Mantle cell lymphoma pathogenesis:

Another turn of the screw to cyclin D1 overexpression

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A aquellos que empezamos juntos

y ahora juntos lo acabamos.

A aquellos que tomaron rumbos

que nos hicieron distanciarnos.

A aquellos que, un día en el mundo,

tuvimos a bien encontrarnos.

A los que el tiempo dejó mudos

pero siempre siento cercanos.

七転び八起き

"Cae siete veces, levántate ocho"

Proverbio japonés

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"Every real story is a never ending story."
-Michael Ende, "The never ending story"

Los seres humanos estamos hechos de historias. En esta vida podremos el punto y final a muchas de ellas. Otras, solo las comenzaremos. Sin embargo, las más grandes, las más épicas, aquellas que están destinadas a cambiar la tierra; ni las veremos empezar ni tampoco las acabaremos.

La Ciencia es uno de esos grandes relatos que nadie sabe cómo empezó y que nadie sabe cómo terminará. Gracias a todos aquellos que me habéis dado las herramientas, la inspiración, la motivación y la fuerza para poder escribir este pequeño capítulo en la mayor de las Historias Interminables.

En toda aventura, los personajes acaban cruzando oscuros pantanos, desfiladeros de vértigo o áridos desiertos. Gracias **Pedro** por haberme salvado en los momentos en los que renunciar era una opción. Gracias por recordarme qué hago aquí, porqué lo hago, porqué merece la pena continuar. Gracias por haberme dado la oportunidad de aprender a caminar en la cuerda floja, a crecer como persona y como científico.
Encara no s'ha escrit una història on el protagonista no tinga un exemple, un model del que aprendre i a qui seguir. Gràcies **Elies** per donar-me l'oportunitat de ser part d'aquesta gran família on metges, biòlegs, tècnics i molts altres professionals tractem de millorar la vida dels pacients, amb l'ideal romàntic de poder posar, algun dia, el punt i final a la història del càncer.

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En qualsevol història hi ha un moment on el grup, cansat, fart i derrotat, es reuneix al voltant d'una foguera. Al CEK no hi podem fer foc, però si hem viscut situacions molt semblants. És en aquestos moments quan et vull agrair, **Magda**, el teu humor i la teua espontaneïtat que tant m'han reconfortat. Perquè les bones històries no s'acaben en un *paper*, ni les acaba un editor, ni tan sols una co-IP més bruta de lo desitjable.

Gracias a **Noe** y a **Cristina,** que en este viaje profesional han sido más que compañeras, sino verdaderas protagonistas del trabajo que presento. Porque sin el tiempo que habéis dedicado a esta aventura no hubiera sido ni la mitad de intensa, ni la mitad de interesante ni la mitad de divertida. Gracias **Santi** por haber aparecido en esta historia hace ya unos años y haber contribuido a que se pueda acabar de escribir este capítulo.

Però una bona narrativa no es fonamenta en el camí que l'autor recorre, sinó en tot l'univers que acompanya al relat. Parle de tots els altres personatges que mai de la vida podria considerar com a secundaris, ja que han donat mil matisos i colors a un conte que, d'altra forma, només seria una crònica gris.

El regne d'Oncomorfologia funcional humana i experimental (també conegut com "Elieslandia") era un regne dirigit per grans senyores nascudes de molt bona casa. Gent com la Comtessa del Mantell (res a vore amb una tal Laura Conde), gran amant de la xocolata negra i aficionada a fer unes cròniques exhaustives sobre els habitants del països que després regalava en les festes de Nadal. O la Duquesa de EsSox11, un territori ben ampli que va des d'aquesta proteïna fins a llindar amb el veí territori de ciclina D1. Algunes de les mes famoses dames, de fet, varen anar guanyant importància amb el pas dels anys, mentre poblaven el regne dels seus hereus, com la delicada (especialment, els seus ulls) Lady Immunoglobulines; la Dama del FISH, sempre vestida per a no desentonar al barri de Gràcia; o la Pubilla de Terrassa i guardiana de l'inventari.

En el regne també vivien dos místics que guiaven la vida espiritual del país. Per una banda, el misteriós anacoreta Fray Long-non-coding-RNA, que des dels cims més alts vigilava com s'organitzaven els habitants del regne (i els seus pardals!). Por otra parte, el maestro de meditación, yogui y guía espiritual Epiñaki. Sus descubrimientos atraían a curiosos de todo el mundo y muchos de sus seguidores acababan viendo la triple hélice del DNA y algún que otro *paradigm shift* tras probar sus famosas hierbas (estamos hablando del *yogui tea* que repartía a aquellos que venían buscando su sabiduría, por supuesto).

Però vet aquí que una vegada, un jove cavaller va a arribar (o naufragar, segons parlen altres escrits) a aquestes terres. Va ser una princesa de pell blanca (menys a l'estiu, que es ficava com una gamba) i gran amant dels *baby elephants* qui va apareixer i qui li va donar la benvinguda mes gastronòmica possible, doncs tant el cavaller com la princesa eren amants del bon menjar (i algunes llengües diuen que també del bon beure). Poc temps després va arriba altra princesa (*princess* total), que va decidir compartir el seu amor pels jocs de taula i els seus brownies no-suficientment-perfectes-per-a-MasterChef amb aquestos protagonistes. Els tres varen ser feliços junts molt de temps, fins que la princesa-repostera va ser catapultada al lavabo de minusvàlids de la -2. Però el seu amor era tan intens que ni això ni el fet de que haguessin de marxar a altres països, va fer que la seua amistat es trenqués. Gràcies a elles el cavaller va poder suportar mil penúries per acabar sempre amb un somriure als llavis.

El regne d'Elieslandia creixia ric i pròsper i molts altres varen arribar a aquestes terres. Però no tot era dolç en aquest països i la gesta que el nostre cavaller es va proposar, acabar amb el ferotge Drac-Tesi, no era cosa senzilla (dit d'altra forma, era cosa complexa). Per això, va haver de contactar amb homes i dones fortes i amb habilitats especials que l'ajudaren a acabar amb el monstre. De fet, encara hui sobreviuen com a soldats de fortuna i, si vostè te cap problema, potser podria contractar-los.

El grup estava encapçalat per la Rodri, la "vilanovina" (es broma, que és de Barcelona!), la més experimentada de tot el grup. En un capítol de la història, el protagonista va descobrir que era la seua germana bessona, però nascuda amb anys de diferència. El seu coneixement d'Eurovisión, música casposa i humor àcid feiaen d'ella una enemiga letal. No volies creuar-te-la en llocs foscos com la sala de revelar Western ni demanar-li cap buffer! Poc després s'uní Skaleator, un esportista inesgotable amb una estranya afició per pujar parets a totes hores. La seua aportació al grup es centrava en el subministrament de productes artesans com cervesa, formatge, limoncello o macarons de dubtòs aspecte. La seua arma definitiva? El pure amb tonyina, una massa informe que deixava KO al enemic.

Conforme s'apropaven a matar al Drac, s'adonaren de que necessitarien reforços. Vilarrasa "la que arrasa", una lliutadora inesgotable que coneix més de 2467293 formes de matar-te amb la mirada, va ser reclutada per a la feina. Sempre amb el seu millor amic, el bazoka al que ella anomena "Little loop", s'ha criat entre els bars hipsters de Sants i les barres del Sonar. Conforme la fama del grup va anar en augment, es va haver d'incorporar el cèlebre joglar Costitxer I per a que cantés totes les aventures amb el seu ukelele. Però no hi havia que subestimar-lo per la seua joventut i manca de pèl a la cara (en breus arribarà a la majoria d'edat!): les versions de Despacito amb la guitarreta feien ballar a qualsevol enemic, que quedava desintegrat pels projectils de "Little loop". Tots feien un grup fantàstic, i junts varen salvar al nostre protagonista en milers d'ocasions. De fet, varen ser totes aquestes aventures que visqueren junts les que varen fer que, més que companys, el nostre cavaller els anomenés amics.

Altres, molts altres, apareixeren a meitat del camí. Alguns fins i tot apareixereren i despareixerren diversos cops durant els anys que durà l'aventura. Com el Senyor Nadeu, famòs cavaller entre conserges del regne i guardià del pont de la CLL. O la Senyora de Can Verdaguer, una important terratinent catalana que va guiar al grup en múltiples excursions (bueno, el grup la seguia com de lluny perquè a vore qui caminava tan ràpid com ella!). Alguns dels personatges xerraven llengües estranyes que el nostre protagonista no acabava d'entendre, com el Senyor-més-enllà-del-Mar. Aquest noble venia d'unes terres riques en *tardeo*, però on es menjaven els coloms com si foren una *delicatessen*! I com oblidar-nos de quan el nostre protagonista vivia a prop de Dama Russinyol, una poderosa (i temible) maga amb la que passava hores discutint dels seus temes preferits: ChIP-Seq, política i descobrir qui havia furtat les pipetes. Y como no recordar a una de las últimas incorporaciones a este reino, la Princesa Balsas, que sacrificó su lugar en la oficina como post-doc para juntarse con la plebe de monos amaestrados. ¿Su archienemigo? La web de Renfe, con la que durante años tuvo que lidiar para reunirse con sus seres queridos en lejanas tierras.

El destino tuvo a bien que nuestro protagonista se cruzara con gente de todos los rincones de la tierra. Desde las frías tierras polacas, llegaron cálidos ánimos, flores y corazones. Desde Turín, se oyeron promesas de tiramisú (¡qué aun no han sido cumplidas, recuerda el narrador!). Inesperados espíritus holandeses que hicieron aparecer waffles que endulzaron la aventura. O incluso caballeros de las vascongadas que llegaron al reino montados, no en un caballo, sino en un *skate*. In fact, some of the people that assisted this knight came from the very same Japan, helping not only with

their work, but also with their smiles. Others came from Greece, but they became rapidly passionate supporters from Elieslandia's football team: Força Barça! De todos ellos (Anna, Anna, Annaaaaaa), hubo alguien que tuvo que estar durante años aguantando las canciones en bucle de nuestro protagonista (Mira que cosha maish linda!). Alguien muy especial que un día, casi con lo puesto y viendo una oferta de última hora, se marchó para dar la vuelta al mundo. Algunos historiadores se plantean que quizás fue la necesidad de huir de alguien tan pesado y negado para hablar portugués lo que más influyó en la decisión de partir.

Todos ellos (y muchos más) han hecho esta historia realidad. Algunos de ellos, aunque apartados de estos dominios científicos, han hecho posible que esta empresa llegue a buen puerto. Mis **padres**, los primeros. Gracias por tantas cosas que no hay libro que pueda recogerlas. Porque vosotros habéis sido los primeros en escuchar mis historias y animarme a hacerlas realidad. Aunque el camino esté marcado y sean mis pies quienes lo recorran, es vuestra fuerza la que ha permitido tanto que empezara como que ahora lo esté acabando. También a ti, **Maria Pilar**, gracias por ser la mejor hermana que alguien pueda describir. Si alguien puede entender "el estrés de los Albero", esa eres tú. Gracias por el amor y la comprensión de estos años, que no hay distancia en la tierra que pueda enfriar. Gracias a mi **yayo**, a mi **yaya**, a mi **abuelo** y a mi **abuela**. Porque entre las gráficas de biología molecular, entre los párrafos en inglés y entre las proteínas de los modelos; si mi tesis es algo, es un reflejo del amor que siento hacia vosotros.

Y claramente con claridad, gracias a todo/as vosotros/as. Mis amigo/as, mis hermano/as de armas, mis compañero/as de lucha. Los que hemos protagonizado tantas historias diferentes. Los que hemos estado en el Japón feudal, en la Tierra Media, en las mansiones de la locura. Las juergas con vosotros, nuestras lágrimas (a veces de alegría y otras...no tanto) y vuestros abrazos me han hecho ser quien soy. No puedo nombraros a todos, pero esta tesis tiene varios capítulos que escribí mientras os sentía a mi lado. A la científica que ya ha acabado la tesis (**Marta**) y a aquellas que la acabarán después (**Eva**, **Sara** y **Meri**). Porque no hay nada más reconfortante que escuchar noticias de otros reinos, de otros proyectos, de otros dragones que vencer. A ti, **Cesc**, que me has puesto el lápiz en la mano cada vez que lo tiraba al suelo. Gracias por hacerme mejor persona. A vosotros, **Chuso** y **Nita**. Porque al despertar por las mañanas, y cada noche tras cerrar el documento de tesis, veía nuestra foto en el ordenador para no olvidarme de lo feliz que alguien puede llegar a ser. Por último, no solo de tesis vive el doctorando. Contamos historias de grandes hombres y mujeres para recordar que nosotros también podemos serlo. Gracias, **Pau**, **Helena**, **Noel** por todas esas historias que hemos vivido y creado juntos tantas veces.

Con todos vosotros espero vivir muchas otras historias. Pero como ya alguien escribió...

"But that is another story and shall be told another time."

-Michael Ende, "The never ending story"

ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive lymphoid neoplasm derived from mature B cells genetically characterized by the presence of the t(11;14)(q13;q32) translocation that leads to the the overexpression of Cyclin D1. However, secondary alterations are necessary for cancer progression, usually targeting proliferation and genomic instability pathways. Cyclin D1 plays a well-established role in G1/S progression, although other functions includingtranscription or DNA damage response (DDR) can be regulated by this cyclin. Non-canonical functions of cyclin D1 are widely unexplored in human cancers, despite being one of the most frequently amplified oncogenes. Therefore, the main goal of this thesis is the characterization of the cyclin D1 non-canonical function in MCL and lymphoid cell lines. Firstly, we performed *in vitro* studies focused on the characterization of the role of cyclin D1 as transcription regulator (Study 1) and its role as inductor of DNA replication stress (Study 2). Secondly, we aimed to investigate wheather the DNA damage response was constitutively active (Study 2) in primary MCL samples. We also analyzed the methylation profiles (Study 3) in primary tumors, correlating our results with available clinical and molecular data.

In Study 1, we analyzed the genomic binding of endogenous cyclin D1 in four MCL cell lines, showing widespread occupancy around the transcription start site of active promoters. Overexpressed cyclin D1 in lymphoblastic cell lines binds to similar regions that endogenous cyclin D1 and causes a global transcription downmodulation. Concordantly, cyclin D1 silencing in MCL cell lines caused an increase in RNA content. We corroborated the effects of cyclin D1 levels on transcription using MCL and multiple myeloma (MM) cell lines, showing that higher levels of cyclin D1 correlated to lower transcription outputs. This global downregulation was detected also by digital quantification of mRNAs. To explain how the whole transcriptome can be downregulated, we analyzed Pol II occupancy by Pol II ChIP-Seq and found that cyclin D1 colocalized with Pol II. Cyclin D1 upregulation was associated with an increased RNA polymerase II pausing and decreased elongation. This phenotype agrees with a global interference of CDK9 functions and we showed that endogenous cyclin D1 can bind to CDK9 in MCL cells. Cyclin D1 overexpressing cells showed higher sensitivity to a CDK9 inhibitor, revealing a synthetic lethality interaction. We validate the therapeutic potential of this interaction using triptolide, a transcription inhibitor used in preclinical studies. As expected, MCL and MM cell lines displaying higher levels of cyclin D1 were more sensitive to the effects of the drug. Therefore, this mechanism expands the oncogenic cyclin D1 functions in transcription and places the transcription machinery as a potential therapeutic target in cyclin D1 overexpressing tumors.

The aim of study 2 was to study the capacity of cyclin D1 to induce DNA replication stress in lymphoblastic cell lines. Cyclin D1 overexpression caused increased cell proliferation,especially the mutant form cyclin D1 T286A that codifies for a more stable protein. However, cyclin D1 overexpressing cells displayed a slower progression through S-phase. The analysis of DNA fibers showed that cyclin D1 overexpression caused DNA replication stress, which determined a slower fork progression, the activation of a higher number of new origins, and a higher proportion of stalled forks together with an important rate of fork asymmetry. Cyclin D1 overexpression also

hampered replication stress recovery induced by drugs, increasing apoptosis and G2/M blocking. Indeed, constitutive overexpression of cyclin D1 led to basal DDR activation, which was detected by the induction of γH2AX and pCHK2 phosphoproteins. These results led us to wonder if MCL can display a high, constitutive hyperactivation of DDR in primary samples. More than thirty samples were analyzed by immunohistochemistry and 66% showed positive γH2AX staining. 33% of all cases showed a concomitant pCHK2expression. The activation of DDR correlated to worse survival, more chromosome abnormalities, higher proliferation and genetic alterations of genes involved in the DNA damage response.

