca el polo vegetal (Hyman, 1951), que corresponderá con el polo posterior de bilaterales (Imai et al., 2000; Logan et al., 1999; Wikramanayake et al., 2003). Estudios morfológicos previos en embriones de planaria sugieren que el oocito de planarias presenta una polaridad primaria (Anderson y Johann, 1958; Benazzi, 1950; Stevens, 1904). No obstante, durante la segmentación del zigoto no se ha detectado ningún tipo

de polaridad y la primera asimetría clara se detecta en la larva con la formación de la faringe embrionaria. La faringe confiere a la larva una asimetría radial y no bilateral. Estudios de expresión génica apuntan hacia la misma dirección, ya que la expresión de los genes sFRP1 y wnt1, que en adultos se expresan en el polo anterior y posterior respectivamente (Gurley et al., 2010), no presentan ningún patrón AP en la larva (Martín-Durán et al., 2010). Además, solo se expresan en blastómeros aislados de la larva y nunca en la faringe embrionaria. Aunque los elementos de la vía wnt no se encuentren polarizados, nuestros resultados muestran que la βcatenina-1 se nucleariza en la faringe y en su primordio. Si se tiene en cuenta que la βcatenina-1 se nucleariza en la región donde se forma el endomesodermo definiendo el polo posterior (Wikramanayake et al., 1998), nuestros datos sugieren que la faringe embrionaria puede ser el polo posterior de la larva. Esta hipótesis está de acuerdo con la función conservada de βcatenina en la especificación de la identidad posterior a lo largo de la evolución (Petersen y Reddien, 2009b).

Durante la metamorfosis, la larva se aplana adquiriendo una polaridad AP y DV. A partir del estadio 5-6 del desarrollo se detectan de forma polarizada como en el adulto varios elementos de la vía Wnt y BMP, como wnt1, sFRP1 o BMP (Martín-Durán et al., 2010). Sin embargo la distribución de βcatenina-1 en el embrión no se encuentra en forma de gradiente, a diferencia del adulto (Sureda-Gómez et al., 2016). Nuestros resultados muestran una acumulación preferencial de βcatenina-1 en la región ventral del embrión (Figura 50). Aunque esto pueda sugerir que la βcatenina-1 especifica región ventral respecto a dorsal, cabe recordar que en este momento el sistema nervioso se está desarrollando en la región ventral (Monjo y Romero, 2014). Y en adultos y embriones hemos demostrado que la βcatenina-1 se nucleariza en este sistema y que ejerce una función durante la regeneración (Sureda-Gómez et al., 2016). Así, hipotetizamos que esta acumulación ventral de βcatenina-1 está asociada al sistema nervioso y no directamente con polaridad.

Cabe recordar que al comienzo del estadio 4, cuando va a dar comienzo la metamorfosis, observamos divisiones asimétricas en términos de distribución de βcatenina-1. En otros organismos como *Platynereis* o *C. elegans*, se ha descrito que la polaridad en el embrión no se especifica a través de un gradiente de actividad de βcatenina, sino a través de la distribución asimétrica de βcatenina durante las divisiones (Rocheleau et al., 1999; Schneider y Bowerman, 2007). Sin embargo en nuestro estudio, la distribución de la βcatenina-1 es a nivel del citoplasma y no a nivel nuclear como en *Platynereis* y *C. elegans*. Sería necesario un estudio más preciso de estas divisiones asimétricas, ya que desconocemos si después de la división, la βcatenina-1 se nucleariza en la célula hija.

3.3 ¿Es la regeneración la recapitulación de la embriogénesis?

El hecho de que en las planarias adultas las diferentes vías de señalización estén continuamente activadas, tal y como ocurre durante el desarrollo embrionario de otros organismos, lleva a pensar que la regeneración es una recapitulación de la embriogénesis. Por ejemplo, la vía Wnt y la vía BMP especifican los ejes AP y DV respectivamente en múltiples organismos durante el desarrollo embrionario (Niehrs, 2010), de igual forma

que lo hacen durante la regeneración y la homeostasis de las planarias (Almuedo-Castillo et al., 2012; Molina et al., 2011).

Sin embargo cabe preguntarse si la regeneración de las planarias recapitula su propia embriogénesis. Y en concreto, si los mecanismos moleculares que controlan diversos procesos en el adulto como la identidad axial o la organogénesis, también son los mismos durante la embriogénesis. Diferentes estudios en embriones de planarias apoyan la idea de que la regeneración es una recapitulación de la embriogénesis, especialmente desde la metamorfosis de la larva. Así, la caracterización molecular durante los estadios 5-7 del sistema visual, el nervioso o el digestivo sugieren que los mismos genes que actúan durante la regeneración lo hacen también en la embriogénesis (Martín-Durán y Romero, 2011; Martín-Durán et al., 2012; Monjo y Romero, 2014).

En cuanto a la βcatenina-1, hemos demostrado que durante la regeneración y la homeostasis esta proteína ejerce múltiples funciones, ya que especifica posterior y controla la organogénesis del cerebro y el mantenimiento de las células germinales (Sureda-Gómez et al., 2016). Los datos obtenidos de βcatenina-1 durante la embriogénesis de planaria apoyan a la recapitulación de su papel durante la organogénesis de diversos órganos, ya que se nuclearíza en los primordios del cerebro y la faringe durante la metamorfosis. Además en el estadio 2 del embrión, también se nuclearíza en el primordio de la faringe embrionaria apoyando su rol durante la formación de órganos (Figura 50).

Sin embargo, su función en el establecimiento de la identidad posterior durante la embriogénesis es una cuestión más difícil de abordar. En el adulto de planaria, la βcatenina-1 especifica identidad posterior (Gurley et al., 2008; Iglesias et al., 2008; Petersen y Reddien, 2008). Además hemos demostrado que durante la homeostasis la distribución de la βcatenina-1 es en forma de gradiente AP (desde prefaringe a la cola) y que durante la regeneración se acumula preferencialmente en blastemas posteriores (Sureda-Gómez et al., 2016). En cambio, durante la metamorfosis de la larva, no observamos un gradiente de distribución AP, sino una acumulación en la región ventral respecto a la dorsal. Como ya hemos mencionado, esto puede ser debido a su papel en la organogénesis del sistema nervioso pudiendo enmascarar una posible distribución en forma de gradiente AP.

Una posible pregunta es si en los primeros estadios, durante la formación de la larva, la βcatenina-1 especifica el eje AP. Sin embargo, debido a que la larva presenta una simetría radial, en caso de promover axialidad no sería exactamente una recapitulación de un proceso del adulto.

4. Modulación de la vía Wnt canónica: el caso de las βcateninas inhibidoras.

La demostración de la existencia de un gradiente de actividad βcatenina-1 ha sido uno de los puntos importantes de esta tesis. Pero, bajo mi punto de vista, la contribución más importante de esta tesis es la demostración de que la βcatenina-1 ejerce diversas funciones aparte de especificar posterior. Aunque ya se sabía que la βcatenina era una proteína multifuncional (Valenta et al., 2012), al inicio de esta tesis la única función descrita de la βcatenina-1 en planarias era especificar posterior. Además, demostrar que la βcatenina-1 participa en el establecimiento del polo anterior, la regeneración del cerebro, la formación de las gónadas, el mantenimiento y la regeneración de los ojos no es una cuestión trivial a nivel metodológico en nuestro modelo y era impensable unos años atrás.

Esta nueva visión de la βcatenina-1 como proteína multifuncional en planarias se debe en gran medida a la posibilidad de conocer la localización de la proteína y su presencia en el núcleo en multitud de células de la planaria adulta, gracias a la producción de un anticuerpo específico. La observación de la nuclearización de βcatenina-1 en tantos tejidos distintos en planarias adultas, durante su regeneración y durante su embriogénesis nos empujó a indagar si ejercía una función en dicho tejido. Además, este hecho nos llevó a preguntarnos cómo podía regularse la actividad nuclear de βcatenina-1 en los distintos tipos celulares, ya que en prácticamente todas las células que expresan βcatenina-1 ésta se encuentra nuclear. Se habían descrito diferentes mecanismos extracelulares e intracelulares de regulación de la βcatenina-1 en planarias (Gurley et al., 2008; Iglesias et al., 2011; Petersen y Reddien, 2008; Petersen y Reddien, 2011), pero no existían datos sobre su regulación en el núcleo.

Aunque la regulación de la βcatenina en el núcleo no se ha estudiado en profundidad en organismos modelo, existen diferentes proteínas descritas que pueden modular su actividad. Por ejemplo, los factores nucleares Bcl-9 o ICAT pueden unirse a βcatenina provocando que sea más o menos activa (Han y Wang, 2014; Valenta et al., 2012; Xu y Kimelman, 2007). Además también se han descrito factores de transcripción que pueden competir con βcatenina por la unión a TCF, como es Sox (Han y Wang, 2014).

En las planarias, también pueden actuar estas proteínas para modular la βcatenina-1. Sin embargo, en esta tesis doctoral hemos descrito un nuevo mecanismo de regulación en el núcleo. Hemos identificado dos nuevas βcateninas, la βcatenina-3 y 4. Ambas poseen dominios de unión a TCF (transcripción) y de adhesión. No obstante, su dominio de transactivación está truncado, lo que sugiere que pueden actuar como inhibidores de la βcatenina-1 por competición. Mediante estudios *in vitro* utilizando el sistema "TOPFLASH reporter" y de co-immunoprecipitación hemos demostrado que las βcateninas 3 y 4 no pueden activar la transcripción, aunque sí unirse a TCF. Además, confirmamos que inhiben la acción de la βcatenina-1 al ser cotransfectadas. Por tanto, *in vitro*, la βcatenina-3 y 4 actúan como inhibidores de la actividad de βcatenina-1 por competición.

En las planarias, hemos podido demostrar *in vivo* que al menos la βcatenina-4 puede inhibir por competición a la βcatenina-1 por la unión a TCF-2, como se ha explicado

anteriormente. Asimismo, la existencia de otros parálogos o formas de splicing alternativo en otros organismos indican que este mecanismo puede haber aparecido de forma recurrente a lo largo de la evolución. La duplicación de βcateninas no es algo aislado en planarias. Por ejemplo, en *C. elegans* la βcatenina se ha duplicado y subfuncionalizado (Korswagen et al., 2000). En vertebrados la Plakoglobina, que proviene de una duplicación de βcatenina, posee un dominio C-terminal muy modificado y puede ser nuclearizada (Klymkowsky, 1999). Además, en *Drosophila*, Armadillo presenta una forma de splicing alternativo que produce un Armadillo sin el dominio de transactivación, y que es expresada en el sistema nervioso, neural armadillo (Narm) (Loureiro y Peifer, 1998). Nuestros estudios in vitro indican que tanto la Plakoglobina como Narm pueden inhibir la función de βcatenina. Estos datos sugieren que una forma truncada de βcatenina podría ser una forma de regular la actividad de βcatenina en el núcleo en tejidos específicos, por ejemplo en el nervioso, posibilitando su complejidad.