Study 3 was centered on elucidating the potential contribution of altered DNA methylation in the development and/or progression of MCL. We performed genome-wide methylation profiling of a large cohort of 132 primary MCL tumors, MCL cell lines and normal lymphoid tissue samples using the Infinium HumanMethylation27 BeadChip. DNA methylation was compared to gene expression, chromosomal alterations and clinicopathological parameters. Primary MCL displayed a heterogeneous methylation pattern dominated by DNA hypomethylation when compared to normal lymphoid samples. A total of 454 hypermethylated and 875 hypomethylated genes were identified as differentially methylated in at least 10% of primary MCL. Annotation analysis of hypermethylated promoters recognized pathways related to cell proliferation. The promoters of WNT pathway inhibitors and several other tumor suppressor genes were shown frequently methylated. A substantial fraction of the genes with promoter hypermethylation showed a significant downregulation of their transcription levels. Furthermore, we identified a subset of tumors with extensive CpG methylation that had an increased proliferation signature, higher number of chromosomal alterations and poor prognosis. Our results suggest that a subset of highly proliferative MCL cases displays a dysregulation of DNA methylation characterized by the accumulation of CpG hypermethylation that may influence the clinical behavior of the tumors.

Overall, we have characterized for the first time the new functions that cyclin D1 performs in transcription and replication stress in MCL. The elucidation of these mechanisms may be useful not only for a better understanding of the tumor, but also for improving diagnostic and treatment of MCL patients. A subset of aggressive cases displayed dysregulated DDR and higher methylation levels and were associated with higher proliferation. Our findings suggest that cyclin D1, in addition to its canonical role in cell cycle regulation, plays other functions that may be important for MCL lymphomagenesis.

RESUM

Les neoplàsies limfoides són un grup heterogeni de tumors que, en molts casos, es caracteritzen per un esdeveniment genètic inicial i l'acumulació de canvis moleculars secundaris que condicionen la progressió tumoral. Freqüentment, les alteracions genètiques primàries són translocacions cromosòmiques que provoquen la sobreexpressió aberrant d'un oncogen. Aquest primer esdeveniment oncogènic altera la proliferació, l'apoptosi o la diferenciació normal linfoide que determinen de forma essencial la biologia del tumor.

El limfoma de cèl·lules del mantell (LCM) és un subtipus de neoplàsia limfoide madura amb un curs clínic en general poc favorable i baixa supervivència. Aquest limfoma es caracteritza genèticament per la translocació t(11; 14)(q13; q32) i la conseqüent sobreexpressió de ciclina D1. De fet, l'estudi immunohistoquímic de la cicina D1 s'ha convertit en una eina imprescindible per a realitzar el diagnòstic diferencial d'aquest limfoma, atès que l'expressió d'aquest oncogen en neoplàsies limfoides es limita a la pràctica totalitat de casos de LCM i un percentatge baix de casos de mieloma múltiple (MM) i la tricoleucèmia. S'han descrit dues variants citològiques principals de LCM: clàssica i blastoide. Les formes blastoides generalment presenten una major proliferació i cariotips més complexos. La identificació en els últims anys de casos de LCM que no mostren els criteris convencionals ha complicat la classificació dels LCM fent necessari un diagnòstic basat en criteris clínics, histomorfológics, citogenètics i moleculars. Dins d'aquest grup s'enquadra un conjunt de pacients amb LCM i que presenten un curs clínic indolent sense necessitat de tractament durant un temps relativament llarg. Aquesta variant específica de LCM s'anomena limfoma de célules del mantell leucèmic no-nodal. La identificació d'aquest grup de pacients és important perquè es podrien beneficiar d'aproximacions terapèutiques més conservadores sense que es produeixi un impacte negatiu en la seva supervivència global.

A més de la t(11; 14)(q13; q32) com alteració oncogènica inicial, la majoria de casos presenten, en comparació amb altres limfomes, un nombre elevat d'alteracions cromosòmiques secundàries. Els gens dianes de moltes d'aquestes alteracions cromosòmiques s'han identificat i en molts casos corresponen a gens implicats en el control del cicle cel·lular i en els mecanismes de resposta i reparació de l'ADN.

El gen que codifica per a la proteïna ciclina D1, *CCND1*, és un dels oncogens més freqüentment amplificats en tumors humans, especialment en tumors de mama o de vies respiratòries. A més, tumors hematològics tenen mecanismes genètics que també causen la sobreexpressió d'aquest oncogen, com la translocació t(11; 14) en MCL i MM. Així mateix, ciclina D1 es troba sobrexpresado en una gran varietat de càncers diferents, com fetge, colon, melanoma o pàncrees; mitjançant altres mecanismes no genètics. Funcionalment, ciclina D1 juga un paper essencial en la transició G1 / S en el cicle cel·lular. Ciclina D1 s'expressa com a resposta a estímuls mitogènics. La seva unió amb les quinases dependents de ciclina (CDK) 4 i 6 determina l'activació d'aquestes últimes, que s'encarreguen de la fosforilació de la proteïna RB. Quan RB no està fosforilat, s'uneix i inactiva els factors de transcripció (TF) de la família E2F. No obstant això, la fosforilació de RB canvia la conformació de la proteïna i permet que les

proteïnes E2F activin la transcripció de gens de fase S i que se superi el punt de restricció en el cicle cel·lular. Per això es considera que la principal funció oncogènica de ciclina D1 estaria relacionada amb el seu paper en la progressió a la fase S, que determinaria un augment de la proliferació cel·lular.

No obstant això, cada vegada són més les evidències que mostren que ciclina D1 pot estar participant en altres funcions, moltes d'elles independents a la seva unió amb CDK4 / 6. S'han trobat més de 30 proteïnes que interaccionen amb ciclina D1, regulant processos com la transcripció, la reparació del ADN, apoptosi, migració i metabolisme mitocondrial. No obstant això, molts dels estudis utilitzen la sobreexpressió de ciclina D1 exògena i / o han estat únicament validats en pocs models. A més, no està clar si aquestes funcions tenen lloc tant en cèl·lules neoplàsiques com teixit normals, o fins i tot si són pròpies de determinats teixits.

L'objectiu principal d'aquesta tesi doctoral és la caracterització de funcions no canòniques que ciclina D1 pogués exercir durant la linfomagènesi del LCM. En aquest projecte de tesi ens hem centrat en l'estudi del possible paper de ciclina D1 com a regulador de la transcripció (Estudi 1) i de la capacitat d'induir estrès replicatiu i dany a l'ADN (Estudi 2). L'alteració d'aquests processos, juntament amb la d'altres mecanismes ja descrits en el LCM, poden ser tant causa com conseqüència de la desregulació epigenòmica en cèl·lules tumorals. Per tot això, també és un objectiu d'aquesta tesi la caracterització dels canvis de metilació en una cohort àmplia de casos de LCM (Estudi 3).

En l'estudi 1 es va analitzar el patró d'unió que ciclina D1 mostra en quatre línies cel·lulars de LCM mitjançant tècniques d'immunoprecipitació de cromatina i seqüenciació (ChIP-Seq). Inesperadament, identificarem més de 40.000 regions genòmiques que van mostrar interacció amb ciclina D1 endògena. Aquestes regions d'interacció amb ciclina D1 van mostrar estar enriquides en seqüències promotores. També analitzem diverses marques d'histones i els llocs de sensibilitat a ADNsa I en els promotors units per ciclina D1. Amb aquestes anàlisis identifiquem que ciclina D1 s'uneix d'una manera global a tots els gens amb transcripció activa. Aquesta conclusió va ser corroborada amb dades de seqüenciació de RNA (RNA-Seq). De fet, unions més fortes de ciclina D1 amb un promotor correlacionaven amb nivells més alts de transcripció del gen. Aquest patró d'unió és molt similar al que fa uns anys s'havia descrit per l'oncogen *MYC*, que es va definir com un amplificador transcripcional. Es va observar que Myc també s'unia especialment a una gran quantitat de promotors, correlacionava amb els nivells d'expressió i amb marques epigenètiques d'activació.

La sobreexpressió de *MYC* causava l'amplificació del contingut global de RNA, de manera que nosaltres decidirem quantificar l'efecte de ciclina D1 sobre els nivells de RNA total cel·lular. Sorprenentment, els nivells de RNA disminuïen en models linfoblàstics quan es sobreexpressava la forma normal de ciclina D1 o la variant amb la mutació T286A, que li atorga major estabilitat i provoca majors nivells de ciclina D1 nuclear. Aquest efecte sobre la transcripció ho confirmarem mitjançant el silenciament de ciclina D1 en línies de LCM, que va causar un augment en la quantitat total de RNA. A continuació, vam comprovar en línies de LCM i MM que la concentració de ciclina D1 correlacionava amb nivells més baixos de transcripció, indicant que la ciclina

D1 endògena estaria comportant-se igual que l'exògena. Mitjançant la utilització de mètodes de quantificació digital confirmarem que la sobre expressió de ciclina D1 també determinava una disminució dels nivells de RNA missatger.

El nostre següent objectiu es va centrar en determinar el mecanisme pel qual ciclina D1 exerceix el seu efecte sobre la transcripció. Per a això ens centrem en l'estudi de la maquinària transcripcional i la seva relació amb la sobreexpressió de ciclina D1. Mitjançant anàlisi de ChIP-Seq de la polimerasa II (Pol II) vam determinar que els nivells de ciclina D1 en els promotors correlacionava amb els de Pol II. De fet, varem trobar que la sobreexpressió de ciclina D1 incrementava la parada de la polimerasa, especialment en aquells gens que unien més quantitat de ciclina D1 al seu promotor. Aquesta parada correlacionava amb un canvi en el patró de fosforilació de la polimerasa II, observant una disminució significativa de la fosforilació Ser5 de Pol II. Aquesta fosforilació s'associava amb l'activació de l'elongació i en conseqüència observarem un augment de l'índex de parada de la Pol II. Com la fosforilació Ser5 és depenent de CDK9, vam decidir comprovar si l'efecte de ciclina podria ser a través la unió a CDK9. Després d'observar que ciclina D1 s'unia a aquesta proteïna, vam comprovar si la sobreexpressió de ciclina D1 en models linfoblàstics augmentava la sensibilitat al 5, 6-dicloro-1-beta-D-ribofuranosylbenzimidazole (DRB) , un inhibidor específic de CDK9. La nostra hipòtesi era que podria existir una letalitat sintètica en aquells casos amb més ciclina D1 i, per tant, menors nivells de transcripció. Conseqüentment, vam comprovar que línies amb majors nivells de transcripció i nivells inferiors de ciclina D1 eren menys sensibles a l'inhibidor. Atès que la inhibició de CDK9 s'aconsegueix a dosis molt altes de DRB i, per tant, l'inhabilita per a la seva administració terapéutica, utilitzarem una droga anomenada triptolide utilitzada en assaigs clínics i que produeix una inhibició de la transcripció. Com esperàvem, en línies cel·lulars de LCM i MM, la inhibició de la transcripció és un bon candidat per a desenvolupar noves estratègies terapèutiques contra tumors de baix potencial transcripcional / alta concentració de ciclina D1.

L'objectiu de l'estudi 2 va ser estudiar *in vitro* l'efecte de ciclina D1 sobre la replicació en el limfoma de les cèl·lules del mantell. Ciclina D1 va demostrar ser capaç d'augmentar la proporció de cèl·lules en fase S, però inhbitint la seua progressió durant la fase S. Les cèl·lules amb sobreexpressió de ciclina D1 mostraven clars defectes durant la fase S. Primer, vam comprovar que la fase S era més lenta en aquelles cèl·lules ciclina D1 positives. A més, vam detectar per primera vegada que ciclina D1 estava causant problemes en la progressió de les forquilles de replicació en models de cèl·lula B. Entre aquests problemes destaquem la disminució de la velocitat de progressió de la forquilla de replicació, un increment del nombre de nous orígens activats, la reducció del percentatge de forquilles amb elongació activa i l'augment de forquilles bloquejades. A més, també vam detectar la presència d'una població de forquilles asimètriques en el cas de la sobreexpressió de ciclina D1. També hem observat que la sobreexpressió de ciclina D1 pot comprometre la recuperació de les cèl·lules a un estrès que generi una parada de les forquilles, per exemple després del tractament amb hydroxyurea. Les cèl·lules que sobrexpressaven ciclina D1 van mostrar major apoptosi que les ciclina D1 negatives. Tot això ens va fer concloure que els nivells de ciclina D1 causen estrès replicatiu en línies cel·lulars limfoblàstiques.

Tots els resultats antreriors ens fan pensar que el paper de ciclina D1 en la linfomagènesi del LCM va més enllà que el seu efecte en l'increment de la proliferació. L'estrès replicatiu pot causar inestabilitat genòmica i activació dels mecanismes de resposta al dany al ADN, per tant vam estudiar els efectes després d'una setmana d'inducció de ciclina D1, observant que s'incrementava la quantitat de proteïna H2AX i CHK2 fosforilades, marcadors d'aquesta activació del dany causada per ciclina D1. A banda, ciclina D1 augmentava significativament la proporció de cel·lules tetraploids.

A continuació, donat que el LCM es caracteritza per alts nivells de ciclin D1, volguerem analitzar si els casos primaris d'aquest càncer expressaven marcadors de dany a l'ADN i dels mecanismes de resposta. Estudiarem mitjançant immunohistoquímica l'expressió de les formes fosforilades de H2AX i CHK2 en mostres primàries de MCL.24/37 (64.9%) del casos tenien activació de H2AX, mentre 14/24 (58.3%) tenien activació concomitant de CHK2. Això ens permet distingir casos amb alta activació de la resposta a dany al ADN (ambdues proteïnes fosforilades) o amb baixa / nul·la activació de la resposta a dany al ADN. El grup amb major dany a l'ADN presentava més anormalitats cromosòmiques, menor supervivència i més alteracions en gens supressors de tumors com *CDKN2A* o *TP53*. Així mateix, aquests casos també eren els més proliferatius (major Ki67).

La caracterització de les alteracions epigenómicas al LCM es va desenvolupar en profunditat en l'estudi 3. Es va realitzar un estudi de metilació amb la plataforma HumanMethylation27 BeadChip de 132 casos primaris de MCL i 6 línies cel·lulars. En aquest estudi vam poder observar que el LCM és un limfoma molt heterogeni i que mostra un gran nombre d'anormalitats epigenètiques quan es compara amb un teixit normal. Curiosament, els fenòmens de hipermetilació i hipometilació *de novo* van mostrar diferents comportament. La hipometilació es concentrava en regions intergèniques, mentre la hipermetilació apareixia freqüentment associada a promotors. De fet, es van identificar un total de 454 gens amb promotors hipermetilats i 875 gens amb promotors hipometilats en almenys el 10% dels casos. En el nostre estudi vam observar que la hipermetilació s'associava amb una reducció de l'expressió, afectant freqüentment a gens supressors de tumors. Així, els promotors hipermetilats corresponien a gens que regulaven processos com la proliferació cel·lular i altres vies de senyalització com la via de WNT, de la qual molts dels seus inhibidors estaven *de novo* hipermetilats. Aquest fenomen suggeriria la inactivació oncogènica mitjançant hipermetilació de gens supressors de tumors en casos primaris de LCM.

L'anàlisi dels fenòmens d'hipermetilació ens va permetre caracteritzar un subgrup de casos que presentaven un major nombre de ecanvis epigenètics, un major percentatge d'alteracions genètiques i una menor supervivència global. Especialment destacable és el fet que aquest grup de casos mostraven també una elevada signatura de proliferació. Consistent amb aquests resultats, el gen supressor de tumors *CDKN2A* es trobava hipermetilat i inactivat en un gran nombre de casos. Globalment, els nostres resultats suggereixen que la desregulació de l'epigenoma al LCM pot ser una conseqüència d'una proliferació descontrolada, en part per la sobreexpressió de ciclina D1, i l'adquisició de determinades epimutacions que poden participar en la progressió tumoral.

Les conclusions d'aquest projecte de tesi doctoral han estat:

1) Ciclina D1 mostra un patró global d'unió al la cromatina, unint-se preferentment a promotors de gens actius i que correlaciona de manera significativa amb el nivell de transcripció del gen.

2) Ciclina D1 es comporta com un regulador negatiu global de la transcripció tant en cèl·lules de MCL com en models linfoblàstoids.

3) La sobreexpressió de ciclina D1 incrementa la parada de la Pol II al promotor i dificulta l'elongació, probablement a través de la seva unió inactivant amb CDK9.

4) Els inhibidors de la transcripció induixen apoptosis en línies cel·lulars de MCL, MM i en models linfoblàstoids de sobreexpressió de ciclina D1, suggerint que aquesta letalitat sintètica pot representar una nova estratègia terapèutica per al tractament de limfomes agressius amb nivells alts de ciclina D1.

5) Ciclina D1 augmenta la proliferació cel·lular i l'entrada a fase S quan és expressada en línies limfoblàstiques.

6) La inducció de ciclina D1 provoca un augment significatiu moderat en la proporció de cèl·lules tetraploides en línies limfoblàstiques.

7) Les cèl·lules que sobrexpressen ciclina D1 presenten defectes en fase S i signes d'estrès replicatiu, manifestant parades de les forquilles de la replicació, activació de nous orígens, ralentiment de la replicació i requereixen de més temps per completar la replicació del ADN. En conseqüència, la sobreexpressió de ciclina D1 dificulta la recuperació cel·lular després d'un estrès replicatiu.

8) Temps llargs d'inducció de ciclina D1 activen els mecanismes de resposta al dany a l'ADN, fosforilant les proteïnes CHK2 i H2AX en cèl·lules limfoides.

9) El limfoma de les cèl·lules del mantell és un càncer caracteritzat per alts nivells d'activació de marcadors de resposta al dany a l'ADN, com γH2AX i pCHK2.

10) Els casos de MCL amb alts nivells d'activació de la resposta al dany a l' ADN tenen pitjors taxes de supervivència i s'associen amb una major inactivació de gens supressors de tumors, amb morfologies més agressives i amb un índex de proliferació més gran.

11) L'anàlisi de la metilació de casos primaris de MCL indica que la hipermetilació es dirigeix essencialment a silenciar els promotors de gens supressors de tumors relacionats amb proliferació, per exemple les inhbidors de la via de WNT.

12) Els casos de MCL que tenen majors nivells de CpGs hipermetiladas s'associen amb un pitjor pronòstic, major nombre d'anormalitats cromosòmiques i major proliferació.

RESUMEN

Las neoplasias linfoides son un grupo heterogéneo de tumores que, en muchos casos, se caracterizan por un evento genético inicial y la acumulación de cambios moleculares secundarios que condicionan la progresión tumoral. Frecuentemente, las alteraciones genéticas primarias son translocaciones cromosómicas que provocan la sobrexpresión aberrante de un oncogen. Este primer evento oncogénico altera la proliferación, la apoptosis o la diferenciación normal linfoide que determinan de forma esencial la biología del tumor.

El linfoma de células del manto (LCM) es un subtipo de neoplasia linfoide madura con un curso clínico en general poco favorable y baja supervivencia. Este linfoma se caracteriza genéticamente por la translocación t(11;14)(q13;q32) y la consecuente sobreexpresión de ciclina D1. De hecho, el estudio inmunohistoquímico de la cicina D1 se ha convertido en una herramienta imprescindible para realizar el diagnóstico diferencial de este linfoma, dado que la expresión de este oncogen en neoplasias linfoides se limita a la práctica totalidad de casos de LCM y un porcentaje bajo de casos de mieloma múltiple (MM) y tricoleucemia. Se han descrito dos variantes citológicas principales de LCM: clásica y blastoide. Las formas blastoides generalmente presentan una mayor proliferación y cariotipos más complejos. La identificación en los últimos años de casos de LCM que no muestran los criterios convencionales ha complicado la clasificación de los LCM haciendo necesario un diagnostico basado en criterios clínicos, histomorfológicos, citogenéticos y moleculares. Dentro de este grupo se encuadra un conjunto de pacientes con LCM y que presentan un curso clínico indolente sin necesidad de tratamiento durante un tiempo relativamente largo. Esta variedad específica de LCM se llama linfoma de las células del manto leucémico no-nodal. La identificación de estos pacientes es importante porque se podrían beneficiar de aproximaciones terapéuticas más conservadoras sin que se produzca un impacto negativo en su supervivencia global.

Además de la t(11;14)(q13;q32) como alteración oncogénica inicial, la mayoría de casos presentan, en comparación con otros linfomas, un número elevado de alteraciones cromosómicas secundarias. Se han identificado los genes diana de muchas de estas alteraciones cromosómicas y en muchos casos corresponden a genes implicados en el control del ciclo celular y en los mecanismos de respuesta y reparación del ADN.

El gen que codifica para la proteína ciclina D1, *CCND1*, es uno de los oncogenes más frecuentemente amplificado en tumores humanos, especialmente en tumores de mama o de vías respiratorias. Además, tumores hematológicos tienen mecanismos genéticos que también causan la sobrexpresión de este oncogen, como la translocación t(11;14) en MCL y MM. Asimismo, ciclina D1 se encuentra sobrexpresado en una gran variedad de canceres diferentes, como hígado, colón, melanoma, páncreas mediante otros mecanismos no genéticos. Funcionalmente, ciclina D1 juega un papel esencial en la transición G1/S en el ciclo celular. Ciclina D1 se expresa como respuesta a estímulos mitogénicos. Su unión con las quinasas dependientes de ciclina (CDK) 4 y 6 determina la activación de estas últimas, que se

encargan de la fosforilación de la proteina RB. Cuando RB no está fosforilado, se une e inactiva los factores de transcripción (TF) de la familia E2F. Sin embargo, la fosforilacion de RB cambia la conformación de la proteína y permite que los factores E2F activen la transcripción de genes de fase S y que se supere el punto de restricción en el ciclo celular. Por ello se considera que la principal función oncogénica de ciclina D1 estaría relacionada con su papel en la progresión a la fase S, que determinaría un aumento de la proliferación celular.

Sin embargo, cada vez son más las evidencias que muestran que ciclina D1 puede estar participando en otras funciones, muchas de ellas independientes a su unión con CDK4/6. Se han encontrado más de 30 proteínas que interaccionan con ciclina D1, regulando procesos como la transcripción, la reparación del ADN, apoptosis, migración y metabolismo mitocondrial. Sin embargo, muchos de los estudios utilizan la sobrexpresión de ciclina D1 exógena y/o han sido únicamente validados en pocos modelos. Además, no está claro si estas funciones tienen lugar tanto en células neoplásicas como en tejido normales, o incluso si son específicas de determinados tejidos.

El objetivo principal de esta tesis doctoral es la caracterización de funciones no canónicas que ciclina D1 pudiera ejercer durante la linfomagénesis del LCM. En este proyecto de tesis nos hemos centrado en el estudio del posible papel de ciclina D1 como regulador de la transcripción (Estudio 1) y de la capacidad de inducir estrés replicativo y daño al ADN (Estudio 2). La alteración de estos procesos, junto con la de otros mecanismos ya descritos en el LCM, pueden ser tanto causa como consecuencia de la desregulación epigenómica en células tumorales. Por todo ello, también es un objetivo de esta tesis la caracterización de los cambios en metilación en una cohorte amplia de casos de LCM (Estudio 3).

En el estudio 1 se analizó el patrón de unión al ADN que ciclina D1 muestra en cuatro líneas celulares de LCM mediante técnicas de inmunoprecipitación de cromatina y secuenciación (ChIP-Seq). Inesperadamente, identificamos más de 40.000 regiones genómicas que mostraron interacción con ciclina D1 endógena. Estas regiones de unión a ciclina D1 mostraron estar enriquecidas en secuencias promotoras. También analizamos varias marcas de histonas y los sitios de sensibilidad a ADNsa I en los promotores unidos por ciclina D1. Con estos análisis identificamos que ciclina D1 se unía de una manera global a todos los genes con transcripción activa. Esta conclusión fue corroborada con datos de secuenciación de RNA (RNA-Seq). De hecho, uniones más fuertes de ciclina D1 con un promotor correlacionaban con mayores niveles de transcripción del gen. Este patrón de unión es muy similar al que hace unos años se había descrito para el oncogen *MYC*, que se definió como un amplificador transcripcional. Se observó que Myc también se unía especialmente a una gran cantidad de promotores, correlacionaba con los niveles de expresión y con marcas epigenéticas de activación.

La sobrexpresión de *MYC* causaba la amplificación del contenido global de RNA, por lo que nosotros decidimos cuantificar el efecto de ciclina D1 sobre la cantidad de RNA total celular. Sorprendentemente, los niveles de RNA disminuían en modelos linfoblastoides cuando se sobreexpresaba la forma normal de ciclina D1 o la variante

con la mutación T286A, que le otorga mayor estabilidad y provoca mayores niveles de ciclina D1 nuclear. Este efecto sobre la transcripción lo confirmamos mediante el silenciamiento de ciclina D1 en líneas de LCM que causó un aumento en la cantidad total de RNA. A continuación, comprobamos en líneas de LCM y MM que los niveles de ciclina D1 correlacionaban con niveles más bajos de transcripción, indicando que la ciclina D1 endógena estaría comportándose igual que la exógena. Mediante la utilización de métodos de cuantificación digital confirmamos que la sobrrexpresión de ciclina D1 también determinaba una disminución de los niveles de RNA mensajero.

Nuestro siguiente objetivo se dirigió a determinar el mecanismo por el que ciclina D1 ejerce su efecto sobre la transcripción. Para ello nos centramos en el estudio de la maquinaria transcripcional y su relación con la sobreexpresión de ciclina D1. Mediante análisis de ChIP-Seq de la polimerasa II (Pol II) determinamos que los niveles de ciclina D1 en los promotores correlacionaba con los niveles de Pol II. De hecho, nuestro estudió encontró que la sobrexpresión de ciclina D1 incrementaba la parada de la polimerasa, especialmente en aquellos genes que unían más cantidad de ciclina D1 a su promotor. Esta parada correlacionaba con un cambio en el patrón de fosforilación de la polimerasa II, caracterizado por una disminución significativa de la fosforilación Ser5 de Pol II. Esta fosforilación se correlaciona con la activación de la elongación y en consecuencia observamos un aumento del índice de parada de la Pol II. Como la fosforilación Ser5 es dependiente de CDK9, decidimos comprobar si el efecto de ciclina podría ser a través la unión a CDK9. Tras observar que ciclina D1 se unía a esta proteína, comprobamos si la sobrexpresión de ciclina D1 en modelos linfoblástoides aumentaba la sensibilidad al 5, 6-dicloro-1-beta-D-ribofuranosylbenzimidazole (DRB) , un inhibidor específico de CDK9. Nuestra hipótesis era que podría existir una letalidad sintética en aquellos casos con más ciclina D1 y, por tanto, menores niveles de transcripción. Consecuentemente, comprobamos que líneas con mayores niveles de transcripción y niveles inferiores de ciclina D1 eran menos sensibles al inhibidor. Dado que la inhibición de CDK9 se consigue a dosis muy altas de DRB, lo que lo inhabilita para su administración terapéutica, utilizamos una droga llamada triptolide, utilizada en ensayos clínicos y que también produce una inhibición de la transcripción. Como esperábamos, en líneas celulares de LCM y MM, la inhibición de la transcripción es un buen candidato para desarrollar nuevas estrategias terapéuticas contra tumores de bajo potencial transcripcional/ alta concentración de ciclina D1.

El objetivo del estudio 2 fue estudiar *in vitro* los efectos de ciclina D1 sobre la replicación en el linfoma de las células del manto. Ciclina D1 demostró ser capaz de aumentar la proporción de células en fase S promoviendo la progresión a fase S. Sin embargo, las células con sobrexpresión de ciclina D1 mostraban claros defectos durante la fase S. Primero, comprobamos que la fase S era más lenta en aquellas células ciclina D1 positivas. Además, detectamos por primera vez que ciclina D1 estaba causando problemas en la progresión de las horquillas de replicación en modelos de célula B. Entre estos problemas destacamos la disminución de la velocidad de progresión de la horquilla de replicación, un incremento del número de nuevos orígenes activados, la reducción del porcentaje de horquillas con elongación activa y el aumento de horquillas bloqueadas. Además, también detectamos la presencia de una población de horquillas asimétricas en el caso de la sobrexpresión

de ciclina D1. También hemos observado que la sobreexpresión de ciclina D1 puede comprometer la la recuperación de las células a un stress que genere una parada de las horquillas, por ejemplo tras el tratamiento con hydroxyurea. Las células que sobrexpresaban ciclina D1 mostraron mayor apoptosis que las ciclina D1 negativas. Todo esto nos hizo concluir que los niveles de ciclina D1 causan estrés replicativo en líneas celulares linfoblásticas.

Estos resultados nos inducen a pensar que el papel de ciclina D1 en la linfomagénesis del LCM va más allá que su efecto en el incremento de la proliferación. El estrés replicativo puede causar inestabilidad genómica y activación de los mecanismos de respuesta al daño al ADN, por tanto estudiamos los efectos tras una semana de inducción de ciclina D1, observando que se incrementaba la cantidad de proteína H2AX y CHK2 fosfosriladas, marcadores de esta activación del daño causada por ciclina D1. Además, ciclina D1 aumentaba de forma significativa la proporción de células tetraploides.

A continuación, dado que el LCM se caracteriza por altos niveles de ciclina D1, quisimos analizar si los casos primarios de este cáncer expresaban marcadores de daño al ADN y de los mecanismos de respuesta. Estudiamos mediante inmunohistoquímica la expresión de las formas fosforiladas de H2AX y CHK2 en muestras primarias de MCL.24/37 (64.9%) de los casos tenían activación de H2AX, mientras 14/24 (58.3%) tenían activación concomitante de CHK2. Esto nos permite distinguir tumores con alta activación de la respuesta a daño al ADN (ambas proteínas fosforiladas) o con baja/nula activación de la respuesta a daño al ADN. El grupo con mayor daño al ADN presentaba más anormalidades cromosómicas, menor supervivencia y más alteraciones en genes supresores de tumores como *CDKN2A* o *TP53.* Asimismo, estos casos también eran los más proliferativos (mayor Ki67).

La caracterización de las alteraciones epigenómicas en el LCM se desarrolló en profundidad en el estudio 3. Se realizó un estudio de metilación con la plataforma HumanMethylation27 BeadChip de 132 casos primarios de MCL y 6 líneas celulares. En este estudio pudimos observar que el LCM es un linfoma muy heterogéneo y que muestra un gran número de anormalidades epigenéticas cuando se compara con un tejido normal. Curiosamente, los fenómenos de hipermetilación e hipometilación *de novo* mostraron diferente comportamiento. Por ejemplo, la hipometilación se concentraba en regiones intergénicas, mientras la hipermetilación *de novo* aparecía frecuentemente asociada a promotores. De hecho, se identificaron un total de 454 genes con promotores hipermetilados y 875 genes con promotores hipometilados en en al menos el 10% de los casos. En nuestro estudio observamos que la hipermetilacion se asociaba con una reducción de la expresión, afectando frecuentemente a genes supresores de tumores. Así, los promotores hipermetilados correspondían a genes que regulaban procesos como la proliferación celular y otras vías de señalización como la vía de WNT, de la que muchos de sus inhibidores estaban metilados. Este fenómeno sugeriría una inactivación oncogénica mediante hipermetilación de genes supresores de tumores en casos primarios de LCM.