En el futuro se debería estudiar si estas formas truncadas de βcateninas (Plakoglobina y Narm) pueden actuar como inhibidores *in vivo*, para poder confirmar que nos encontramos ante un caso de convergencia evolutiva.

CONCLUSIONES

Las conclusiones principales de esta tesis son:

1. Los Wnts posteriores Wnt1, Wnt11-2 y Wnt11-5 ejercen funciones específicas en el control de la identidad central y posterior, probablemente a través de la señalización por βcatenina1. Wnt11-1 controla la elongación de la región posterior de forma independiente de βcatenina-1.

2. Existe un gradiente de actividad βcatenina-1 a lo largo del eje anteroposterior de la planaria. Sus niveles más altos se encuentran en el extremo posterior (la cola); los más bajos no se encuentran en el extremo anterior de la cabeza sino en la región prefaríngea.

3. Existe una actividad local de βcatenina-1 en anterior que controla la expresión del gen notum, regulador de la vía Wnt, durante la regeneración y la homeostasis.

4. Durante la regeneración, la βcatenina-1 se nuclearíza en blastemas anteriores y posteriores. Solamente la nuclearización de βcatenina-1 en el blastema posterior depende de Wnt-1.

5. La βcatenina-1 tiene un papel esencial durante la organogénesis. Se encuentra altamente nuclearizada en el sistema nervioso, muscular, digestivo, visual y reproductor, durante la regeneración y la homeostasis. La βcatenina-1 es necesaria para la correcta regeneración del cerebro y de los ojos, y para la maduración del sistema reproductor.

6. Las planarias presentan duplicaciones y especialización funcional de las βcateninas. Las βcateninas 3 i 4 presentan el dominio C-terminal (de transactivación) truncado y 'in vitro' actúan como inhibidoras de la actividad transcripcional de la βcatenina-1.

7. La βcatenina-4 es necesaria para la especificación de las células fotoreceptoras de los ojos de planarias, gracias a su actividad como inhibidora de la βcatenina-1, por competición a la unión al TCF-2.

8. La Plakoglobina y Neural Arm (forma de splicing alternativo de Armadillo que pierde el dominio C-terminal) inhiben in vitro la actividad transcripcional de la βcatenina. La existencia de formas de βcatenina sin el dominio C-terminal podría ser un mecanismo conservado evolutivamente para controlar la actividad nuclear de la βcatenina.

9. Durante la embriogénesis de planaria, la βcatenina-1 se encuentra nuclearizada en algunas células vitelinas, sugiriendo un papel instructor para éstas. Por su morfología y posición, los primeros blastómeros con βcatenina-1 nuclear podrían corresponder a las primeras células migratorias con destino mesoendodérmico, tal y como se ha descrito en múltiples modelos animales. La nuclearización de βcatenin1 en el primordio de la faringe embrionaria corrobora esta hipótesis.

10. Durante la metamorfosis de la larva, la βcatenina-1 se nuclearíza de forma masiva, especialmente en los primordios de los nuevos órganos, de forma similar a la situación descrita en las planarias adultas, sugiriendo su importancia durante la especificación del destino celular en ambos momentos del desarrollo.

11. La función de especificación del eje AP de la βcatenina-1 durante el desarrollo embrionario de las planarias no es patente tras la observación de su localización subcelular, posiblemente al quedar enmascarada por la alta actividad de βcatenina-1 requerida en la organogénesis.

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ANEXOS

Anexo 1

Revisión 1:

Wnt signaling in planarians: new answers to old questions.

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Resumen en castellano:

La vía de señalización Wnt en planarias: nuevas respuestas para antiguas preguntas

Los ligandos Wnts son unas glicoproteínas secretables implicadas en una gran variedad de procesos celulares esenciales, como el control de la proliferación, la migración y la determinación de la identidad celular. En los últimos años, se han realizado muchos esfuerzos en dilucidar el papel de la vía de señalización Wnt durante la regeneración y la homeostasis de las planarias. Las planarias tienen la increíbles peculiaridad de que son capaces de regenerar cualquier fragmento amputado mimetizando a la perfección su identidad y proporción. En esta revisión, vamos a resumir toda esta información obtenida sobre la función de las vías Wnt canónica o βcatenina dependiente y Wnt no canónica o βcatenina independiente en planarias. También presentamos e intentamos poner en contexto todos los datos obtenidos que sugieren un papel de estas vías durante la embriogénesis de planarias. Finalmente, proponemos un modelo para integrar todos los datos obtenidos hasta el día de hoy y resaltamos aquellas preguntas que permanecen sin respuesta.

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Wnt signaling in planarians: new answers to old questions

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ABSTRACT Wnts are secreted glycoproteins involved in a broad range of essential cell functions, including proliferation, migration and cell-fate determination. Recent years have seen substantial research effort invested in elucidating the role of the Wnt signaling pathway in planarians, flatworms with incredible regenerative capacities. In this review, we summarize current knowledge on the role of canonical (β-catenin-dependent) and non-canonical (β-catenin-independent) Wnt signaling in planarians, not only during regeneration, but also during normal homeostasis. We also describe some of the preliminary data that has been obtained regarding the role of these pathways during embryogenesis. Models are proposed to integrate the different results which have been obtained to date and highlight those questions that still remain to be answered.

KEY WORDS: *planarian, Wnt signalling, regeneration, development, axial polarity*

Introduction

Wnt ligands are a family of secreted glycoproteins involved in cell-cell communication events that control every major developmental process, including cell-fate determination, cell proliferation, polarity, adhesion, motility, apoptosis, and hence, patterning and morphogenesis (reviewed in Mikels and Nusse 2006). Historically, Wnt ligands have been classified as canonical or non-canonical, depending on whether or not they lead to the activation of β-catenin. However, this classification may be artificial and the function of a specific Wnt ligand should not be understand as an intrinsic property of the ligand, but rather as a context-specific result of its interaction with different receptors (reviewed in van Amerongenen and Nusse, 2009). Although the best known Wnt receptors belong to the Frizzled (Fz) family, other receptors, such as the tyrosine kinases Ror2 and RYK, have begun to be identified (Angers and Moon 2009).

The best understood Wnt signal-transduction cascade is the canonical pathway, in which binding of Wnt proteins to Frizzled/LRP5-6 receptors causes the recruitment and activation of Dishevelled, responsible for the disassembly of the β-catenin destruction complex, mainly composed of Axin, APC and GSK-3. As a consequence, β-catenin is stabilized and enters the nucleus, where it associates with Lef/Tcf transcriptional repressors, causing the derepression of transcriptional targets (reviewed in Huang and He, 2008). Thus, canonical Wnt signaling directly targets the nucleus, and it is broadly used to regulate cell fate, proliferation and self-renewal of stem and progenitor cells in any tissue and at any stage of metazoan life.

Despite the broad range of activities, β-catenin signaling is a strikingly conserved mechanism to pattern the antero-posterior (AP) axis in nearly all animals examined (reviewed in Petersen and Reddien, 2009). It controls specification of posterior identities in most bilaterian embryos (reviewed in Niehrs, 2010), and this role is conserved in adult stages during regeneration, as demonstrated in cnidarians (Hobmayer *et al.*, 2000) and in our current model, planarians.

By definition, Wnt signaling that does not lead to β-catenin activation is referred to as non-canonical or β-catenin independent. A number of non-canonical Wnt signaling pathways exist, including the planar cell polarity (PCP) pathway and the calcium pathway. PCP refers to the coordinated polarization of cells or structures in the plane of a tissue (Goodrich and Strutt, 2011). For instances, in *Drosophila* wing imaginal discs, where this phenomenon was first discovered, Dvl is recruited by a Fz receptor, promoting the asymmetric localization of the PCP core proteins within the cell, becoming the Fz-Dvl-Diversin/Diego (Div/Dgo) complex localized oppositely to the Strabismus/Van-Gogh (Stbm/Vang)-prickle (Pk) complex. The disruption of these protein asymmetries is manifested by the miss-orientation of wing hairs. Nowadays, β-catenin-independent pathways are linked to a highly diverse set of processes in both vertebrates and invertebrates. These include assembly and patterning of neural circuitry, convergent extension movements during gastrulation, orientation of cell division and planar polarization of cells and tissues (reviewed in Goodrich and Strutt, 2011). Although all these events occur in different biological contexts and they can

Abbreviations used in this paper: ap, antero-posterior; Fz, Frizzled; pcp, planar cell polarity.

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be viewed as independent from each other, their basis may be common: a concerted intracellular rearrangement controlled by regulating actin filaments, which models cell and tissue architecture.

The planarians are a particularly interesting model in which to explore the role of Wnt signaling in events as diverse as cell proliferation and patterning. These freshwater flatworms can regenerate a whole animal from almost any piece of their body. Although other species such as *Dugesia japonica*, are used by several research groups, *Schmidtea mediterranea* is now the most widely studied planarian species since its genome has been sequenced and several EST and transcriptomic databases have been generated. Since technology is also available for functional analysis using methods such as RNA interference (RNAi), it is well suited to experiments designed to unravel the molecular control of processes such as the re-establishment of tissue polarity during regeneration. In this review, we present the current data on the roles of the different planarian Wnt signaling elements in development and regeneration and propose models to integrate the findings that have been obtained to date.