El análisis de los fenómenos de hipermetilación en LCM nos permitió caracterizar un subgrupo de casos que presentaban un mayor número de epimutaciones, un mayor

porcentaje de alteraciones genéticas y una menor supervivencia global. Especialmente destacable es el hecho de que este grupo de casos mostraban también una elevada firma de proliferación. Consistente con estos resultados, el gen supresor de tumores *CDKN2A* se encontraba hipermetilado e inactivado en un gran número de casos. Globalmente, nuestros resultados sugieren que la desregulación del epigenoma en el LCM puede ser una consecuencia de la proliferación descontrolada mediada en parte por la sobrexpresión de ciclina D1 además de la adquisición de determinadas epimutaciones que pueden participar en la progresión tumoral.

Las conclusiones de este proyecto de tesis doctoral han sido:

1) Ciclina D1 muestra un patrón global de unión a la cromatina, uniéndose preferentemente a promotores de genes activos que correlacionan de forma significativa con el nivel transcripción del gene.

2) Ciclina D1 se comporta como un regulador negativo global de la transcripción tanto en células de MCL como en modelos linfoblastoides.

3) La sobrexpresión de ciclina D1 incrementa la parada de la Pol II en el promotor y dificulta la elongación, probablemente a través de su unión inactivante con CDK9.

4) Los inhibidores de la transcripción provocan una gran respuesta apoptótica en líneas celulares de MCL, MM y en modelos linfoblastoides de sobrexpresión de ciclina D1, sugiriendo que esta letalidad sintética puede representar una nueva estrategia terapéutica para el tratamiento de linfomas agresivos con niveles altos de ciclina D1.

5) Ciclina D1 aumenta la proliferación celular y la entrada a fase S cuando es expresada en líneas linfoblásticas.

6) La inducción de ciclina D1 provoca un incremento moderado significativo en la proporción de células tetraploides en línias linfoblásticas.

7) Las células que sobrexpresan ciclina D1 presentan defectos en fase S y signos de estrés replicativo, manifestando paradas de las horquillas de la replicación, activación de nuevos orígenes, ralentización de la replicación y requieren más tiempo para completar la replicación del ADN. En consecuencia, la sobrexpresión de ciclina D1 dificulta la recuperación celular tras un estrés replicativo.

8) Tiempos largos de inducción de la ciclina D1 activan los mecanismos de respuesta al daño al ADN, fosforilando las proteínas CHK2 y H2AX en células linfoides.

9) El linfoma de las células del manto es un cáncer caracterizado por altos niveles de activación de marcadores de respuesta al daño al ADN, como γH2AX y PCHK2.

10) Los casos de MCL con altos niveles de activación de la respuesta al daño al ADN tienen peores tasas de supervivencia y se asocian con una mayor inactivación de

genes supresores de tumores, con morfologías más agresivas y con un índice de proliferación mayor.

11) El análisis de la metilación de casos primarios de MCL indica que la hipermetilación se dirige esencialmente a silenciar los promotores de genes supresores de tumores relacionados con proliferación, por ejemplo la vía de WNT.

12) Los casos de MCL que tienen mayores niveles de CpGs hipermetiladas se asocian con un peor pronóstico, mayor número de anormalidades cromosómicas y mayor proliferación.

LIST OF SELECTED ABBREVIATIONS

AR: Androgen receptor
BCR: B-cell receptor
CDK: cyclin-dependent kinases
CKI: Cyclin kinase inhibitor
ChIP: Chromatin immunoprecipiation
CIN: Chromosomal instability
CIMP: CpG island methylator phenotype
Cyc: Cyclin
CTD: Carboxy-terminal domain
DDK: Dbf4-dependent kinase
DDR: DNA damage response
DOX: Doxycycline
DRB: 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DSB: Double strand break
ER: Estrogen receptor
FISH: Fluorescent *in situ* hybridization
GC: Germinal center
GEP: Gene expression profile
HDAC: Histone deacetylases
HDA: High DDR activation
HMP: High methylation profile
HR: Homologous recombination
HU: Hydroxyurea
Ig: Immunoglobulin
IHC: Immunohistochemistry
IP: Immunoprecipitation
IR: Ionizing radiation
LDA: Low DDR activation
LMP: Low methylation profile
MCM: Minichromosome maintenance
MM: Multiple mieloma
MRN: Mre11-Rad50-Nbs1
OIRS: Oncogene-induced replication stress
Pol: RNA polymerase
RSR: Replication stress reponse
RT: Room temperature
SSB: Single strand breaks
UTR: Unstranslated region
UV: Ultraviolet
TAF: TATA box binding protein associated factor
TAM: Transcription-associated mutagenesis
TAR: Transcription-associated recombination
TBP: TATA box-binding protein
TCR: Transcription-coupled repair
TF: Transcription factor
TMA: Tissue microarray
Tript: Triptolide
TSG: Tumor suppressor gene
TSR: Transcription stress response
TST: Transcription start site
TTS: Transcription termination site

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INTRODUCTION

1. Non-Hodgkin B cell lymphomas

Hematological neoplasms comprise a large number of entities with different biological and clinical features mainly affecting blood, bone marrow and lymphoid organs. They are classified according to their morphologic, clinic, genetic and immunophenotypic characteristics (NAVARROSwerdlow et al., 2016). The WHO classification stratifies these cancers primarily according to cell lineage: myeloid, lymphoid and hystocytic/dendritic lineages. Assigning a normal counterpart for each neoplasm can explain, to some extent, the biology and clinical manifestation of those tumors. In addition, particular molecular mechanisms fostered by cancer cells contribute to the development of the disease. (Jaffe et al., 2008).

Lymphoid neoplasms are clonal tumors of mature and immature B cells, T cells or natural killer cells. Lymphomas are classified into two major groups, Hodgkin and non-Hodgkin lymphomas. Among the non-Hodgkin lymphomas, B-cell lymphomas accounted for more than 90 % of cases (Morton et al., 2007). Non-Hodgkin B cell lymphomas consist of multiple entities with different clinical course, symptomatology and treatment. In fact, its molecular heterogeneity is a hallmark of this diverse group of tumors.

The broad spectrum of non-Hodgkin neoplasms makes difficult the study of all entities as a whole. This doctoral thesis has focused on one of the most aggressive B cell cancers, mantle cell lymphoma (MCL). This introduction aims to serve as a state-of-the-art of MCL, revisiting the main aspects of its pathogenesis.

1.1 General concepts of B-cell non-Hodgkin's lymphomas

Non-Hodgkin B-cell lymphomas represents a heterogeneous group of neoplasms derived from B cells at distinct stages of cell differentiation. The immunophenotypic, histological and transcription heterogeneity responds to two biological aspects. Firstly, the functions and pathways active in the normal counterpart cells from which the neoplasm rises (LeBien and Tedder, 2008; Swerdlow et al., 2016). Secondly, *de novo* acquired molecular changes that cause the transformation and the expansion of the malignant clone (Scott and Gascoyne, 2014; Shaffer et al., 2002). Therefore it is of paramount importance to keep in mind the basics of B-cell development in order to understand the pathogenesis of non-Hodgkin B-cell lymphomas.

1.1.1 Normal B-cell differentiation

Hematopoiesis is a biological multi-step process oriented to the production of all mature cells of the hematopoietic system. Hematopoietic stem cells show self-renewal properties that allow the production and maintenance of all the immune cells. During development, they first become multipotent progenitors and eventually give rise to myeloid and lymphoid lineages. Lymphoid lineage includes B cells, T cells and natural killer cells (Chao et al., 2008; Monroe and Dorshkind, 2007).

The B-cell maturation stages, from precursor to effector B cells, are defined by specific immunophenotypes, characterized by the expression of cell surface markers and rearrangements of the immunoglobulin gene (Figure 1). B-cell differentiation is oriented to the production of effective B cells and to create a broad repertoire of B-cell antigen receptors. First steps take place in the bone marrow and are aimed to generate the antigen-recognition structure of the B-cell receptor (BCR) , by the recombination of the variable (V) , diversity (D) and join (J) segments of the immunoglobulin (Ig) genes (Pieper et al., 2013). In the genome there are numerous V, D and J segments, but they are randomly rearranged to obtain an unique segment, a process that is globally called V (D) J recombination. Heavy (H) -chain variable region contains the three mentioned segments, whereas the light (L)-chain presents only V and J segments. This process finishes with the rearrangement of the constant (C) segment of the Ig gene. Two high chains and two light chains linked by covalent unions compose a functional immunoglobulin, the antigen-recognition structure of the BCR (LeBien and Tedder, 2008). Eventually, naïve B cells expressing mature surface Ig (IgM+ and IgD+) leave from the bone marrow to the bloodstream.

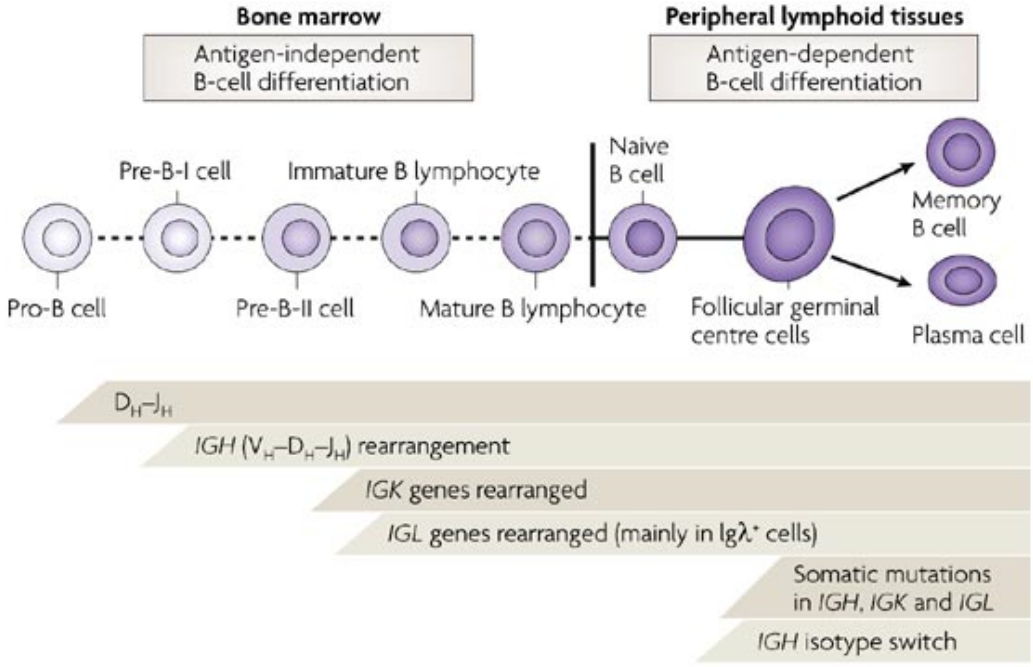


Figure 1. Schematic representation of the different stages in B-cell development. Gene rearrangement, somatic mutations and IGH class switch are also shown (Jares et al., 2007).

Naïve B cells are small resting lymphocytes that visit secondary lymphoid organs, where they can be stimulated by the recognition of an antigen through the BCR. This activation induces proliferation and notable changes, including somatic hypermutation and Ig-class switch that determine its maturation into antibody-secreting plasma cells or memory cells. Although B-cell activation is triggered by antigen recognition, additional signals must be provided by non B-cells (Bretscher and Cohn, 1970). According to the nature of the cells producing these secondary signals, we distinguish two responses: T cell-dependent or independent pathways.

The T-cell dependent B-cell activation determines important changes in the architecture of the lymphoid organs. A few days after the antigen challenge, clusters of proliferating B cells expand in the central region of the lymphoid follicles region (Jacob et al., 1991; Nieuwenhuis and Opstelten, 1984). These transient structures are called germinal centers (GCs), and they are areas of B cell proliferation, BCR diversification and selection of those B cells with higher affinity for the antigen. Proliferative germinal center B cells, called centroblasts, diversify the BCR mainly through somatic hypermutation and RNA editing in the so-called dark zone of the germinal centre (Kurosaki et al., 2015). They end up migrating to the light zone of the GC, where they compete for survival signals derived from T cells and follicular dendritic cells. At this stage, these B-cells are smaller and called centrocytes. In the light zone it also takes place the class switch recombination, a process through which the constant segment of the Ig changes from mainly expressed IgM to other isotypes (IgA, IgE and IgG, mainly). Different isotypes have particular effector functions and class switch recombination does not affect the variable regions of the immunoglobulin (Zarrin et al., 2004). In order to enable these error-prone processes of mutations and recombination, DNA damage-related proteins must be downregulated in the GC (Starczynski et al., 2003). Eventually, B cells will egress the GC transformed in plasma cells, highly specialized cells in the production of antibodies; or memory B cells. Memory B cells are long-lived, resting B cells which can be rapidly activated to proliferate and differentiate upon the recognition of the cognate antigen (Maruyama et al., 2000). Secreting plasma B cells will remain in the primary and secondary lymphoid organs, while memory B cells can migrate to bloodstream (Kurosaki et al., 2015).

T-independent activation occurs without the generation of GC structures. In that case, the recognition of the antigen occurs in the outermost region of the lymphoid follicle, the marginal zone. Marginal zone B cells can directly interact with antigens presented by macrophages. These interactions may activate B cell somatic hypermutation and class-switch recombination, but less efficiently than in the GC(Cerutti et al., 2013). Antibodies generated by this extrafollicular response are largely IgM and display comparatively low affinity (Bortnick et al., 2012); Obukhanych and Nussenzweig 2006).

1.1.2 Initial events in lymphomagenesis

B-cell maturation stages are characterized by particular expression patterns of differentiation markers and by a regulated occupancy of histological structures in the lymphoid tissue. The observation of malignant clones as "stalled" at a particular maturation step makes possible the classification of B-cell lymphomas according to their immunophenotypical characteristics compared to normal counterparts (Greaves, 1986; Kuppers et al., 1999).

The main genetic abnormalities driving early lymphomagenesis are chromosomal translocations (Figure 2). Genes located at the breakpoint sites are often oncogenes whose expression is upregulated as a consequence of the rearrangement leading to dysregulation of proliferation, apoptotic response, growth or other tumor characteristics. These translocations can take place in "dangerous" stages of the differentiation process, like during the Ig recombination in the pre-B cell stage. B cells may continue the maturation process and they could be blocked in a more advanced stage (Shaffer

et al., 2002). Alternatively, this initial oncogenic event may also cause a maturation arrest of the oncogenic clone. These aberrant events are not usually sufficient to cause cell death neither initiate tumor progression. For example, these translocations can be detected in healthy individuals in very small clonal expansions (Lecluse et al., 2009). Therefore, several other changes must be acquired in cancer progression, which involve a vast range of abnormalities with different implications in the pathogenesis (Swerdlow et al., 2016). From a clinical point of view, some of these translocations are specific of certain disease (Campo et al., 2011), allowing a precise differential diagnosis.