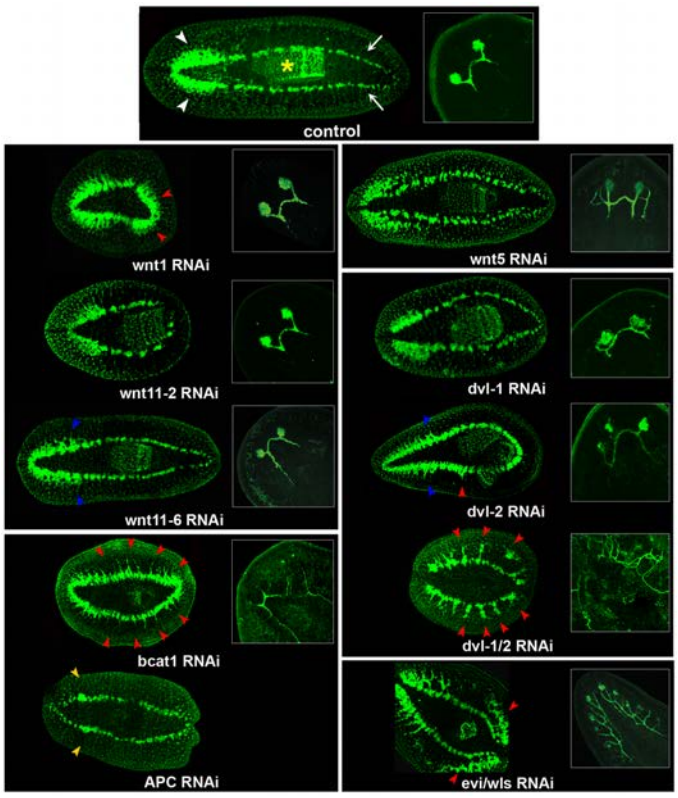

Fig. 1. Comparative analysis of the phenotypes generated after silencing different Wnt signaling elements. Immunohistochemistry with anti-synapsin (CNS) and anti-arrestin (VC-1) (visual system, within white squares) shows the CNS organization and paths of visual axons after different genetic ablations. *In the control animal, the brain and the ventral nerve cords are labeled with white arrowheads and arrows, respectively. The pharynx is labeled with a yellow asterisk. In the silenced animals, the ectopic brain is labeled with red arrowheads, the expansion towards posterior of the brain is labeled with blue arrowheads and the two ectopic brain primordia are labeled with orange arrowheads. All animals correspond to trunk pieces after 12-20 days of regeneration. All images are confocal z-projections. Anterior is shown to the left.*

Canonical/β-catenin-dependent Wnt signaling specifies posterior identity in adult planarians

Before the molecular era, generation of two-headed planarians was not a strange event. Morgan and Child observed that when a planarian is cut into extremely thin fragments, 'two-headed' or 'janus headed' planarians often develop (Morgan 1898; Child 1911). These observations were central to their proposal of a gradient, which Child supposed to be a 'metabolic gradient', responsible for AP axis patterning. According to this hypothesis, when the size of the sectioned planarian is very small, the difference in the activity of the 'metabolic gradient' between both edges is insufficient to instruct different fates in the two edges (reviewed in Blackstone 2006). The idea of a gradient underlying the bipolar anatomy of planarians has remained broadly accepted since its initial proposal by Morgan and Child (reviewed in Meinhardt 2009; Adell *et al.*, 2010). This parallels development in experimental embryology, where gradients have long been thought to be responsible for regulating axial development. It has now been demonstrated that the patterning of the AP axis (primary axis in prebilaterians) involves Wnts and β-catenin activity in almost all metazoans (reviewed in Petersen and Reddien, 2009; Niehrs, 2010).

The first and the most striking evidence for the essential role of canonical Wnt signaling in the specification of the planarian AP axis came from experiments involving silencing of *βcatenin-1*. The *S. mediterranea* genome contains at least three β-catenins. *βcatenin-1* seems to be both necessary and sufficient to transduce the canonical Wnt signal (Gurley *et al.*, 2008; Petersen and Reddien 2008; Iglesias *et al.*, 2008), whereas *βcatenin-2* functions exclusively in cell-cell adhesion (Chai *et al.,* 2010). Although other organisms such as *C. elegans* and vertebrates also have genomic duplications of β-catenin, only planarians are known to exhibit a clear functional specialization between different paralogs. Thus, silencing of *βcatenin-1* results in the complete loss of posterior and central identities and the generation of a fully anteriorized animal (Fig. 1 and 2). The planarians generated by *βcatenin-1* silencing were referred to 'radial-like hypercephalized' animals because of their almost radial symmetry. Such a striking transformation of the body plan as a consequence of targeting a single gene had no precedent. Consistent with the role of *βcatenin-1* as an effector of a morphogenetic signal, decreasing doses of *βcatenin-1* dsRNA generate a range of AP phenotypes. The strongest phenotype is the 'radial-like hypercephalized' planarian, followed by 'two-headed' planarians with multiple ectopic eyes, 'two-headed' planarians without ectopic eyes, and finally 'tailless' planarians, in which the tail has a rounded shape and the animals have two ventral nerve cords (VNCs) that fuse with a rounded morphology instead of connect-

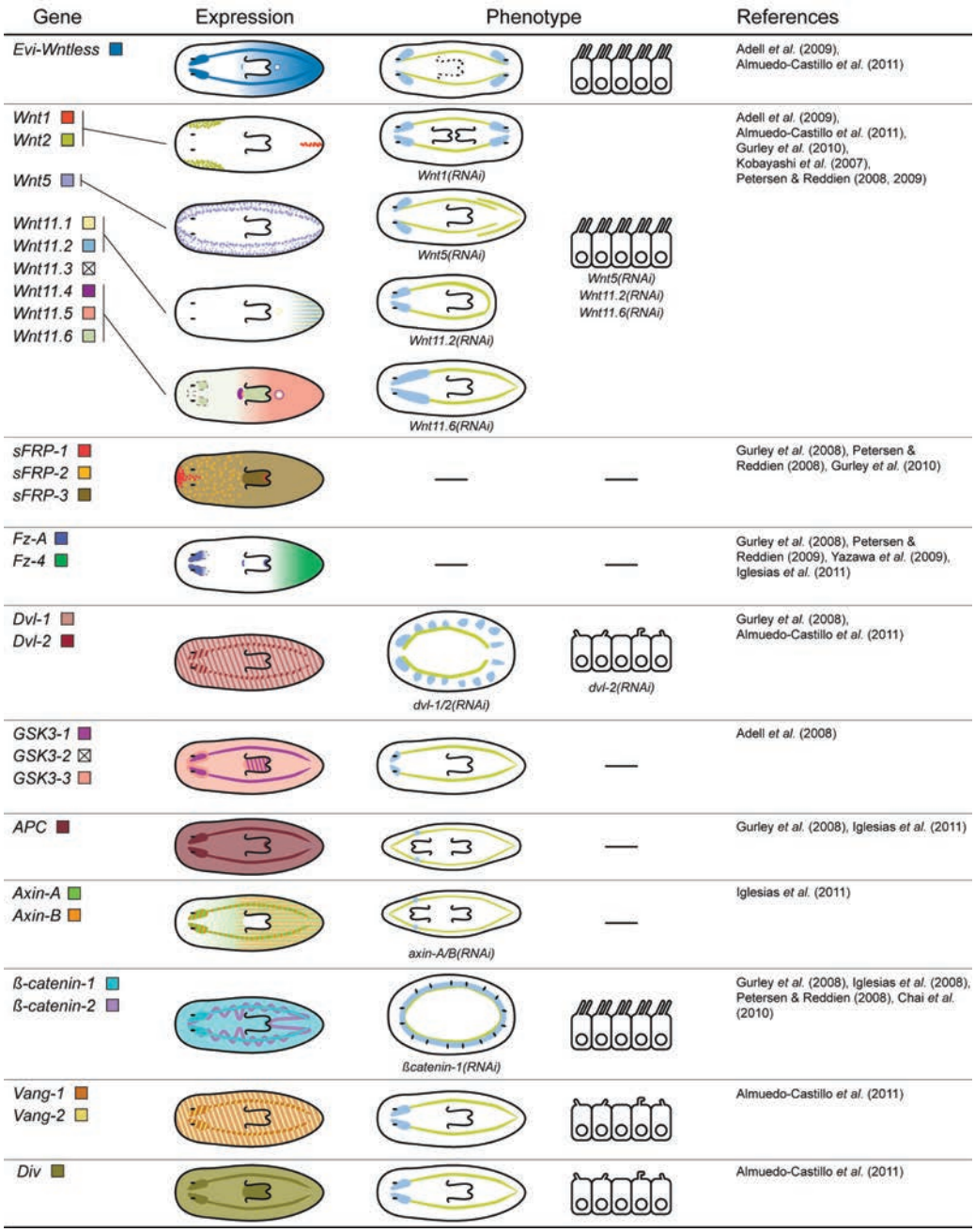

Fig. 2. Schematic summary of the expression patterns of Wnt elements and the phenotype generated after they are silenced. *Gene expression is depicted in stripes when different paralogs are expressed in the same region. The features displayed in the phenotypes are the pattern of the CNS (brain in blue and ventral nerve cords in green), the eyes, the number and position of the pharynx and the organization of the epidermal cilia (apically located and well oriented, as in a wild-type situation, or internalized and with less density, after knockdown of components of the planar cell polarity pathway). In the case of the Wnt ligand family, only the ones for which a phenotype is obtained upon silencing are shown. The phenotype of* vang-1/2 *and* div *RNAi planarians corresponds to a control phenotype apart from the positioning of the cilia.*

ing in the tip of the tail (Iglesias *et al.,* 2008; Adell *et al.,* 2010). Silencing of both *S. mediterranea dvls (dvl-1* and *-2*) also results in the complete loss of posterior identity (Gurley *et al.*, 2008; Almuedo-Castillo *et al.*, 2011). Moreover, both paralogs seem to be functionally specialized, and only *dvl-2* is clearly involved in β-catenin-dependent signal transduction (Almuedo-Castillo *et al.*, 2011) (Fig. 1 and 2). Furthermore, silencing of *S. mediterranea APC* or *axins* (*axin-A and -B*), two elements of the β-catenin destruction complex, leads to posteriorized or 'two-tailed' planarians (Gurley *et al.,* 2008; Iglesias *et al.,* 2011) (Fig. 1 and 2).