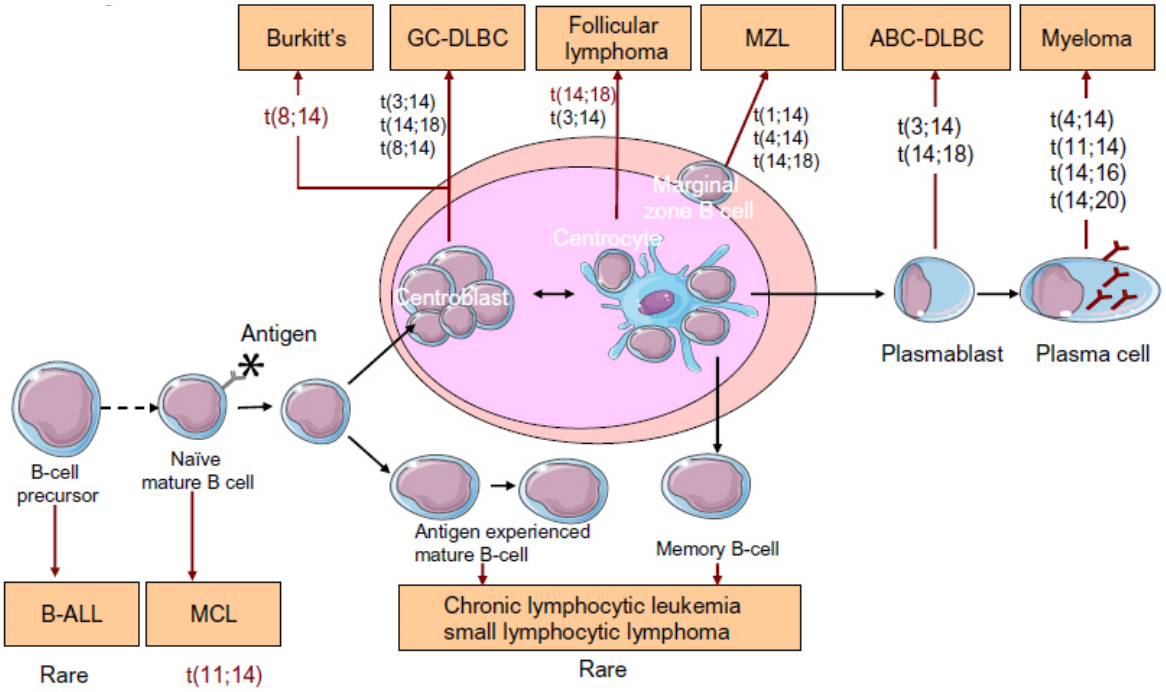


Figure 2. Cell of origin of different B-cell neoplasms and the main translocations involving the IGH locus they harbor (the disease-defining translocations are shown in red) B-ALL: B-Acute lymphoblastic leukemia; GC-DLBC: Germinal Center-Diffuse large B cell lymphoma, ABC-DLBC: Activated B-cell-like- Diffuse large B cell lymphoma; MZL: Marginal zone lymphoma(Boyle E. M. , 2014)

Although cellular morphology and immunophenotype have been traditionally used to define the cellular origin of lymphomas (Figure 2), some lymphomas have heterogeneous characteristics which make difficult the assignation of a particular cell of origin. Traditional studies have been complemented by molecular biology techniques, including large-scale gene-expression profiling**.** For instance, the presence of somatic hypermutation is an indicator of the post-GC origin of the lymphoma (Stevenson, 1998). A GC B-cell gene-expression signature was found associated with follicular lymphoma, Burkitt's lymphoma and a subset of diffuse large B-cell lymphomas (Alizadeh et al., 2000) (Hoefnagel et al., 2005). Moreover, other molecular approaches have been shown useful to accurately explain the cell of origin of B-cell lymphomas, for example using DNA methylation profiling to study the cell of origin of chronic lymphocytic leukemia (Kulis et al., 2015) or MCL (Queiros et al, 2016).

1.2 Mantle Cell Lymphoma: Clinical and biologic characteristics

Mantle cell lymphoma is an aggressive mature B-cell neoplasm which stands for the 7-9% of non-Hodgkin lymphomas in Europe. Short median survival of 4–5 years makes MCL one of the most aggressive lymphomas, due to common relapses and failure to current treatments (Vose, 2013). MCL is considered an incurable disease and patients need urgent treatment at the moment of diagnosis (Herrmann et al., 2009). MCL is usually diagnosed above 60 years old (Zhou et al., 2008). Although prevalence of non-Hodgkin lymphoma is greater among men than women, MCL shows a surprisingly high ratio male-to-female (2.3:1 in Europe) (Sant et al., 2010). Typical clinical presentation entails generalized lymphadenopathy, hepatosplenomegaly and bone marrow involvement at the moment of diagnosis, with frequent involvement of peripheral blood (Bosch et al., 1998). However, other extranodal sites can also be affected (Ferrer et al., 2008; Montserrat et al., 1996; Ruskone-Fourmestraux and Audouin, 2010; Samaha et al., 1998).

Morphologically, MCL presents two main cytologic variants, classical and blastoid/pleomorphic types. Classical MCL is characterized by a monotonous population of small/medium B cells with irregular nuclei and inconspicuous nucleoli. Blastoid/pleomorphic morphology is present in 10-20% of MCL cases, and it is associated with poorer outcome. These cases are frequently tetraploid and have increased proliferation rates (Jares and Campo, 2008). Immunophenotypic criteria for the diagnosis of MCL is based on the expression of B cell mature markers such as CD19+, CD20+, CD22+ and CD79a+ and with the expression of CD5, although some cases can be negative for CD5. (Pérez-Galán et al., 2011). MCL cells express surface immunoglobulins (IgM/IgD) and they lack markers such as CD23 or proteins expressed in GC such as CD10 or BCL6 (Jares et al., 2007). Aggressive cases often show inactivation of tumor suppressor genes (TSGs), such as *TP53* or *CDKN2A* (Izban et al., 2000).

In the last years, it has been discovered a subset of patients with an indolent clinical course that show longer overall survival (7-10 years) even without the need of therapy (Campo and Rule, 2015; Royo et al., 2012). At the moment of diagnosis, this subgroup of patients usually presents lymphocytosis and frequent splenomegaly with minimal or absent lymphadenopathy. Over the last decade, this subgroup has been named as leukemic non-nodal MCL (Royo et al., 2012; Swerdlow et al., 2016). Fernandez and colleagues confirmed, using an unsupervised gene expression analysis, that this subgroup presents a gene expression profiling more similar to conventional MCL than to other lymphomas. In addition, a differential gene expression analysis identified a specific 13-gene signature overexpressed in conventional MCL compared to non-nodal MCL subtype. (Angelopoulou et al., 2002; Fernandez et al., 2010).

Recently, one of the genes present in this signature, SOX11, has shown a prognostic and diagnostic value in MCL (Ek et al., 2008). This transcription factor belongs to the high mobility group protein family and, particularly, it is implicated in neurogenesis and neural differentiation. In cancer, the function of *SOX* genes is not well known, although SOX11 is highly expressed in gliomas epithelial ovarian tumors and breast cancer (Brennan et al., 2009; Sernbo et al., 2011; Weigle et al., 2005) and its expression

correlates with worse prognosis in breast cancer (Zvelebil et al., 2013). Several works have clarified the oncogenic role of SOX11 in MCL lymphomagenesis. SOX11 promotes tumor growth *in vivo* and has a major effect blocking B cell differentiation (Vegliante et al., 2013). Moreover, SOX11 expressing B cells increase tumor angiogenesis and modify tumor microenvironment (Palomero et al., 2014). These oncogenic effects are consistent with the better clinical outcomes displayed by SOX11 negative cases (Royo et al., 2012).

Pre-germinal centre naïve B cells have been traditionally considered the normal counterpart of MCL cases based on their immunophenotypic characteristics, its topographic distribution in the mantle zones and the lack of somatic hypermutated *IGHV* (Jares et al., 2012). However, a subgroup of MCL (around 15%–40%) that carries *IGHV* hypermutations has been recently characterized, suggesting that they may have been originated from cells that had experienced GC maturation. Recently, a significative association between the mutational status and the overall survival has been reported (Navarro et al., 2012).

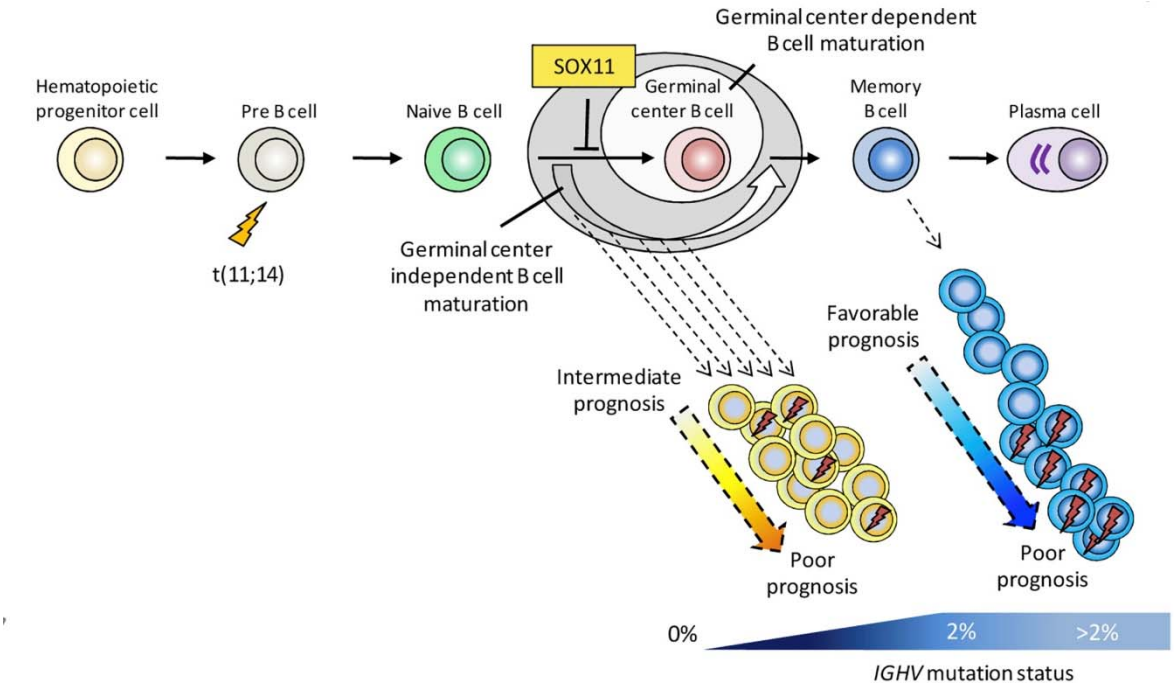


Figure 3. Proposed model of MCL pathogenesis. Conventional MCL cases (yellow) would arise from GC-independent B cells, with intermediate to poor prognosis. GC- dependent B cell maturation would give rise to non-nodal MCL (blue cells). The acquisition of mutations and copy number alterations would lead to poor prognosis in both types. (Modified from (Queiros et al., 2016)

The existence of two subtypes of MCL with different clinical and molecular characteristics may reflect different cell of origin. Conventional MCL cases with unmutated IGHV may be derived from naïve B cells with oncogenic expression of SOX11, which blocks B cell differentiation and the entrance to GCs. However, SOX11 negative cases would enter GC and undergo hypersomatic mutation of IGHV (Kolar et al., 2007; Sims et al., 2005). Concordantly, leukemic non-nodal MCL present more frequently mutated *IGHV* genes, supporting the post-germinal origin of these MCL cases (Navarro et al., 2012; Royo et al., 2012). Both non-nodal and classic cases can

progress to more aggressive variants through the accumulation of secondary genetic alterations. (Navarro et al., 2012; Nygren et al., 2012)(Figure 3).

1.3 Primary oncogenic event in MCL: cyclin D1 dysregulation

The translocation t(11;14)(q13;q32) is considered the primary oncogenic event in MCL (Bosch et al., 1994; de Boer et al., 1995). This rearrangement juxtaposes the gene *CCND1* on 11q13 with the enhancer of the IGH at 14q32, resulting in aberrant overexpression of cyclin D1. Although it is difficult to ascertain, the known cyclin D1 role in cell cycle progression support the idea that this dysregulation is the initial oncogenic event in MCL lymphomagenesis (Wiestner et al., 2007). Conventional cytogenetics allows its detection in 65% of MCL patients, but fluorescent *in situ* hybridization (FISH) techniques enable the detection of the translocation in virtually all MCL cases (Vaandrager et al., 1996). Strikingly, cyclin D1 is the only D-type cyclin that is not expressed in normal B lymphocytes (Teramoto et al., 1999).

Analysis of the breakpoint regions revealed that the translocation occurs in an early pre-B cell stage during B-cell differentiation in bone marrow, as a consequence of an aberrant recombination during the V(D) J initial maturation event (Welzel et al., 2001). Interestingly, the *IGHV* and *CCND1* locus are physically adjacent in the nucleus of immature lymphoid B cells, a common phenomenon also observed in other translocated oncogenes in other lymphomas (Roix et al., 2003). Nevertheless, the mature nature of tumor B cells in MCL clearly indicates B cells with t(11;14) translocation continue B cell differentiation at least to the naïve stage. This suggests that further gain of secondary alterations is required in order to get the full oncogenic potential of MCL cells. Consistent with this scenario, cyclin-D1 behaves as a weak oncogenic factor in multiple tissues, unable to induce transformation directly (Barnes and Gillett, 1998; Bates and Peters, 1995). In addition, the t(11;14) translocation has been detected in healthy individuals, in a subpopulation of naïve cells that persists beyond the normal expected life-span of a circulating B cell. More strikingly, the recombination junction site found in those individuals were exactly the same found in MCL cancer cells (Lecluse et al., 2009). Despite the weak oncogenic potential of cyclin D1, its dysregulation may unlock particular oncogenic-prone pathways and enhance oncogenic activity of secondary abnormalities (Beltran et al., 2011). Recently, an early molecular stage of MCL has been reported. This comprises a group of lesions known as *in situ* mantle cell neoplasia with very limited malignant potential and without any clinical manifestations. Molecularly, they carry the t(11;14) translocation and express cyclin D1. In addition, some of them may express the SOX11 aggressive marker, indicating SOX11 can be activated in early phases of the disease (Christian et al., 2010)

Although more than two decades have passed from the discovery of cyclin D1 overexpression in MCL, many questions remain unclear around cyclin D1 role in MCL pathogenesis. One of the most intriguing issues is the identification in MCL cases of several additional mechanisms that promote cyclin D1 increment over the pathological levels that are reached as consequence of the translocation (Explained in detail in the section 2.4.1 Cyclin D1 and hematologic tumors). For example, secondary chromosomal rearrangement of the *CCND1* locus or mutations in the long 3'

untranslated region (UTR) lead to the expression of truncated cyclin D1 transcripts that are missing these destabilizing regions (Wiestner et al., 2007). These higher stable transcripts have been correlated to tumor aggressiveness and proliferation (Slotta-Huspenina et al., 2012; Wiestner et al., 2007). Eventually, Cyclin D1 expression levels have been linked to overall survival (Rosenwald et al., 2003). Altogether, these findings indicate that cyclin D1 upregulation is a key event in MCL pathogenesis.

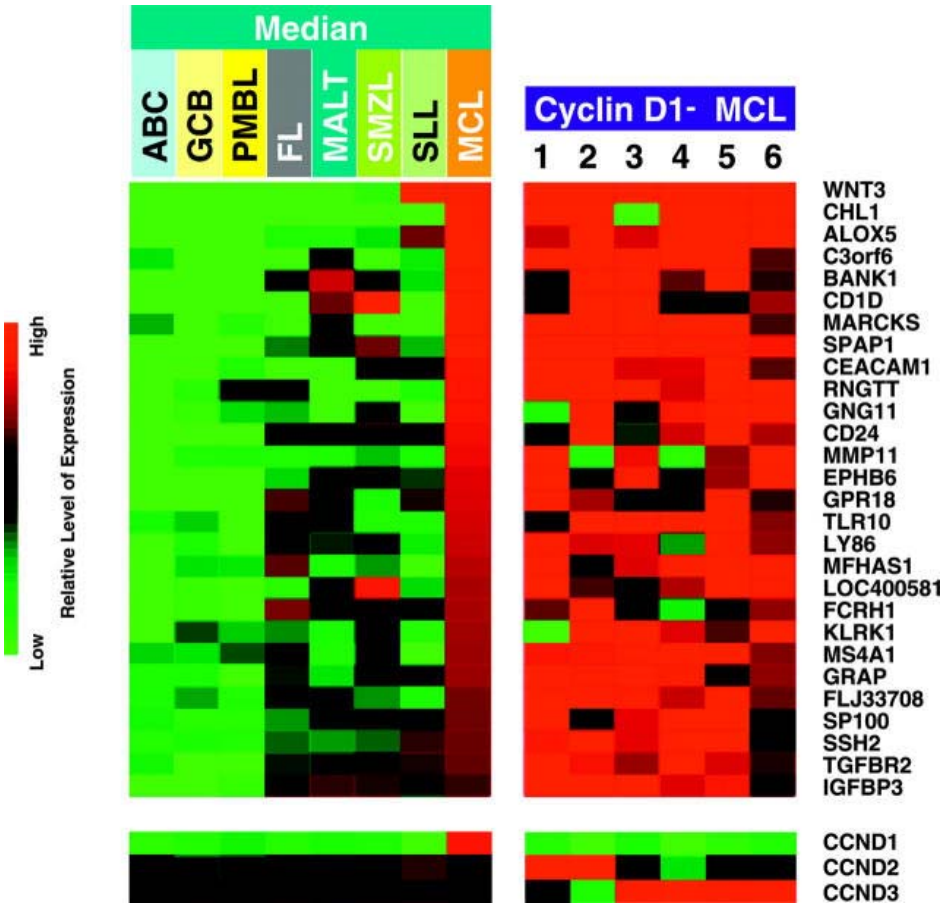


Figure 4. Expression of MCL signature in 6 cases of cyclin D1-negative MCL. The specific MCL signature was generated comparing MCL cases with primary cases of diffuse large B-cell lymphoma (cases of activated B-cell-like (ABC), germinal center B-cell-like (GCB) and primary mediastinal (PMBL) variants), follicular lymphoma (FL), extranodal marginal zone lymphoma MALT type (MALT), splenic marginal zone lymphoma (SMZL) and small lymphocytic lymphoma (SLL) (median expression levels of the MCL signature genes in these entities are shown). For the cyclin D1-negative MCL, each column represents a single lymphoma specimen and each row represents the level of expression of a single gene in the MCL signature. In the bottom panel, the gene expression levels of the D-type cyclins in the various entities and the 6 cases of cyclin D1-negative MCL are shown. (Extracted from Fu et al, 2005)

Some cyclin D1-negative MCL showed overexpression of cyclin D2 or D3. Subsequent studies were able to detect eminently cases with translocations between *CCND2* and *IG* loci (Gesk et al., 2006; Herens et al., 2008; Shiller et al., 2011), although few cases with *CCND3* have been reported (Wlodarska et al., 2008). However, routine immunohistochemistry (IHC) of cyclin D2 or D3 may not be useful to clearly identify cyclin D1-negative MCL, due to the fact these cyclins are expressed in other B-cell lymphomas. On the contrary, SOX11 IHC is a selective marker identifying MCL cyclin D1-negative cases (Mozos et al., 2009; Quintanilla-Martinez et al., 2009).

In recent years, there has been an ongoing debate after pathologists identified a small subset of lymphomas resembling conventional MCL both morphologically and phenotypically but lacking the t(11;14) translocation. This group of patients represents about the 1% of the total MCL (Royo et al., 2011). One of the first studies, conducted by Fu and colleagues in 2005, showed six cases of non-classified lymphomas with morphologic, pathologic and clinical features resembling typical MCL. Using gene expression profiling (GEP) , these cases showed a transcription program similar to conventional MCL despite lacking the t(11;14) (Fu et al., 2005a), (Figure 4). These cases were defined as cyclin D1-negative MCL. A comprehensive characterization of a large series of cyclin D1 negative confirmed these results, showing no differences in the profile of secondary events between cyclin D1-negative and cyclin D1-positive MCL (Salaverria et al., 2013).

To sum up, cyclin D1 overexpression caused by t(11;14) translocation is the main common feature in MCL cases. However, minor subtypes overexpressing other D-type cyclins pose intriguing questions around the role of cyclin D1 in the pathogenesis and the redundancy of the D-type cyclins. Furthermore, mechanisms that increase cyclin D1 expression even beyond the levels reached by translocation agree with an important role of cyclin D1 in MCL pathogenesis. The cooperation/interaction between cyclin D1 dysregulated pathways and the secondary alterations that necessarily must be acquired during MCL lymphomagenesis could be an essential point to clarify its role in MCL pathogenesis.

1.4 Secondary alterations in MCL

The modeling of cancer progression and the secondary events acquired during cancer evolution is not as well characterized in lymphoid neoplasms as in solid tumors. Cyclin D1 overexpression is a weak oncogene with limit capacity to drive oncogenesis (Wang et al., 1994). Consequently, secondary events must contribute crucially to MCL lymphomagenesis (Jares et al., 2012), which could be divided in genetic alteration events and epigenetic dysregulated mechanisms.

1.4.1 Genetic alterations

The use of genome wide technologies enabled the characterization of the molecular alterations governing MCL lymphomagenesis. Comparative genomic hybridization from different studies manifested that many copy number alterations in MCL were in common with other lymphomas, but displayed higher incidence. Loss of material is usually related to the inactivation of TSGs, while amplified regions may cause oncogene overexpression. For some of these chromosome alterations the target genes have been found, although some remain unclear (Table 1). More than 90% of the MCL cases showed at least one alteration and the total number per case was also higher than in other lymphoid neoplasias (Royo et al., 2011). Furthermore, the presence of karyotypes in the tetraploid range can be observed, especially in the blastoid variants (Au et al., 2002; Espinet et al., 2010; Wlodarska et al., 1999). On the contrary, secondary translocations are rare events, being the translocation of *MYC* with *IG* genes the most representative aberration in few cases with bad prognosis (Royo et al.,

2011) The high extent of genomic instability in MCL genomes has become one of the hallmarks of its molecular pathogenesis (Bea and Campo, 2008).

Table 1. Recurrent secondary genomic alterations in MCL detected by CGH (Royo et al., 2011)

Chromosomal region	% cases (Monni et al., 1998)	% cases (Bea et al., 2009)	%Cases (Salaverria et al., 2008)	Minimal region	Target genes
Loss 1p	33	24	52	1p21-p22	*CDKN2C FAF1*
Loss 3p	4	7	5	3p13-p14	
Loss 6q	30	27	20	6q21-q22; 6q25-q26	*TNFAIP3*
Loss 8p	7	7	13	8p22-p21	
Loss 9p	30	16	18	9p21	*CDKN2A/B MTAP*
Loss 9q	15	13	21	9q21-q22	
Loss 10p	–	18	3	10p14-p15	
Loss 11q	30	22	28	11q22-q23	*ATM*
Loss 13q	41	40	17	13q13-q14; 13q33-q34	
Loss 17p	19	16	13	17p13	*TP53*
Gain 3q	52	49	32	3q27-q28	
Gain 7p	15	27	8	7p22	
Gain 7q	7	9	4	7q11-q21	
Gain 8q	30	21	11	8q24	*MYC*
Gain 10p	7	9	1	10p12-p13	*BMI1*
Gain 12q	11	20	6	12q13-q14	*CDK4*
Gain 13q	7	16	3	13q31-q32	
Gain 15q	26	11	11	15q22-q24	
Gain 18q	7	18	11	18q21-q22	*BCL2*

Interestingly, mutational genome wide studies of MCL did not show a higher number of global somatic mutations compared to other lymphoid neoplasms (Beà et al., 2013). Different studies using whole-genome and whole-exome sequencing showed the heterogeneous landscape of mutations, especially in SOX11 positive cases (Beà et al., 2013). *ATM* gene is the most recurrently mutated gene, with an incidence that goes from 40 to 50% of cases. *CCND1* mutations in exon 1 are also very frequent in MCL cases (Beà et al., 2013; Zhang et al., 2014). Recent studies indicate these mutations contribute to increase cyclin D1 stability (Mohanty et al., 2016). Strikingly, these CCND1 mutations appears more frequently in SOX11-negative cases than in SOX11 positive cases (86% vs 18%) (Beà et al., 2013). *TP53* mutations are also recurrently found in MCL cases (13-31% of cases). Different studies have shown a group of genes mutated in MCL with low frequencies (<10-15% of cases), such as NSD2, MLL2, MLL3, NOTCH1/2 or BIRC3 (Beà et al., 2013; Rahal et al., 2014; Zhang et al., 2014). However, mutations and genetic alterations target recurrent functional pathways, suggesting MCL pathogenesis should be studied globally instead of focusing on particular gene alterations (see 1.5).

1.4.2 Epigenetic alterations

Molecular genetics of MCL have been broadly studied for decades. However, only few epigenetic studies have been published up to date. Initial studies focused on the analysis of DNA methylation dysregulation in specific promoters. Classically, promoter hypermethylation occurs in TSGs and it silences gene expression (Figure 5). On the other hand, aberrant hypomethylation can lead to genome instability. Initial works in the field studied the epigenetic dysregulation of certain promoters in MCL. For instance, it was reported that the promoter of development genes, in particular homeobox transcription factor genes such as HLXB9 and HOXA13, were highly hypermethylated in MCL (Halldorsdottir et al., 2012). Enjuanes and collaborators (2011) found a signature of hypermethylated genes that correlated to survival in MCL cases (*SOX9, HOXA9, AHR, NR2F2,* and *ROBO1*). Aberrant hypermethylation of these promoters also correlated to higher proliferation and increased number of chromosomal abnormalities (Enjuanes et al., 2011). Leschenko and collaborators in 2010 performed the first genome-wide methylation analysis and identified aberrant methylation patterns in CDKN2B, MLF-1, PCDH8, and HOXD8 promoters, leading to downregulation of these transcripts (Leshchenko et al., 2010). Moreover, the authors identified hypomethylation of CD37, HDAC1, NOTCH1, and CDK5 promoters that showed pathological overexpression that was confirmed by IHC methods

Recently, a genome-wide characterization of MCL epigenetic has been published (Queiros et al., 2016). Using DNA microarrays and whole-genome bisulfite sequencing, the authors showed a global loss of DNA methylation mostly occurring at gene body and enhancers. The methylome of MCL primary cases was compared with the methylation patterns associated with the different stages of B cell maturation. This study confirmed the hypothesis that MCL may derived from two different cells of origin. However, this study does not address the analysis of MCL epigenetically dysregulated pathways.

Despite the recent progress in understanding the role of epigenetics in MCL pathogenesis, there are still many unanswered questions about epigenetic secondary events and their role in MCL. First of all, it is not clear how some of the most recurrent mutations taking place into genes controlling DNA methylation (*NSD2, MLL2* or *MLL3*) are affecting epigenetic dysregulation in MCL cells. Secondly, there are not fully characterized the pathways targeted by these secondary alterations. Lastly, it is not known the origin of the secondary epigenetic alterations. One of the aims of this thesis is framed within this context, investigating the dysregulation in the proliferation induced by cyclin D1 overexpression and how it may be related to the accumulation of DNA hypermethylation in MCL.

1.5 Altered molecular pathways in MCL

The search for potential genes targeted by genetic and epigenetic alterations in MCL cells has revealed that most of them are involved in three common pathogenic pathways: cell cycle, genomic instability and apoptosis evasion (Jares et al., 2007). However, many studies support the idea that MCL presents many defects in

intracellular signaling pathways that contribute to MCL aggressiveness, such as resistance to apoptosis.

First of all, cell cycle dysregulation is pivotal in MCL pathogenesis (Figure 6). The primary oncogenic event t(11;14) gives rise to cyclin D1 overexpression, a protein with a classical role in the progression through the cell cycle, increasing cell proliferation. Other cell cycle genes, especially in *INK4A/CDK4/RB1* and *ARF/MDM2/TP53* pathways are frequently altered in MCL patients (Figure 6, (Jares and Campo, 2008)). The alterations of genes in the *INK4A/CDK4/RB1* concomitant with cyclin D1 overexpression support the importance of the G1/S phase transition dysregulation in MCL pathogenesis. One of these alterations is the epigenetic or genetic inactivation of *CDKN2A* on 9p21 (Hutter et al., 2006). This locus codifies for the protein p16, a CDK4 inhibitor. Alternative mechanisms include amplification of CDK4 or BMI1, an inhibitor of p16 (Bea et al., 2001; Jacobs et al., 1999). In some aggressive cases, mutations in *RB1* gene causing homozygous inactivation have been detected (Pinyol et al., 2007). All these alterations impact on the progression of the disease. Consistent with these data, the proliferation signature, that may integrate the effect of all the cell cycle genetic alterations, remains one of the best prognostic factors. (Rosenwald et al., 2003)

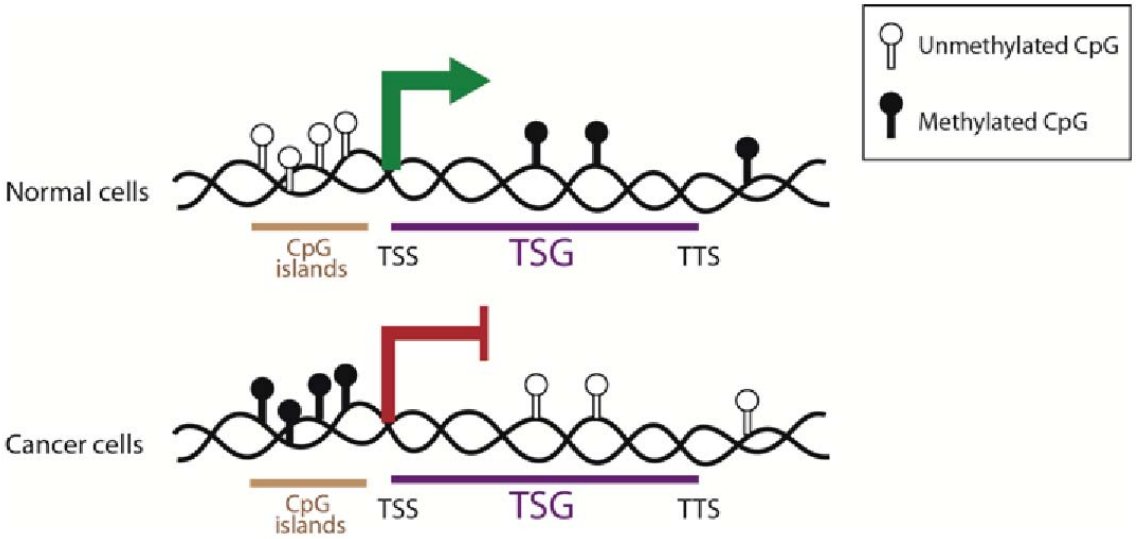


Figure 5. Abnormal methylation of tumor suppressor genes causes its transcription downregulation in cancer cells. CpG islands of tumor suppressor genes are demethylated in normal tissues, permitting its transcription. Gene body CpGs and non-promoter CpGs are usually methylated. In cancer cells, CpG hypermethylation silences TSGs. Hypomethylated CpGs in cancer cells can lead to an increment of genomic instability. TSG genes schematic representation goes from transcription start site (TST) to the transcription termination site (TTS).

Chromosomal instability is a remarkable characteristic of MCL, suggesting that DNA damage response can be altered in MCL cases. *ATM* and *TP53*, two key genes in chromosomal stability maintenance, are the first and the third more frequently mutated genes in MCL (Beà et al., 2013). Deficient response to double strand breaks (DSBs) in lymphoid cells might produce genomic instability facilitating the development of lymphomagenic alterations and increased aggressiveness (Camacho et al., 2003; Kuppers and Dalla-Favera, 2001). *CHK1* and *CHK2*, which are downstream targets

of DNA damage sensors ATR and ATM, are also occasionally deregulated in MCL (Tort et al., 2005; Tort et al., 2002). Although *CHK2* mutations have been reported, *CHK1* has only been downregulated in some cases. On the other hand, some studies in MCL cases point out that cyclin D1 correlates with a licensing unbalance, indicating that cyclin D1 may have a direct role in replication problems in MCL. (Pinyol et al., 2006). Interestingly, unbalanced licensing also correlated to a higher number of genetic alterations, indicating that cyclin D1 may be increasing chromosomal instability in MCL cases. In this regard, some studies have described that cyclin D1 overexpression promotes rereplication and induces mitotic problems (Aggarwal et al., 2007). Besides, some microtubule-associated proteins are targets of genetic and epigenetic alterations and may have an impact on microtubule dynamics and, consequently, on chromosomal instability (Vater et al., 2009)

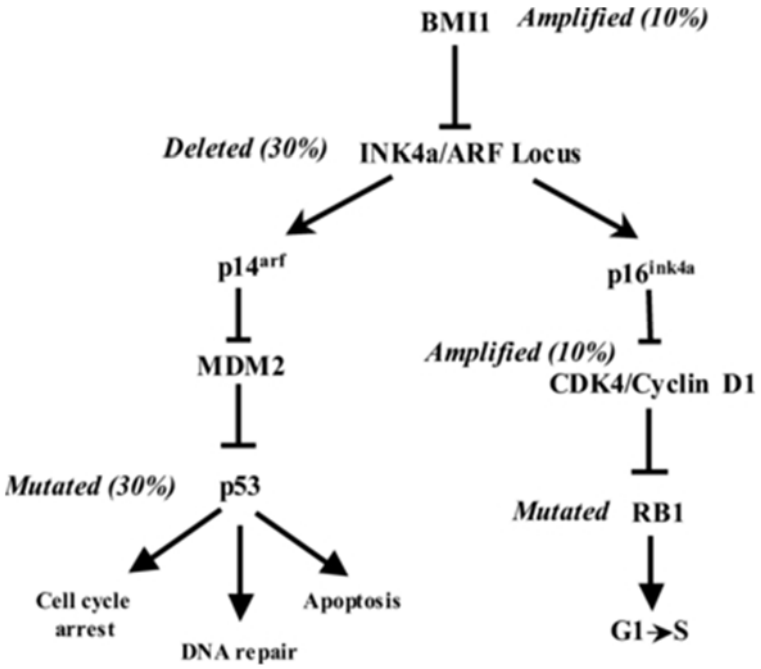


Figure 6. Integration of genetic secondary events related to G1/S cell phase transition in MCL pathogenesis. Modified from (Jares and Campo, 2008).