The *S. mediterranea* genome contains a large Wnt family comprising 9 members (Petersen and Reddien 2008; Adell *et al.*, 2009; Gurley *et al.*, 2010). Although their phylogenetic relationship has not been definitely solved, 6 of them have been classified in the Wnt11 subfamily (*wnt11-1* to *-6*) and the other three each belongs to a different subfamily: Wnt-1 (*wnt1*); Wnt-2 (*wnt2*), and Wnt-5 (*wnt5*). With the exception of *wnt11-3* (formerly known as *wntP-4*), for which expression has never been detected, all of the *S. mediterranea* Wnts display a very specific expression pattern (Fig. 2). *wnt11-6* (formerly *wntA*, based on homology to the first reported *D. japonica* WntA) is expressed in the posterior part of the brain and in the pharynx, and causes posterior expansion of the brain when silenced (Kobayashi *et al.,* 2007; Adell *et al.,* 2009). *wnt11-4* (formerly *wntP-3*) is expressed in clusters of cells in the esophagus and also in isolated cells in the posterior part of the animals (Petersen and Reddien 2008; Gurley *et al.,* 2010), and *wnt-2* is detected laterally in the head region. However, no phenotype has been reported after silencing of these genes. *wnt5* is expressed from the most external region of the CNS to the lateral edges of the planarian (Adell *et al.,* 2009; Gurley *et al.,* 2010; Almuedo-Castillo *et al.,* 2011). Its role will be discussed in the corresponding section, as it clearly functions as a non-canonical Wnt in our model.

In this section we will focus on the 'posterior' Wnts, expressed in the posterior part of the intact planarians. *wnt1* (formerly *wnt-P1*) is expressed in a row of discrete cells exclusively in the posterior dorsal midline (Petersen and Reddien 2008; Gurley *et al.,* 2010), *wnt11-1* and *2* in a broader domain in the tail (Petersen and Reddien 2008; Adell *et al.,* 2009), and *wnt11-5* (formerly *wnt-P2)* as a gradient from the tail to the prepharyngeal region (Petersen and Reddien 2008; Petersen and Reddien 2009) (Fig. 2). Interestingly, during regeneration, their mRNA appears gradually. The first Wnt expressed is *wnt1* in individual cells in both anterior and posterior blastemas. In fact, it has been shown to be activated in response to any wound as early as 6 hours after amputation (Petersen and Reddien 2009; Gurley *et al.,* 2010). At 48 hours, its expression is restricted to the posterior midline, resembling the pattern in the intact animals. *wnt11-1* and *2* are not detected until 48 hours after amputation, when they appear exclusively in the posterior blastema (Adell *et al.,* 2009; Gurley *et al.,* 2010). *wnt11-5* expression is known to be triggered by *wnt1* and *β-catenin* at posterior wounds at 24 h post-amputation (Petersen and Reddien 2009). As is already expressed in the trunk region of intact animals, it does not disappear after amputation but moves posteriorly as regeneration proceeds, in parallel to the morphogenetic changes and establishment of new AP identities (Fig. 3). It is interesting to note that the wound induced *wnt1* expression and the dynamics of *wnt11-5* expression are not affected after ablation of neoblasts by irradiation, suggesting that, although proper morphallactic remodeling cannot take place, the first global response to the new positional identities are stem cell independent (Petersen and Reddien 2009; Gurley *et al.,* 2010).

Functional analysis of the 'posterior' Wnts by RNAi demonstrates that *wnt1* signals through a canonical pathway, since its silencing produces 'two-headed' planarians (Fig. 1 and 2). However, the loss of polarity is achieved in a very low percentage of animals, and after silencing of *wnt1*, most of the animals appear with the same 'tailless' phenotype already reported to be generated after low-level silencing of *βcatenin-1* and *dvl-2* (Adell *et al.,* 2009; Almuedo-Castillo *et al.,* 2011). Planarians in which *wnt11-2* has been silenced also display the 'tailless' phenotype, but never a reversal of polarity (Fig. 1 and 2). No phenotype is reported after individual silencing of the other posterior Wnts. Based on their expression pattern and functional data, it has been suggested that synergistic effects of several posterior Wnts account for the graded activation of β-catenin along the planarian AP axis, as it has also been proposed in cnidarians (reviewed in Guder at al., 2006). Specifically, *wnt1* is thought to activate *wnt11-5* through *βcatenin-1* and both would be responsible for patterning the posterior end. This relationship is supported by the observation that silencing of *wnt11-5* together with *wnt1* increases the proportion of 'two-headed' planarians (Peteresen and Reddien 2009). It has also been proposed that *wnt11-2* could be a target of *βcatenin-1* (Yazawa *et al.,* 2009), but synergistic effects have never been demonstrated. Remarkably, *wnt11-5* expression appears just after *wnt1* and before *wnt11-1/2* during regeneration of head fragments, which normally do not express posterior Wnts (Peteresen and Reddien 2009). Thus, current data suggest that *wnt1* and *wnt11-5* act through *βcatenin-1*. However, the canonical role of *wnt11-1* and *2* remains to be demonstrated. After silencing of *wnt11-2*, genes expressed in the midline of planarians fail to properly localize along the posterior midline of the animal, but are detected as dispersed cells around the rounded tail. It has therefore been proposed that *wnt11-2* is required for recruitment of midline cells rather than for establishment of posterior identity (Gurley *et al.*, 2010). It is possible that *wnt11-2* controls the cell migration or the oriented cell division required to extend the posterior axis, similar to the non-canonical function of Wnt-11 and Dishevelled in AP elongation of the epiblast during vertebrate gastrulation (Gong *et al.,* 2004).

Interestingly, 'radial-like hypercephalized' planarians are never generated when *wnts* are silenced alone or in combination (Adell *et al.,* 2009; Petersen and Reddien 2009). To explain this observation it could be proposed that inputs from different Wnts could control activation of *βcatenin-1*. Although some Wnt ligands are more relevant than others in targeting a specific response, receptors are the key components to initiate the different cascades (van Amerongen and Nusse, 2009). We can thus expect some degree of rescue by the other Wnt ligands that preferentially signal through different receptors. An alternative explanation is that RNAi targeting of a single intracellular element, which probably has a short half-life, is much easier than silencing several more stable secreted proteins. Finally, the available data also suggest that the position within the pathway at which the silenced gene acts can be decisive. For instance, silencing of *S. mediterranea* dishevelleds, an intracellular element common to every Wnt branch and that directly controls β-catenin degradation, produces a rapid and complete anteriorization of treated planarians (Fig. 1 and 2). In contrast, silencing of *evi/wls*, a transmembrane protein required for secretion of Wnts, only produces bipolar 'two-headed' animals with multiple eyes, even when two rounds of injection/regeneration

are performed (Adell *et al.,* 2009; Almuedo-Castillo 2011) (Fig. 1 and 2). As expected, silencing of those elements also produces β-catenin independent defects, which will be discussed in the corresponding section.

Canonical Wnt activation is responsible for posterior specification during regeneration but also during homeostasis in intact planarians. Injection of β*catenin-1* or *dvl-1/2* dsRNA generates fully anteriorized animals even when no injury has been induced (Peteresen and Reddien 2008; Iglesias *et al*., 2008; Almuedo-Castillo *et al*., 2011). This is not surprising if we consider that the mechanisms of pattern formation should always be active in planarians, which are continuously renewing their tissues and setting them to new proportions, for instance when adjusting their size to food availability. However, silencing of other elements of the pathway such as *wnt1* during homeostasis does not generate "two-headed" planarians, supporting that during regeneration, the early posterior-fate choice of *wnt1* takes place just in response to a wound. Amputation forces the regeneration of new tissues and, thus, de novo synthesis of proteins. In general, quicker and stronger effects are observed in regenerating than in intact animals. However, we should bear in mind that homeostasis and regeneration, particularly during early stages, are unlikely to involve the same molecular inputs and responses. During regeneration, cells must adopt completely different fates with respect to their original position and, thus, very early signals must re-direct their fate in a context of high rates of apoptosis and proliferation. In contrast, the changes in tissue fates during homeostasis are more progressive and follow already established patterns. For that reason it is important to pay attention to the expression pattern of genes that show a polarized expression at specific time points. The current data will be discussed bellow in an attempt to propose a general model for the re-establishment of AP pattern during regeneration.

Alternative mechanisms involved in planarian antero-posterior axis specification

Although canonical Wnt signaling is essential for patterning the AP axis in adult planarians, other pathways, such as the hedgehog (Hh) pathway, are known to be involved in establishing and maintaining polarity (Rink *et al.,* 2009; Yazawa *et al.,* 2009). Silencing of the planarian homologs of Patched, a Hh receptor with inhibitory activities, generates 'two-tailed' planarians, whereas inhibition of the planarian homologs of Hh, Gli-1 or Smo induces anteriorization (Fig. 3). However, inhibition of Hh signaling generates posterior heads in only a few animals, and the effect is only more penetrant when two of the elements are silenced simultaneously or two rounds of regeneration are induced (Yazawa *et al*.2009). In most animals, *Hh* silencing leads to a 'no-tail' phenotype (Fig. 3) that differs from the 'tailless' phenotype generated after β*catenin/wnt1/wnt-11-2* RNAi (Fig. 2). In fact, after *Hh* silencing, the posterior marker Fz4 does not disappear, demonstrating that posterior identity is not completely abolished (Rink *et al.,* 2009). The essential difference between the 'tailless' and the 'no-tail' phenotype is that in the first the VNCs and gut do differentiate but do not end at a posterior tip, while in the second the structures fail to differentiate. Those differences could reflect a secondary role for Hh in proliferation and growth, in addition to the one in fate determination.

Simultaneous silencing of *patched* and *βcatenin-1* produces anteriorized animals, demonstrating that Hh acts upstream of β-catenin (Rink *et al.,* 2009). The expression patterns of the components of the Wnt pathway, together with the observation that Hh transcripts are found not in the posterior blastema cells but in the preexisting VNCs, led Yazawa *et al.,* (2009) to propose a model in which Hh might be transported posteriorly along axons in the VNCs. According to that model, Hh would then be responsible for the activation of *wnt1*, which would lead to the activation of β*catenin-1* and *wnt11-2*. This model is very suggestive, since, after amputation, planarians would regenerate according to the existing AP polarity of the fragment. If a directional signal exists, this is inherent to the existing CNS, and the fact that, after amputation, adjacent cells adopt different fates according to each blastema would be easily explained. As argued by the authors, a recent report demonstrating that voltage-gated Ca^{2+} channels are involved in the acquisition of proper polarity during regeneration (Noggi *et al*., 2009) would agree with this model, as Ca^{2+} channels are essential for microtubule assembly and axonal transport (Rajnicek and McCaig, 1997). However, it should be noted that, at least at

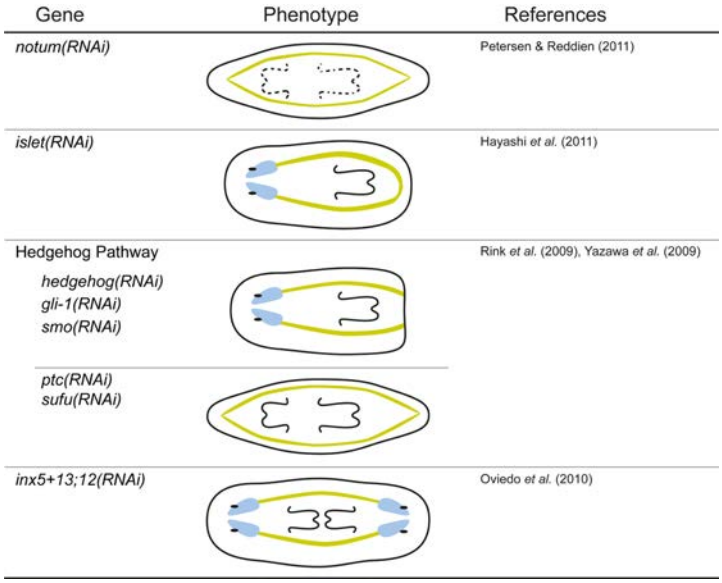

Fig. 3. Schematic representation of the antero-posterior defects generated after modulating "non-Wnt" elements. *The features displayed in the phenotypes are the pattern of the CNS (brain in blue and ventral nerve cords in green), the eyes, and the number and position of the pharynx. Silencing of* notum *or inhibitory elements of the Hh pathway induce "two-tailed" planarians. Silencing of activators of the Hh pathway or of* Hh *itself produces posterior regeneration defects. RNAi of* islet *leads to 'tail-less' regeneration phenotype. Downregulation of innexins generates 'two-headed' planarians. The pharynges of* notum *RNAi planarians are represented by dots because the exact position and number have not been precisely determined.*

the mRNA level, *ptc* expression does not show any asymmetry between 3 and 48 hours (Rink *et al.,* 2009).

Perhaps more interesting is the recent study by Beane *et al.,* (2011), who demonstrated that membrane depolarization is sufficient to drive anterior regeneration, even in posterior wounds, and that calcium signaling is at least one of the target effects of the depolarization. Taken together, these data highlight the essential role of ion transport as an early signal to regulate differential gene expression and to control polarity and morphogenesis.

The existence of long-distance communication mechanisms to rapidly inform cells of changes occurring during planarian regeneration is an attractive model. Further support for this mechanism comes from the work form Oviedo *et al.,* (2009) linking gap junctional communication with the integrity of the VNCs and the transport of signals such as Hh (Oviedo *et al.*, 2009). Gap junctions are plasma membrane channels involved in cell-cell communication via the direct transfer of small molecules between adjacent cells. They are known to be involved in syncytial communication between cell groups and to regulate many aspects of embryonic development (Levin *et al.*, 2007). The *S. mediterranea* genome contains a large family of innexins, the gap junctional components found in invertebrates, (Nogi and Levin 2005), and when their function is blocked, 'two-headed' planarians are generated (Nogi and Levin 2005; Oviedo *et al.,* 2009) (Fig. 3). Oviedo *et al.,* performed multiple experiments to block either innexins or the continuity of the VNCs during the process of regeneration and analyzed the polarity of the regenerated animals. Interestingly, they found that fragments containing the brain always regenerated a normal tail, even if the VNCs were disrupted or gap junctions blocked. However, when the VNCs were disrupted alongside blockade of gap junctions, the AP identity of the blastema was abnormal and ectopic posterior heads developed even in fragments containing the brain. Those results demonstrate an essential role of the pre-existing CNS in polarity specification and suggest that blastemas receive the information by at least two mechanisms: gap junctions and VNCs. Interestingly, both signals act very early and rapidly, as they are critical during the first 3-6 hours.

The strong phenotypes generated when modulating canonical Wnt signaling, suggest that the integration of information essential for AP patterning in adult planarians—irrespective of whether this occurs through long-range signals or local cues—acts at the level of *βcatenin-1* activity, and that other signals or mechanisms involved in AP patterning would function by regulating this activity. The interaction between the Hh and the Wnt pathway is an established mechanism for pattern formation during development. For instance, the absence of Shh signaling in vertebrates results in direct inhibition of β-catenin activity by Gli3R (Ulloa *et al.,* 2007). These findings are clearly consistent with the proposed cross-talk between Wnt and Hh signaling in planarians. On the other hand, it is not known whether Wnts can travel through gap junctions. However, both Innexins and Connexins, in invertebrates and vertebrates, respectively, appear to be regulated by Wnt signaling (reviewed in Bauer *et al.,* 2005). Further studies will now be necessary to determine how these different mechanisms work together to control AP patterning.

Early fate decisions and the role of locally secreted wound-activated signals: proposing models for antero-posterior axis establishment

Based on gene expression pattern and kinetics, we have grouped the molecular events underlying regeneration into three theoretical

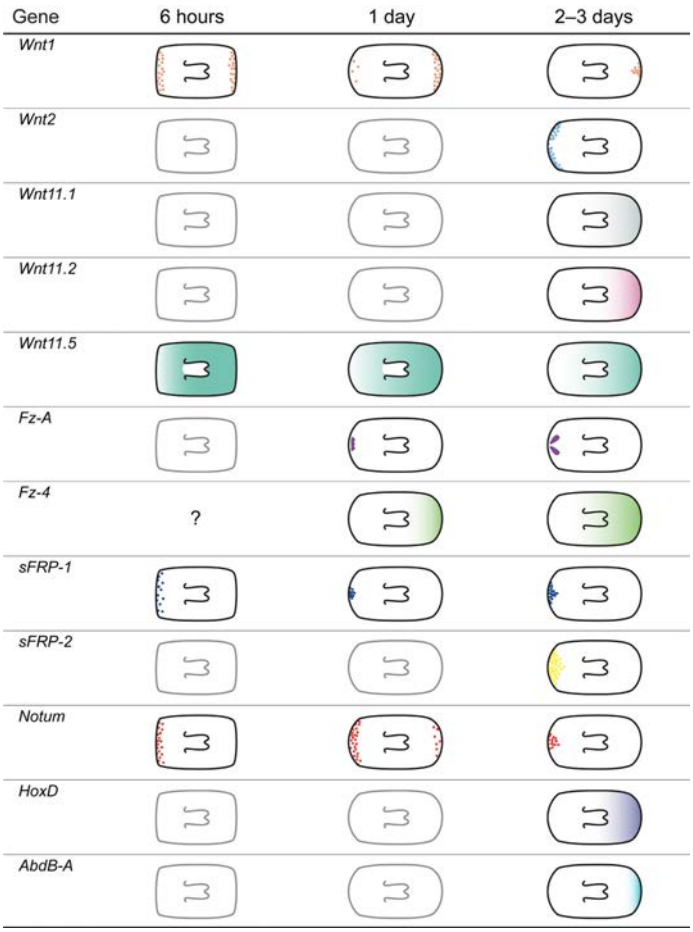

Fig. 4. Schematic representation of polarized gene expression during early regeneration. *The first genes appearing in response to the wound are* wnt1, sFRP-1 *and* notum, *although only* sFRP-1 *exhibits permanent polarize expression in the anterior wounds. One day after amputation,* fzA *and* fz4 *appear in the anterior and in the posterior wounds, respectively. At 3 days, all the known polarized genes are already detected, showing their anterior or posterior location, as well as* wnt1 *and* notum, *which show their final polarized pattern.* wnt11.5 *expression gradually moves posteriorly, in parallel to the re-patterning of the planarian fragment.*

stages. The earliest stage extends from wound healing to 18-24 hours after amputation. During this time, the expression pattern of several genes is "reset" and expression is either undetectable or found in regions that differ from those seen in adult planarians. It is important to note that AP decisions and cell-fate remodeling at this time are independent of stem cell proliferation (Peteresen and Reddien 2009; Gurley *et al*., 2010; Hayashi *et al*., 2011). The second stage is from 18-24 hours to 3 days. During this period, most genes begin to be expressed in a definitive pattern. During the last stage, from 3 days to the end of regeneration, genes are clearly expressed in the primordia of the differentiating tissues and organs.

Currently available data suggest that the critical decisions influencing the establishment of axial polarity and appropriate cell fates are taken during the first stage. At that time, *wnt1* is activated at any wound in a set of isolated cells before becoming restricted to the most posterior dorsal midline during the second stage. The recently identified planarian homolog of Notum, a secreted hydrolase that modulates the range of action of Wnt ligands, is also critical for the acquisition of AP polarity. *notum* is highly upregulated preferentially in anterior wounds as soon as 6 hours after amputation, and later on, at around 2 days, accumulates in the most anterior tip of the blastema; in intact animals it is exclusively expressed in the anterior-dorsal end (Petersen and Reddien 2011) (Fig. 4). Importantly, like *wnt1*, *notum* is expressed in any wound but always preferentially in the anterior edge. Supporting its role in preventing posterior specification, silencing of *notum* generates 'two-tailed' planarians (Fig. 3). *sFRP-1*, one of the three *S. mediterranea* secreted Frizzled-related proteins (sFRP), is the earliest gene to be detected in anterior blastemas, after only 3 hours (Fig. 4). No phenotype has been observed after silencing of *sFRP-1*, however. These wounding-response factors are perfect candidates to mediate the local control cell reprogramming after amputation and specifically launch the different regeneration programs.

A very recent report nicely shows that *D. japonica islet*, a LIM homeobox transcription factor, is essential to maintain *wnt1* expression during the second stage of regeneration, but does not have any effect on the wound-induced *wnt1* expression. During the first two days of regeneration, *islet* mRNA appears in the posterior dorsal midline, like *wnt1* mRNA. Moreover, *islet* RNAi planarians display the 'tail-less' phenotype, similar to the one caused by *βcatenin/wnt1/wnt-11-2* silencing (Fig. 3). The authors suggest that Islet would be inducing stem cells to differentiate into Wnt1-secreting cells. Accordingly, the first stage related *wnt1*-expression is independent of both Islet function and stem cell proliferation (Hayashi *et al*., 2011).