Evading apoptosis is a main hallmark of MCL cases. For instance, amplification/ overexpression of BCL2 or inactivation of *BCL2L11, FAF1* and *TNFAIP3* have been reported (Royo et al., 2011). In fact, disruption of apoptosis and cell survival pathways is characteristic of blastoid variants (Khoury et al., 2003). In this regard, the abnormal activation of different signaling pathways is also often detected in MCL cases. Nuclear factor-κB pathway pathogenic activation is frequently found in MCL (Fu et al., 2006; Martinez et al., 2003; Pham et al., 2003). There are also alterations of the *PI3K/AKT/mTOR* pathway (Peponi et al., 2006; Rizzatti et al., 2005; Rudelius et al., 2006). For instance, high levels of activated AKT, mTOR, and their respective downstream targets have been identified in MCL (Dal Col et al., 2008; Rudelius et al., 2006). BCR signaling is also constitutively active in primary MCL (Pighi et al., 2011; Psyrri et al., 2009; Rinaldi et al., 2006). Pharmacological *in vivo* and *in vitro* studies of the BCR support the view that antigen selection may play a role in the pathogenesis of at least a subset of MCL. JAK/STAT pathway activation has also been reported (Baran-Marszak et al., 2010; Lai et al., 2003). NOTH pathway may be also targeted in MCL

lymphomagenesis. A recent study found *NOTCH1/2* truncating mutations in MCL cases associated with poor survival. Inhibition of this pathway reduced proliferation and induced apoptosis of MCL cells (Kridel et al., 2012). Some studies also suggest a role of the WNT canonical pathway in the molecular pathogenesis of this disease. Concordantly, inactive phospho-GSK3B, a key inhibitor of the pathway has been found in MCL (Chung et al., 2010). However, further studies are needed to confirm and expand these observations.

2. Cyclin D1 in health and disease

Studies over decades have revealed that cell cycle progression is controlled in eukaryotes by a conserved group of kinase proteins named cyclin-dependent kinases (CDKs). Because of the importance of the cell progression process, CDK activity is finely regulated by different strategies. One of the main mechanisms corresponds to the expression control of their regulatory subunits, known as cyclins. CDKs' heterodimerization with their cyclin partner is required for the activation of the kinase complex, which in turn regulate cell cycle transitions. In mammals, different CDKs and cyclins are specialized in the control of specific steps of cell cycle progression. (Figure 7). Hence, cyclins and CDK can be classified according to the cell cycle phase where they develop their roles. In this regard, CDK4, CDK6 and CDK2 play a role in G1/S transcription interacting with cyclins D and E respectively. CDK2 also plays a role in S-phase progression binding to Cyclin A. CDK1 controls G2/M cell cycle phases through binding to cyclin A and B.

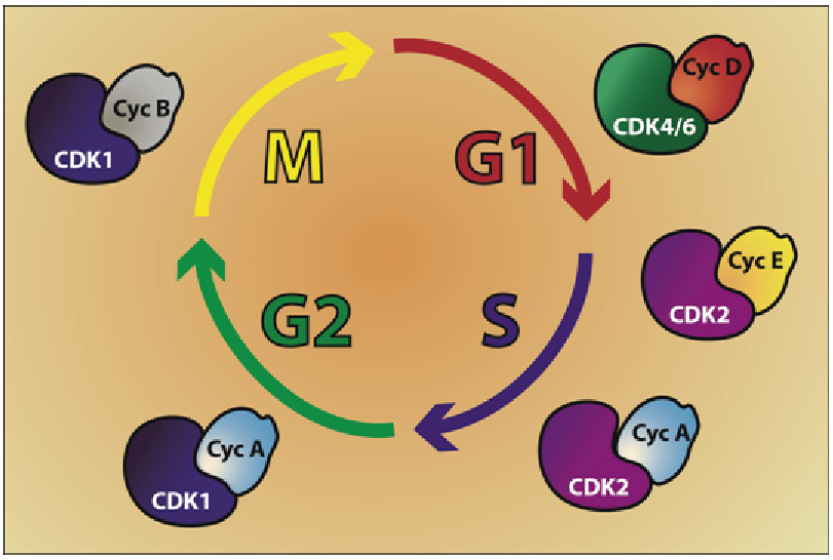


Figure 7. Canonical interactions between cell cycle CDKS and cyclins during cell cycle.

On the other hand, CDKs also play an important, but less understood, role in transcription. Specific cyclins activate a larger number of CDKs (CDK7, CDK8, CDK9, CDK11, CDK12 and CDK13) to control and modulate transcription. Independently of its classification, CDK- cyclins are tightly interregulated. For examples, CAK complex, which consists of CDK7, cyclin H and MAT protein regulate the activation of cell cycle CDKS (Kohoutek and Blazek, 2012).

Cyclin D1 is the best characterized member of the D-type cyclins owing to its widespread presence in human oncogenesis. Although often associated with higher proliferation, in the last decade an increasing number of evidence suggest a role of cyclin D1 in diverse cellular processes. However, some of them remain poorly understood, such as the role in DNA repair and genomic instability. Other functions have been studied only in a limited number of cell types and their actual role in oncogenesis remains unknown.

2.1. Structure of Cyclin D1: gene and protein domains

CCND1 (previously known as *BCL-1* or *PRAD-1* oncogene) is a gene located in the long arm of chromosome 11 (11q13). Structurally, CCND1 is codified by 5 exons, with a remarkably long 3' UTR tail. Two CCND1 mRNA isoforms have been reported (isoforms a and b). These isoforms differ in the mRNA length, (isoform b has 1.7Kb against 4.5 Kb of isoforms a) and codify for different C terminus of the protein (Figure 8. A). The isoform b misses exon 5 due to absence of splicing and retains intron 4, which contains a stop codon and a polyadenilation signal used only by this isoform (Betticher et al., 1995). This shorter mRNA isoform encodes a protein with an altered carboxy-terminal domain.

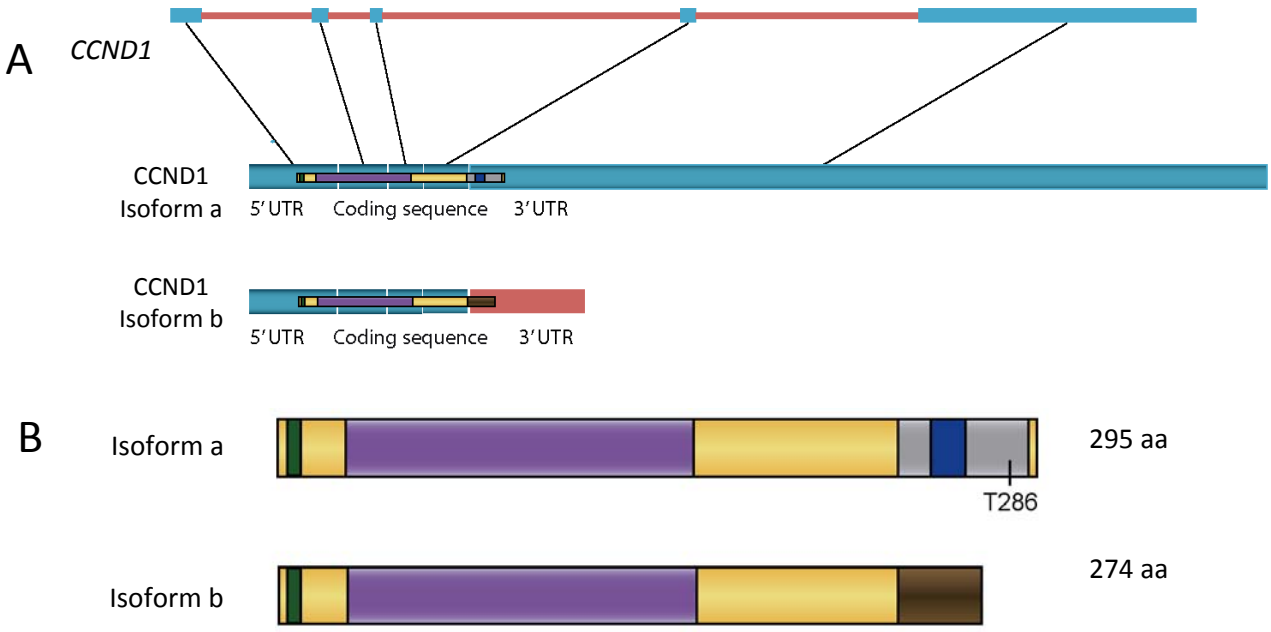


Figure 8. Cyclin D1 gene, transcripts and protein structure. (A) CCND1 gene and proportional length of cyclin D1 isoforms. (B) Protein domains in isoforms a and b. Green: RB-binding domain. Purple: Cyclin box. Blue: Leucine-rich motif. Grey: PEST domain. Brown: Alternative C-terminal sequence found in isoform B transcripts.

The cyclin box is highly conserved among the cyclin family members, but also between metazoan species (Figure 8. B). This domain is critical for the binding and activation of CDKs. Additional domains have been defined, including a RB binding motif in the N-terminus and a PEST domain in the C-terminus. The last one is a common domain found in proteins that are recognized and degraded by ubiquitinases. This domain seems to be critical to achieve the effectively degradation of cyclins in order to ensure the correct regulation of cell cycle phases. The PEST domain is codified by the fifth

exon, so it is absent in the isoform b. A critical residue in the the PEST domain is the threonin 286. This aminoacid is phosphorylated by the kinase GSK3 promoting the export of nuclear cyclin D1 and its degradation by cytoplasmic ubiquitinases (Diehl et al., 1998). Another interesting domain is the C-terminal leucine-rich motif (LLXXXL). It has been hypothesized that this motif could be folding with an amphipathic α-helix conformation. Similar helixes act as activators of some transcription factors (TFs) , so this motif could play a specific role over certain proteins (Coqueret, 2002)

1.2. Canonical function of Cyclin D1 : G1/S checkpoint

From yeast to humans, the restriction point is a process highly conserved during cell cycle initiation. This refers to the time in the G1 phase when cells, in response to positive growth signals, are irreversible committed to cell cycle progression. In mammals, cyclin D1 plays a major role in the regulation of the restriction point or G1 checkpoint through its binding to CDK4 and CDK6.

The main target of CDK4/6-cyclin D1 complex is the retinoblastoma protein. RB protein remains unphosphorylated in G0 and early G1, sequestering the E2F factors and inhibiting their transcriptional functions. Non-phosphorylated RB has been linked to commitment to cell differentiation and maintaining cells in G0. Upon cyclin D1 expression, CDK4/6 form active complexes with this cyclin and phosphorylate RB in early G1. This process changes RB protein structure, disrupting the interaction between RB and E2F proteins (Zhang et al., 2000). Importantly, E2F factors orchestrate the expression of S-phase genes in late G1, such as the transcription of replication factors (Narasimha et al., 2014; Sengupta and Henry, 2015). Remarkably, cyclin E becomes expressed upon RB phosphorylation. Cyclin E binds to CDK2 and enhances its kinase activity targeting RB and results in its hyperphosphorylation. Hyperphosphorylation of RB residues inactivates the RB interaction with chromatin. As a consequence, E2F proteins increase the transcription activity of specific genes and cause S-phase entrance (Dick and Rubin, 2013).

The CDK4/6-cyclin D1 complexes are important drivers of G1/S progression, so cells have developed different strategies to tightly regulate their activity. One of the main regulatory mechanisms relies on the expression of the cyclin-dependent kinase inhibitors (CKIs), a group of proteins that binds and inactivates CDK-cyclin complexes. Based on the specificity of the targeted CDK and their sequence, they are divided into two families: the INK4 family (p15, p16, p18 and p19) or Cip/Kip (p21, p27 and p57). INK4 inhibitors target CDK4/6, while Cip/Kip proteins can bind to all CDK-cyclin complexes (Denicourt and Dowdy, 2004). INK4 CKI family inactivates cell cycle progression by interacting with CDK4/6 and avoiding cyclin D1 binding. Mouse knock-out models show a large redundancy between INK4 members (Roussel, 1999). Although removal of these genes individual or collectively do not lead to cancer formation, recent results indicate they can be cancer-susceptibility genes (Fero et al., 1996; Krimpenfort et al., 2007; Latres et al., 2000; Martin-Caballero et al., 2001; Nakayama et al., 1996; Sharpless et al., 2001). The only exception is the protein p16, an INK4 family member frequently mutated in human cancer. Concordantly, it is the only protein of the family that, if removed, provokes tumor development in mice (Hara et al., 1996; Hirama and Koeffler, 1995). The genomic locus (*CDKN2A*) gives rise to

two different transcripts, both with tumor suppressor characteristics. The protein p16 is encoded by exons 1a, 2, and 3, whereas p14 is encoded by exons 1b and an alternative reading frame of exon 2 (for this reason p14 is also known as alternative reading frame, ARF). Their roles in oncogenesis as TSGs have centered scientific attention over decades, showing their role not only in cell cycle, but also in DNA damage response (Bieging-Rolett et al., 2016; Shapiro et al., 1998). In brief, while p16 inhibits CDK4/6–Cyclin D1 complexes, ARF is activated upon oncogenic-stress and interferes with the activity of MDM2, leading to p53 stabilization and activation of p53-dependent transcription program (Kim and Sharpless, 2006). (Figure 9)

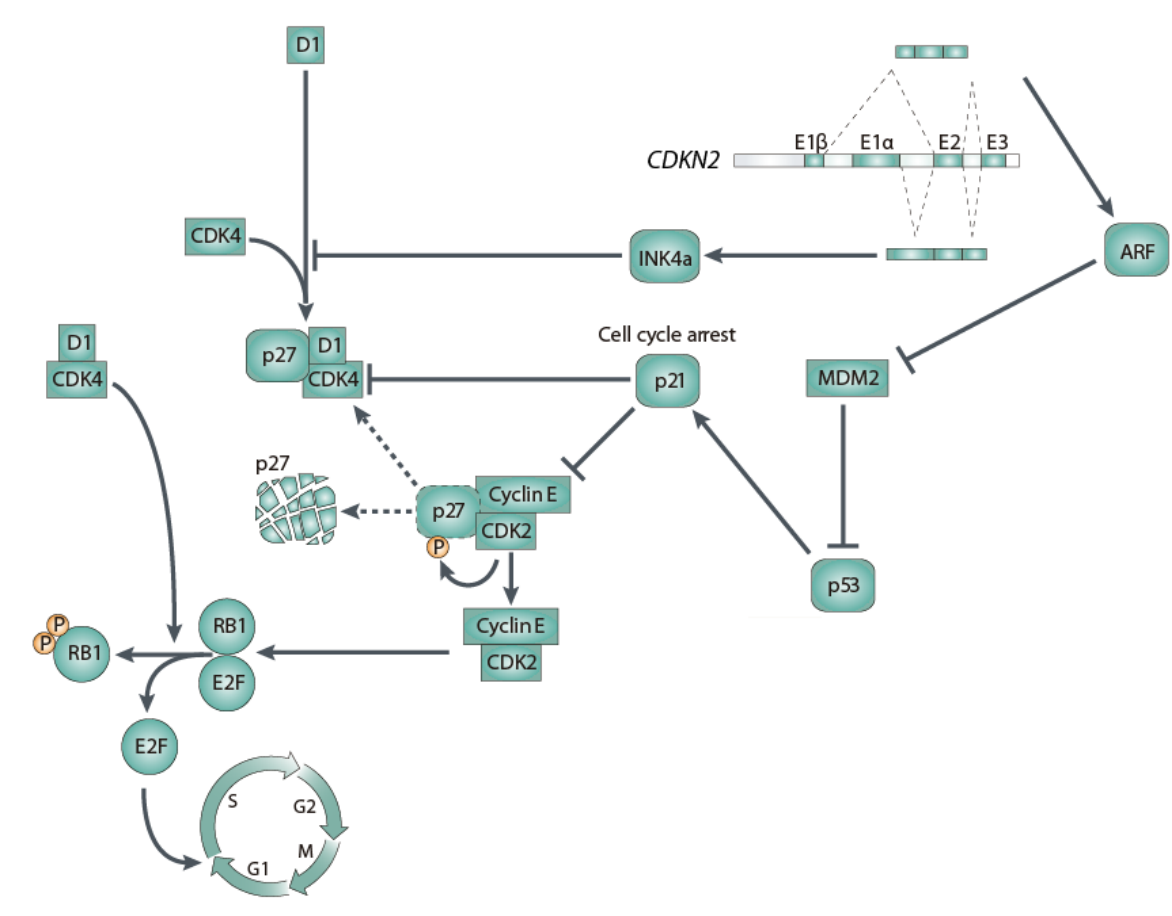


Figure 9. Main regulatory pathways in the G1/S checkpoint regulating cyclin D1-mediated restriction point transition (Modified from (Jares et al., 2007))