Except for *wnt11-5*, whose expression depends on the early posterior wound-induced *wnt1* expression (Petersen and Reddien 2009; Gurley *et al*., 2010), the other 'posterior' Wnts (*wnt11-1* and *11-2*) are detected later on, during the second stage of regeneration, and are stem cell dependent (Gurley at al., 2010). Similar patterns are observed for the only two Fz receptors analyzed to date, out of the 10 found in the *S. mediterranea* genome (Kobayashi *et al*., 2007; Gurley *et al.,* 2008). *fzA*, is expressed in the anterior blastema at 1 day of regeneration with a pattern directly related with the differentiation of the brain primordia. Likewise, *fz4* is expressed in the posterior blastema also after 1 day of regeneration (Rink *et al*., 2009) (Fig. 4). A second sFRP, *sFRP-2,* is detected in the anterior blastema, but not before the second day (Gurley *et al*., 2010) (Fig. 4). It could be hypothesized that the decisions taken in the first stage are permanently established during the second stage. Interestingly, the Hox genes *AbdbA* and *HoxD* are detected specifically in the posterior blastema around 2 days after amputation (Iglesias *et al.,* 2008) (Fig. 4), suggesting a possible role as canonical Wnt targets. From about 3 days after amputation, not only the Wnt elements but most of the genes are expressed in patterns clearly resembling the ones observed in adults. For instance, genes with polarized expression patterns are clearly concentrated at one tip of the animal, genes expressed in the brain appear as two bi-lateral domains in the anterior blastema and genes expressed in the pharynx appear in its primordium.

With all the available data, an integrative and coherent model for early AP fate decisions can be proposed in which wound-induced *wnt1* expression is responsible for activating *βcatenin-1* and its targets, such as *wnt11-5* and *notum* (Petersen and Reddien, 2011) (Fig. 5A). Notum is described as a target of β-catenin in other models, and in fact, its expression in planarians disappears or is upregulated after *βcatenin-1* or *APC* silencing, respectively. Moreover, evidence for an epistatic relationship is strengthened by the observation that silencing of *βcatenin-1* or *wnt1* together with *notum* produces the same phenotype as silencing *βcatenin-1* or *wnt1* alone, that is, 'two-headed' or 'tailless' planarians (Petersen and Reddien, 2011). Thus, in anterior wounds, inhibition of *wnt1* by anterior-specific signals such as *notum* and *sFRP-1* would allow the differentiation of a head. In posterior wounds, *wnt1* expression would be maintained and other *βcatenin* targets such as *wnt11-1/2* (Petersen and Reddien 2009; Yazawa *et al.*, 2009), the posterior Hox genes, and probably *wnt1* itself (Petersen and Reddien 2011) would be activated (Fig. 5A). The observation that notum is transiently expressed in the posterior blastema suggests that an unknown factor must target its inhibition.

Activation of wound-induced *wnt1* expression is known to be independent of *βcatenin-1* and stem-cell proliferation (Petersen and Reddien 2009; Gurley at al. 2010). One possibility is that it is under the control of a general signal that is released by the cells around the injury and is linked to ion transport and depolarization of cell membranes. Once activated in the posterior blastema, maintenance of *wnt1* expression does depend on *βcatenin-1* and very probably on Hh activation (Yazawa *et al.,* 2009). Interestingly, the 'two-tailed' planarians generated after over-activation of Hh have normal levels of *notum* expression (Petersen and Reddien 2011). This capacity of Hh to induce tail differentiation in the presence of *notum,* indicates that Hh is not involved in generating the asymmetric expression of notum and raises the possibility of a late role of Hh in posterior specification. The role of Hh in early regeneration is still an important question to be elucidated. Over-activation of Hh by *ptc* RNAi increases early *wnt1* expression in anterior and posterior wounds, which would agree with the expression of at least *ptc* at that early time point in the wounds (Rink *et al*., 2009). The function of Wnt1 and Hh in anterior wounds also remains unknown, since no defect in anterior regeneration is observed after silencing *wnt1* or *Hh*. Different options can be considered, namely the expression of *wnt1* detected in anterior wounds does not imply a functional readout, or there is an early function of Wnt1 independent of posterior specification that we are not able to detect.

Since modulation of *βcatenin-1* leads to such striking AP defects, it has been widely assumed that its activation must be specific to posterior blastemas. However, this model predicts that *βcatenin-1* must also be active at least transiently in anterior tissues, for instance

to activate notum as early as 6 hours after amputation. In fact, *βcatenin-1* mRNA is detected in any wound, anterior or posterior, although not before 12 hours after amputation (Gurley *et al.,* 2008). Taking into account that βcatenin function is post-transcriptionally regulated, it could be that at very early time points the pre-existing protein levels are sufficient to activate early targets such as *notum*. The possibility that *βcatenin-1* is in fact active in any blastema at any time would explain the high levels of mRNA detected for this gene in both blastemas at all stages of regeneration. This is often overlooked in the literature but is consistent with the essential role of β-catenin in the control of fundamental cell processes. Although the canonical Wnt signal has been conserved for posterior specification, it is also clearly involved in the patterning of any tissue and organ at any developmental stage. For example, in vertebrates, β-catenin is responsible for posterior specification of the embryo, as well as for specifically patterning the liver, the gut (reviewed in Ladde and Monga 2011) and the brain (Kiecker at al., 2001). It therefore seems highly unlikely that the only function of *βcatenin-1* would be in AP axis specification, and evidence suggests that it is also a general mechanism necessary for the proliferation and differentiation of neoblasts at later stages of regeneration (Iglesias *et al*., 2011). In our opinion, the fact that silencing of β*catenin-1*, *axins* or *APC* only appears to result in AP axis defects in planarians merely reflects the crucial importance of *βcatenin-1* activity during the first few hours after amputation. 'Two-headed' planarians, which appear to have two well-patterned brains and a well-differentiated gut, may well occur because the downregulation of *βcatenin-1* is mild or only occurs for a short period of time (or both). However, it remains to be determined whether 'radial-like hypercephalized' planarians have a well-patterned brain or a well-differentiated digestive system. We would argue that *βcatenin-1* is essential in the first few hours of regeneration to re-establish the posterior organizer, and that this explains why we mostly see an anteriorized phenotype after it is silenced, but that it is also essential for patterning of adult tissues such as the CNS. This would explain the ubiquitous expression of *βcatenin-1* in any blastema, throughout intact animals and both in differentiated cells such as neurons and in proliferating cells (Iglesias *et al.,* 2011) (Fig. 2).

Altogether, patterning of the planarian AP axis can be divided into two main phases. The first one, in response to wound healing and independent of stem cells, where AP fate choice takes place; and the second one, which involves stem cell proliferation, growth and identity maintenance (Petersen and Reddien 2009; Yazawa *et al*., 2009; Gurley *et al*., 2010; Petersen and Reddien 2011 and Hayashi *et al*., 2011) (Fig. 5A). Accordingly, only the silencing of proteins that directly act in early posterior identity specification, such as *wnt1* or *βcatenin-1*, leads to the formation of a posterior head, while *wnt 11-1, 11-2* or *islet* RNAi interference cause defects in patterning and elongating posterior tissues, but never inversion of polarity.

Is the planarian antero-posterior (AP) axis also patterned by β-catenin activity during embryogenesis?

The role of the Wnt/β-catenin pathway in the establishment of the animal-vegetal (prospective AP) axis during early embryogenesis has been demonstrated not only in vertebrates but also in spiralians. In the nemertean *Cerebratulus lacteus*, as in most metazoans studied, β-catenin is restricted to the most vegetal cells and is required for proper endomesoderm development (Henry *et al*., 2008). In the annelid *Platynereis dumerilii*, β-catenin is involved in establishing asymmetric sister-cell fate along the animal-vegetal axis (Schneider and Bowerman, 2007). Interestingly, this mechanism is also functional within Ecdysoazoans, in the nematode *C. elegans* (Kaletta *et al*., 1997).

To date, only two reports have presented data on the expression of Wnt elements in planarian embryos, specifically in the planarian species *Schmidtea polycroa*, which is closely related to *S. mediterranea* (Martín-Durán *et al*., 2010; Martín-Durán *et al*., 2011). Despite their phylogenetic position as spiralians, planarians

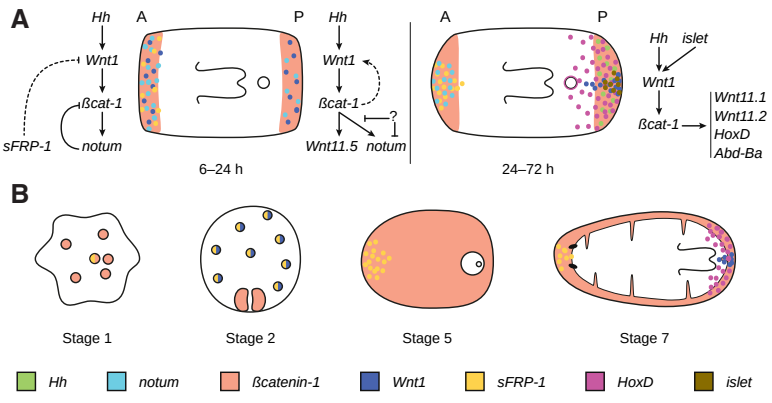

Fig. 5. Proposed model for establishment of posterior identities after amputation and comparison with the expression pattern of Wnt pathway components during embryogenesis. **(A)** *Proposed epistatic model for antero-posterior patterning during planarian regeneration. After wound-healing,* wnt1*, which is detected during the first few hours in any kind of wound, would activate* βcatenin-1*, which would in turn activate very early targets, such as* wnt11-5 *and* notum*. Notum, which normally modulates the range of Wnt activity, could inhibit the local function of* β*catenin-1 in the anterior wounds, but not in posterior ones, where an unknown signal should inhibit its function. Although no functional data have been reported, sFRP1 is predicted to inactivate Wnt1 in the anterior wound. Hh is known to modulate wound-induced* wnt1 *expression. In a second phase, differential programs are already launched in each blastema, but canonical Wnt activation needs to be maintained posteriorly. At this stage, around 2 days after amputation, the effectors of canonical Wnt signaling, such as the posterior Wnts and the posterior Hox genes, are activated. A indicates anterior; P, posterior.* **(B)** *Schematic representation of the expression of the Wnt elements analyzed during* Schmidtea polychroa *embryogenesis.* βcatenin-1 *is detected as early as stage 1, but only by RT-PCR. At stage 2,* βcatenin-1 *is expressed in the embryonic pharynx, and* sFRP1 *and* wnt1 *in the many blastomers around the embryo. At stage 5,* βcatenin-1 *is detected throughout the embryo, but the rest of the elements are not. At stage 7, the Wnt elements show a pattern clearly resembling that found in adults. Note that the expression dynamics of these Wnt elements during regeneration and during embryogenesis share some interesting features, for instance the early co-expression of* wnt1 *and* sFRP1 *and their subsequent polarization.*

show an ectolecithal embryo without any recognizable cleavage pattern and with the early formation of transient structures required to introduce the maternal nutrients. During cleavage, or stage 1 (the first 36 hours), blastomeres are not attached and remain dispersed within the yolk-derived syncytium. At stage 2, a recognizable organization emerges. Some blastomeres move towards the periphery of the syncytium and form a thin embryonic epidermis. Other blastomeres migrate to a point in the syncytium, which may or may not be prespecified by an existing signal, and differentiate to form a provisional embryonic pharynx that will ingest yolk cells (Martín-Durán *et al*., 2010). The embryonic pharynx is the only discernible external feature in planarian embryos until stage 6, when the definitive pharynx and the rest of the organs and tissues clearly differentiate.

βcatenin-1 is expressed at stage 2 exclusively in the embryonic pharynx of the yolk-feeding embryo. At stage 3 and 4, expression disappears, and at stage 5 it is detected ubiquitously in the germ band. By stage 7, embryos display the same expression pattern as that found in the adult, that is, widespread expression in the parenchyma and the nervous system (Fig. 5B). *wnt1* is also detected at stage 2 but in a completely different domain, that is, in the outlying blastomeres but not in the embryonic pharynx. Thereafter it is not detected until stage 7, in the juvenile, in a small cluster of cells at the posterior definitive tip, clearly resembling the adult pattern (Fig. 5B). *sFRP-1* is detected at stage 2 in the outlying blastomeres but not in the pharynx, the same as *wnt1*, but this expression is maintained during stages 3 and 4. At stage 5 it becomes restricted to discrete cells in the germ band on the opposite side to the degenerating embryonic pharynx. This asymmetric distribution is observed until the end of development, when *sFRP-1* is expressed in the anterior tip and in the definitive pharynx in the hatching embryo, again resembling the pattern found in the adult (Martín-Durán *et al*., 2010) (Fig. 5B).

Together, these data suggest that the definitive mechanisms for patterning of the AP axis, involving canonical Wnt signaling, emerge at stage 5, that is, after yolk ingestion and proliferation of the blastomeres in the germ band, and when the embryo begins to flatten and the definitive structures appear. Accordingly, *hoxD* is not detected until stage 6, initially in a strip of cells at the embryonic pole containing the definitive pharynx and spreading later on from the pharynx to the posterior end, resembling the adult pattern (Iglesias *et al*., 2008). Before stage 5, planarian embryos are undergoing patterning as well as differentiating a transient pharynx and beginning to actively feed on the external yolk. Due to its extremely divergent development, it is difficult to discern not only the role of canonical Wnt signaling but also whether axial polarity is established during the early stages of development. For instance, it is unclear whether the expression of *βcatenin-1* in the pharynx at stage 2 indicates a polarizing function of the whole embryo or is simply required for the differentiation of such a complex structure. However, the expression of β-catenin during differentiation of the transient pharynx could reflect an early role in endomesoderm specification, which is linked to posterior fates in most spiralian embryos analyzed (Martín-Durán *et al*., 2010). Nevertheless, the co-expression of *sFRP-1* and *wnt1* in the outlying blastomeres at stage 2, the maintenance of *sFRP1* but not *wnt1* at stage3, and the later restriction of their expression to the anterior and posterior tip of the embryo, respectively, strikingly resembles the situation found during regeneration in the anterior blastema (Fig. 5). However, the relationship between those genes and *βcatenin-1* in both situations is not clear at all, at least from the data available on mRNA expression. It is interesting to note that, despite *βcatenin-1* only being expressed in the pharynx during initial embryonic stages, it has been shown by RT-PCR to be highly upregulated during the first few hours of development. This raises the interesting question of whether in planarians, like in most metazoans, maternal β-catenin is essential for the first polar decisions of the embryo. In most bilaterians, the main body axes are established before gastrulation, and later on they are only refined (reviewed in Niehrs, 2010). If we understand gastrulation as the induction of extensive cell movements in order to differentiate the embryonic germ layers, we would expect that the first axial polarity is established in planarians during stage 1, before differentiation of the transient pharynx. It will be very interesting to analyze Wnt activation and *βcatenin-1* activity during this period to determine whether their roles are conserved not only for refining and re-patterning the AP axis but also for its establishment.

Do planarians have anterior and posterior organizers?

An organizer is often defined as a group of cells with the ability to induce organized cell fates in the surrounding tissue, even when ectopically transplanted. In planarians, like in *Drosophila* imaginal discs or regenerating amphibian limbs, the dorsoventral (DV) boundary seems to act as an organizing region. Interaction between dorsal and ventral epidermal cells is an essential step for planarian regeneration and, moreover, grafting experiments demonstrate that ectopic DV confrontations generate outgrowths with an organized AP pattern (Schilt 1970; Kato *et al.,* 1999; Agata *et al.,* 2007). Those observations suggest the presence of organizing regions at specific positions along the DV boundary, similar to the apical ectodermal ridge, which is responsible for proximo-distal patterning of vertebrate limbs during regeneration (reviewed in Fernandez-Teran and Ros, 2008).

These findings beg the question of whether an anterior and a posterior organizer are also required during planarian regeneration. Current data suggest the existence of a posterior organizer as a source of Wnts and an anterior one as a source of Wnt inhibitors (Adell *et al*., 2010; Meinhardt, 2009), and both of them would be very quickly re-established after amputation. However, this raises two important questions. Firstly, are those organizers also responsible for maintenance of the AP axis in intact animals? Secondly, is the anterior organizer also a source of neural activators?

The induction of different fates along the AP axis is likely to be the result of diffusion of the morphogens (Wnts) and their antagonists (sFRPs) or modulators (notum). However, given the large size of planarians (1- 10 mm), it seems unlikely that so few molecules could account for the patterning of the whole animal. Even during later stages of regeneration, when planarian fragments undergo extensive morphallaxis that requires complete cell re-specification, the range of apical morphogens will need to be very long. The observation that several Wnts and sFRPs are expressed in nested domains along the entire AP axis does support the hypothesis that their integrated activity would account for a graded activation of *βcatenin-1*. The 'intercalary model' of regeneration (Chandebois 1979; Chandebois 1980; Agata *et al.,* 2007) perhaps re-defines the mechanism of patterning more accurately. According to this model, the activity of the signals from the apical organizers would

be restricted to a number of cell diameters and would instruct the identity of tissue edges in the planarian. Intercalary regeneration can function as a general mechanism to re-arrange tissues and organs during regeneration and accounts for the re-specification of central structures. In our opinion, this model is also applicable to homeostasis in planarians. Of course, these models are not mutually exclusive, and canonical Wnt signaling could play an essential role in the process of intercalary regeneration, or 'continuous intercalary re-specification' in the case of intact animals. Nevertheless, it should be noted that the existence of a real gradient of β-catenin activity in planarians, such as the one reported in *Xenopus* embryos (Kiecker *et al.,* 2001), has never been demonstrated.

The potential role of the anterior organizer in the induction of neural fates is an intriguing problem, particularly the question of whether anterior identity can be uncoupled from neural differentiation. Although this issue is far from being resolved, some very interesting preliminary results have been obtained. It has been known for some years that silencing of *nou-darake* (*ndk*), an FGF receptor with inhibitory activities, induces brain differentiation along the length of the planarian body without affecting the AP identities (Cebriá *et al.*, 2002). Interestingly, *ndk* RNAi animals also differentiate a pair of 'brain primordia' in the posterior blastema (Cebriá *et al.*, 2002; Iglesias *et al.*, 2011). Thus, it could be that FGF activation induces neural fates in planarians, as has been described in models of embryonic development (reviewed in Stern, 2005). Very recently it has been reported that the 'two-tailed' animals generated after *APC* or *axins* RNAi also develop a pair of 'brain primordia' in ectopic posterior blastemas, which would correspond to the anterior wounds (Iglesias *et al.*, 2011). Although differentiation of those brain primordia cannot progress in the presence of high *βcatenin-1* activity, this observation demonstrates that neural differentiation can take place in 'posterior' fated tissue. Taken together, these results suggest that early brain determination can be uncoupled from blastema polarity, and that FGF could function as a 'brain activator' secreted from the anterior organizer (Adell *et al.,* 2010; Iglesias *et al.*, 2011). Further evidence that brain differentiation can be uncoupled from positional identify comes from functional analysis of *prep*, a TALE class homeodomain protein that is expressed specifically in the head region (Felix and Aboobaker 2010). Although this gene is required for head formation and anterior markers are dramatically diminished when it is silenced, the ectopic brain induced after *ndk* silencing does not require *prep* function.

Finally, the different phenotypes generated after canonical Wnt signal modulation suggest that the range and nature of the anterior and posterior organizers are essentially different (Meinhardt, 2009). *βcatenin-1* downregulation easily induces completely anteriorized animals with a continuous brain and without any central structures such as the pharynx. In contrast, upregulation of *βcatenin-1* never completely posteriorizes planarians. Instead, it generates 'two-tailed' animals with corresponding pharynges that have opposing orientations. Thus, suppression of the posterior organizer allows continuous differentiation of anterior structures, while induction of an ectopic posterior organizer is only possible at wounds and exerts a short-range effect. More data are required on the activity of Wnt pathway components and other factors that act in wounds and during homeostasis in order to explain these differential responses.

S. mediterranea wnt5 acts through a non canonical/β-catenin-independent pathway to control neural connectivity

Very little is known about the involvement of the β-catenin-independent pathway in the course of planarian regeneration. So far, the non-canonical Wnt pathway has been implicated in two different processes. Firstly, it is known to be involved in controlling neural connectivity during nervous system regeneration and, secondly, it plays a role in the apical docking and planar polarization of the cilia (Almuedo-Castillo *et al.*, 2011). *wnt5*, the only non-canonical Wnt described in planarians, is thought to function as a signal to restrict nervous system growth along the medio-lateral axis (Adell *et al.*, 2009; Gurley *et al.*, 2010). Secretion of Wnt5 is thought to require the transmembrane protein evi/wntless and probably also signals through both Dvls. This is based on the observation that loss of function of these former genes leads to similar defects in terms of lateral displacements and disconnections of the CNS, as well as aberrant projections of the visual axons (Adell *et al.*, 2009; Almuedo-Castillo *et al.*, 2011) (Fig. 1). However, the molecular mechanisms underlying wnt5-dependent positioning of neural structures remain to be defined. Two different options can be considered, namely that wnt5 establishes medio-lateral patterning

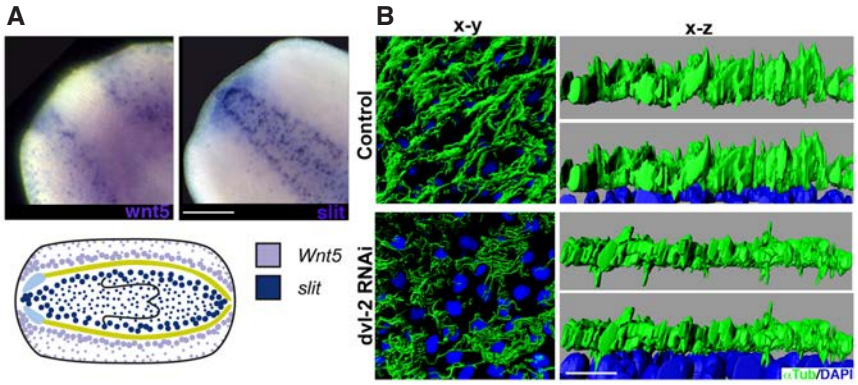

Fig. 6. The non-canonical Wnt pathway during planarian regeneration. (A) *The expression patterns of* wnt5 *and* slit *suggest a shared role in positioning the planarian nervous system along the medio-lateral axis. Animals correspond to trunk pieces 3 days after amputation. Anterior is shown to the left.* **(B)** *Anti-α-tubulin (αTub) immunostaining of control and* dvl-2 *RNAi planarians. Epidermal cilia of* dvl-2*-silenced planarians fail to localize apically and appear disorganized, shorter and embedded in the nuclear layer. x-y and x-z confocal projections are shown. DAPI nuclear staining is shown in blue. A region located approximately 1 mm from the anterior end of the planarian was chosen for imaging ventral cilia. Confocal sections were deconvolved using Huygens Deconvolution Software (Scientific Volume Imaging) and processed using Imaris Software (Bitplane) for 3D reconstructions. Scale bar, 10 μm.*

of the blastema (Gurley *et al*., 2010) or that it restricts the positioning of the regenerating nervous system by regulating migration of the neural precursor cells or axonal growth (reviewed in Endo *et al*., 2007). The second possibility would be consistent with its evolutionarily conserved role as a repulsive cue for growing axons. Notably, *Wnt5* and *slit,* another conserved signal involved in repelling axon growth, show complementary expression patterns in which *Wnt5* is expressed from the outer part of the CNS to the planarian margin and *slit* is expressed from the internal part of the CNS to the midline (Marsal *et al*., 2003; Cebriá *et al*., 2007; Adell *et al*., 2009; Gurley at al., 2010) (Fig. 2). Moreover, the silencing of slit in planarians leads to collapse of the CNS at the midline (Cebrià *et al*., 2007), a phenotype that could be considered opposite to the one generated after silencing *wnt5*, at least in relation to nervous system regeneration. All this may indicate that *wnt5*, together with *slit*, restricts the positioning of the newly differentiated nervous tissues along the medio-lateral axis of the blastema (Almuedo-Castillo *et al*., 2011) (Fig. 6A). Whether or not these two molecules have reciprocal roles in regulating and restricting the expression of the other remains to be determined.

A planar cell polarity network is required for ciliogenesis and docking of apical basal bodies in the planarian epidermis

Planar cell polarity refers to the ability of cells to become asymmetrically organized within the plane of the tissue. This capacity is commonly controlled by a non-canonical branch of the Wnt pathway known as the PCP cascade, which involves the accumulation of specific components to different regions of the cell. Some of the key components of the PCP cascade are known to be involved in ciliogenesis. For instance, the docking and polarization of cilia basal bodies within the epithelial sheet appears to involve a combination of hydrodynamic forces and signaling through PCP proteins (reviewed in Wallingford and Mitchell, 2011).

In planarians, we have found that components of the evolutionarily conserved core PCP pathway—planarian orthologs of dishevelled (*dvl1/2*), van-gogh (*vang1/2*) and diversin (*div*)—are involved at least in the apical positioning of the cilia basal bodies (Almuedo-Castillo *et al*., 2001). In a control situation, cilia emerge from the apical side of the cells, and the actin filaments are located on top of the nucleus (Fig. 6B). Silencing of *dvl-2, vang-1* and *-2*, or *div* leads to an aberrant arrangement of the cilia network, both at the level of the plane of the epithelium and at its apical-basal axis (Almuedo-Castillo *et al*., 2011). Epithelial cilia appear at a lower density and are shorter and disorganized. In addition, the basal bodies of the cilia fail to reach the most-apical part of the epithelial cells (Fig. 6B). Interestingly, this loss of polarization is also observed in the epithelial actin network and a high concentration of empty vesicles and isolated basal bodies is found to be embedded in the cytoplasm of *dvl-2* RNAi planarians. Consistent with this observation, it has been shown that the positioning of the basal bodies in *Xenopus* embryos relies on the functional interaction of Dvl and the actin-remodeling Rho-GTPase, RhoA, in directing vesicle-containing basal bodies towards the apical side of the epithelial cells by active actin cytoskeleton rearrangements (Park at al., 2008). These observations prompt to us to conclude that the defective apical positioning of the cilia observed after silencing core components of the PCP pathway in planarians could be a consequence of abnormal assembly of the actin filaments, leading to aberrant vesicle trafficking of the cilia basal bodies.

Given the phylogenetic position of planarians within the Lophotrocozoa superphylum, the presence of a functional PCP network in these organisms has evolutionary significance. The relationship between PCP and ciliogenesis has been demonstrated in vertebrates (reviewed in Wallingford and Mitchell, 2011), but not in invertebrates, possibly because the most common models, such as *Drosophila*, do not have a ciliated epidermis. The presence of this network within the spiralians thus suggests that it may be an ancient feature in bilaterians. Interestingly, while the role of Wnt ligands as master orientating cues for planar polarity is broadly accepted in vertebrates, there is no evidence for such a role in *Drosophila* (Chen *et al*., 2008; Gao at al.,2011). When we consider that no defects related to cilia docking are found after silencing any Wnt ligand in planarians (Almuedo-Castillo *et al*., 2011) (Fig. 2) these results together suggest that orchestration of planar polarity by a Wnt gradient may be a vertebrate innovation (reviewed in Goodrich 2011).

Finally, although it might be predicted that interference with core components of the PCP would affect processes such as cell migration of different cell types during regeneration, no such defects are observed. In fact, the only defects that have been described are those relating to ciliogenesis. Although we tend to think of all PCP proteins as a complex, however, it has been demonstrated that many features are context specific and vary between the different tissues and organisms under study (reviewed in Goodrich and Strutt, 2011). Thus, further studies are required in order to identify new functions of the PCP network during planarian regeneration.

Concluding remarks

Current evidence from planarians supports an evolutionarily conserved role for canonical Wnt signaling in specifying posterior identity not only during embryogenesis but also in adults. In planarians and cnidarians, the two classical models with extreme regenerative capacities, this mechanism is active both during regeneration and homeostasis. Although very little is known about the activation of canonical Wnt signaling at the adult stage of non-regenerating models, it is tempting to speculate that the constant maintenance of these 'embryonic' signals could be the key to regenerative capacity, as recently discussed in Reddien (2011). A second feature of regenerative animals is the existence of pluripotent and totipotent stem cells. The neoblast population of planarians possesses pluripotent/totipotent stem cells, able to differentiate into any cell type (Wagner *et al*., 2011). It may be that the maintenance of these stem cells in adult animals is possible thanks to the continued activation of the signaling centers. In planarians, the BMP signaling pathway also has an evolutionarily conserved role, as it is required for re-establishment and maintenance of the DV axis during regeneration and homeostasis, respectively (Molina *et al*., 2007; Orii *et al*., 2007; Reddien *et al*., 2007; Molina *et al*., 2011; Gaviño *et al*., 2011). Thus, in adult planarians, as in most metazoan embryos, Wnts and BMPs establish the main body axes that specify a spatial coordinate system to pattern the body. The establishment of both the AP and DV axis must be interdependent because they are always perpendicular. In planarians, it is not known whether a unique organizer could share both functions or, as proposed by Meinhardt (2009), the DV interaction triggers

the formation a first organizer, which establishes DV identities, and then the AP organizer arises to pattern the AP identities in a perpendicular direction.

In contrast to the β-catenin-dependent pathway, the molecular components and interactions that underlie the specific activation of the different non-canonical branches of the Wnt signaling pathway are still far from clear. Interestingly, results in planarians support a conserved role for Wnt5 as a β-catenin-independent input. It remains to be determined whether it regulates cell fate, cell migration or functions as an axon guidance cue. Perhaps more relevant, however, is the discovery that PCP elements have a conserved role in apical positioning of the cilia in planarians, as this had only been reported previously in vertebrates. In planarians, like in *Drosophila*, PCP appears not to depend on Wnt ligand, and components of the PCP pathway appear to exert local effects that do not require morphogenetic signals. Nevertheless, the possibility that Wnts act as modulators of this pathway, possibly through Frizzled binding, cannot be completely ruled out.

Recent years have seen substantial advances in our understanding of the role of the Wnt pathway during planarian regeneration and homeostasis, and evidence has accumulated that its function is conserved from the embryo to the adult. However, as usual, with new information come new questions. Many elements of the pathway remain to be characterized in planarians, for instance the receptors and co-receptors, and the nuclear transcription factors. More importantly, we still do not know which cell types express the different Wnt elements and how they control neoblast differentiation and migration. To advance research in these areas, technical developments will also be required, for instance to obtain reliable readouts of the different branches of the Wnt pathway or analyses at the protein level, such as protein localization, protein-protein interaction, over-expression and live-imaging. Of course, all these methodologies rely on the successful implementation of transgenesis in planarians. Furthermore, it is clear that much more effort should be invested in studying non-standard models across the entire phylogenetic tree and at different developmental stages in order to identify common and derived traits and discover the essential mechanisms that govern cell behavior. Planarians are currently established as an invaluable model for integrative research into body patterning, tissue regeneration and stem cell biology. We therefore expect many of the questions raised in this review to be answered in the coming years.

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