Contrary to INK4 members, the Cip/Kip family relies on binding to different CDK-cyclin complexes in the nucleus in order to inactivate them and restrain cell cycle progression. However, each CDK inhibitor has specific roles in different tissues and cell processes (Sherr and Roberts, 1999). For example, p21 and p27 are ubiquitously expressed, but p57 is restricted to some tissues and plays an important role in development (Lee et al., 1995; Matsuoka et al., 1995). Mice knock-down models also show different phenotypes depending on the depleted gene (Besson et al., 2008). Interestingly, the differential affinity that these proteins display for different CDKs represents a regulatory mechanism. For instance, p27 shows a great affinity for CDK4/6 complexes. However, it has been reported that CDK4/6-cyclin D complexes are little sensitive to inhibition by Cip/Kip proteins (LaBaer et al., 1997) and, strikingly, p27 may increase the stability of CDK4/6-cyclin D1 binding. The increase in CDK4/6-

Cyclin D1 complexes during the restriction point causes p27 sequestration and hampers p27 inhibitory action on CDK2-Cyc E. These active CDK2-cyclin E complexes, in turn, phosphorylate p27 and mark it for degradation (Perez-Roger et al., 1999).

The canonical functions of cyclin D1 can also be performed by two other D-type cyclins: cyclin D2 and cyclin D3 (Qie and Diehl, 2016). Although it is not the main topic of this work, it is interesting to note the high degree of structural homology among them, greater than 73%. Besides, all three form complexes with CDK4/6 and activate G1/S phase progression (Musgrove et al., 2011). Genetic ablation of individual D cyclins led to minimal abnormalities, indicating high redundancy. However, they are not equally expressed in all tissues (Fantl et al., 1995; Sicinska et al., 2003; Sicinski et al., 1996; Sicinski et al., 1995). In this regard, cyclin D1 knock-outs display neurological, retinal and mammary epithelial abnormalities. Cyclin D2 deficient mice have problems in testes and ovarian, while cyclin $D3^{-/-}$ showed imperfect development of immature T cells. Strikingly, mice lacking all three D-type cyclins were not viable, but reached the mid-gestation phase, revealing that the vast majority of embryonic cell types can divide in a cyclin D-independent fashion, with the exception of the hematopoietic stem cells and cells of the myocardium (Kozar et al., 2004). On the other hand, double knock out of CCND1 and CCND2 survived up to 3 weeks post-natally (Ciemerych et al., 2002). As expected, *CCND1* locus changed by *CCND2* reduced CCND1-associated defects in mice (Carthon et al., 2005). In addition, biological differences have been detected among the three D-type cyclins. For example, they display different affinities for other CDKs like CDK2 (Ewen et al., 1993; Higashi et al., 1996). Besides, non-CDK mediated functions may be differently regulated by these three cyclins. For instance, Cyclin D3 activates ATF5-mediated transcription, but not cyclin D1 or cyclin D2 (Liu et al., 2004).

2.3. Non-canonical functions of Cyclin D1

In the past years, increasing evidences suggest broader roles of cyclins D than the ones related to its catalytic activation of CDK4/6 during cell cycle progression. Cyclin D1 has been the main target of these new studies, revealing that cyclin D1 participates in transcription, DNA damage response, apoptosis escape, cell migration and cell bioenergetics (Figure 10). These studies draw a more complex scenario, where some functions are mediated by cyclin D1-CDK4 complexes, although other functions are CDK-independent. Moreover, a lot of questions remain to be answered, for example certain interactions have been just confirmed in particular models (fibroblasts, lymphoblastic models, …), arising the necessity to study these new roles in other tissues to characterize tissue-related functions. Finally, the oncogenic effects of these non-canonical roles are poorly understood in human oncogenesis, since just a few of these functions have been proven in cancer cell models.

2.3.1 Chromatin recruitment and transcription

Cyclin D1 cannot bind directly to chromatin, so the role it performs in transcription must depend on its capacity to interact with other proteins able to bind to DNA. As reviewed in (Coqueret, 2002) and (Musgrove et al., 2011), cyclin D1 may regulate transcription by two main mechanisms. First, cyclin D1 overexpression can occlude the activation domain of the TFs, causing the displacement of their regulators. On the other hand,

cyclin D1 can be recruited to chromatin and act as co-activator/ co-repressor, changing the conformation of the TFs. Nuclear receptors, developmental transcription factors and chromatin remodeling proteins are the main targets of cyclin D1 regulatory binding.

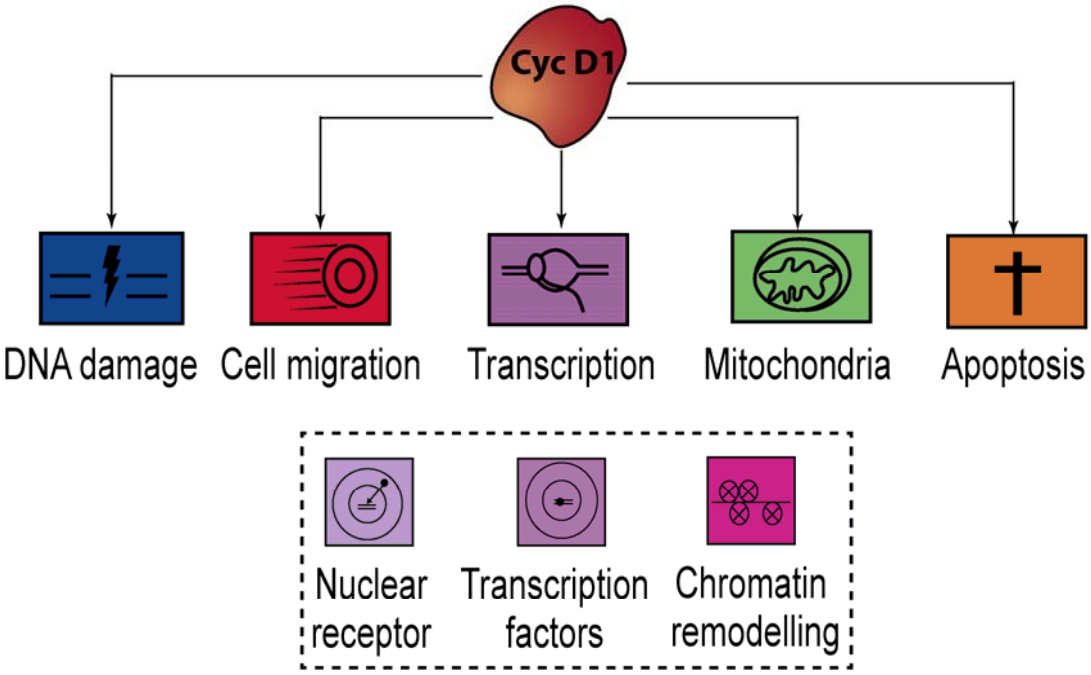


Figure 10. Cyclin D1 participates in the regulation of non-canonical pathways. Cyclin D1 can bind many different proteins and regulate DNA damage, cell migration, transcription, bioenergetics and inhibition of apoptosis. These functions can be dependent or independent to its binding to CDK4/6. Regarding transcription regulation, cyclin D1 may interact with nuclear receptors, development transcription factors and chromatin remodeling proteins.

2.3.1.1 Nuclear hormone receptors

Nuclear receptors are signaling intracellular proteins that directly regulate transcription upon binding to its ligands. As they are not located in the membrane, ligands (usually hydrophobic hormones) must diffuse through cell membrane and induce a conformational change into the nuclear receptor in the cytoplasm, which in turn leads to the re-localization of the nuclear receptor into the nucleus.

One of the best studied interactions of cyclin D1 with nuclear hormone receptors is its binding to androgen receptor (AR). Cyclin D1 is able to interact *in vitro* with AR and the co-activator P/CAF. As a result of this interaction, P/CAF cannot activate AR-response transcription (Reutens et al., 2001). Strikingly, this inhibitory function of cyclin D1 may be related to cyclin levels, since P/CAF overexpression could rescue the activation on AR-responsive promoters despite cyclin D1 overexpression in certain promoters. (Knudsen et al., 1999; Petre et al., 2002). Thyroid hormone receptor also suffers cyclin D1 induced repression caused by the direct binding of cyclin D1 (Lin et al., 2002a).

Cyclin D1 may also activate the transcription capacity of nuclear hormone receptors. For example cyclin D1 positively modulates the activity of estrogen receptor (ER) through its binding to coactivators SRC1 and AIB1. The interaction of Cyclin D1 with

P/CAF leads to ER activation (McMahon et al., 1999). Interestingly, cyclin D1 can activate ER binding even in the absence of estrogens, suggesting a way to substitute the pro-survival signals of this pathway in pathological conditions (Zwijsen et al., 1997). Cyclin D1 activation of ER signaling is independent of CDK4 (Neuman et al., 1997)

2.3.1.2 Chromatin remodeling enzymes

Acetylation and methylation of histones by histone acetyltransferases and histone deacetylases (HDAC) are critical to determine the activation status of chromatin regions. Cyclin D1 may impact on the pattern of histone modification at certain regulated promoters (Hulit et al., 2004). As we described above, it physically interacts with P/CAF, a chromatin remodeling protein, participating in the activation of nuclear receptors (McMahon et al., 1999). In addition, cyclin D1 seems to interact with p300 and inhibits its methyl-transferase activity (Fu et al., 2005c). However, this interaction could not be validated in cyclin D1 overexpressing cell lines(Zwijsen et al., 1998). Transcription repression of certain promoters also occurs through the interaction of cyclin D1 with PRMT5 promoting the activation of its methyltransferase activity (Aggarwal et al., 2010). Cyclin D1 also activates HDAC repression by a direct binding, like the recruitment of HDAC3 to PPARγ-bound promoters (Fu et al., 2005b). It has been reported that cyclin D1 can bind ER and NcoA/SRC1A, a ER cofactor participating in the recruitment of chromatin remodelers, at the same time (Zwijsen et al., 1997). This opens the possibility of cyclin D1 recruiting specific cofactors to TFs.

Taking all the available data together, it seems that cyclin D1 regulation of chromatin activity may be explained according to two different models (Coqueret, 2002). First of all, cyclin D1 can regulate transcription occluding the activation domain of the TFs, like in the case of AR. In excess, cyclin D1 may titrate the regulators away from the promoters. Secondly, cyclin D1 can directly recruit coactivators or corepressors, like in the case of HDAC3 recruitment to promoters or NcoA/SRC1A binding to ER, what would explain activation of ER but repression of many other TFs. In this regard, some authors speculate that the accessibility of the C-terminal LLXXXL motif controls the regulation of transcription in this second way of acting (Figure 11) (Coqueret, 2002).

Despite the evidences of cyclin D1 regulation of histone modifications, many of these experiments have been just analyzed in certain models, the majority of them in transfected cells such as COS-7 or 293T and confirmed by GST-pulldown experiments. For this reason, it will be of interest to determine whether cyclin D1 can interact in normal and/or transformed cells.

2.3.1.3 Conventional transcription factors

More than twenty TFs have been described to be able to bind to cyclin D1. Strikingly, cyclin D1 induces transcription inhibition in the majority of these models. For example, cyclin D1 in CDK4 complexes sequesters MYOD and blocks its transcription program in skeletal myoblasts (Zhang et al., 1999). Cyclin D1 also blocks BETA2/NeuroD effects on the development program of small intestinal epithelium cells. However, some data suggests that this effect is not mediated by direct interaction, but through p300

modulation (Ratineau et al., 2002). Cyclin D1 also inhibits muscle differentiation inactivating the interaction between MEF and GRIP-1, also in a CDK4-dependent way (Lazaro et al., 2002). Cyclin D1 also interacts with INSM1, a transcription factor regulating BETA2/NeuroD, inhibiting its transcription activity and promoting the recruitment of HDACs (Liu et al., 2006). This interaction also participates in cell cycle arrest (Zhang et al., 2009). Overexpressed cyclin D1 binds to RUNX3 and blocks its transcription activity, through directly interfering with the interaction of the p300 activator (Iwatani et al., 2010).

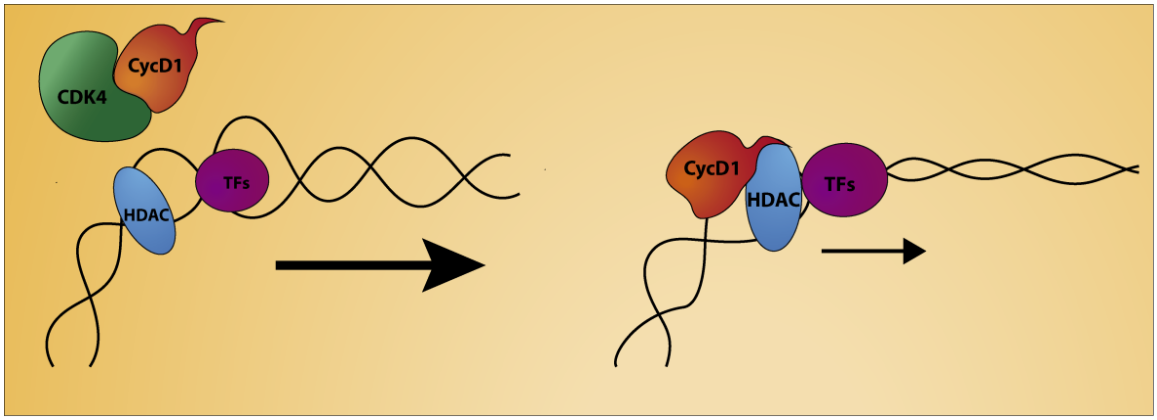


Figure 11. Cyclin D1 transcription repression model. Cyclin D1 may interact with HDAC proteins through its leucine-rich domain, independent of CDK4/6 binding. HDACs are recruited to promoters and inhibit transcription due to the activation of histone deacetylation or the donwregulation of specific TFs. (In the figure, active transcription is represented by black arrows.

Cyclin D1 can also participate in cell cycle regulation through direct regulation of transcription, independently of its binding to CDK4. For example, cyclin D1 interacts with Myb proteins, like B-Myb or DMP1(Horstmann et al., 2000a). In fact, expression of any D-type cyclin inhibits the transcription activity of DMP1 in a CDK-independent manner (Hirai and Sherr, 1996; Inoue and Sherr, 1998). Myb proteins participate in cell cycle regulation, but also regulate the expression of G1/S checkpoint proteins. It also binds and inhibits STAT3 upon interleukin-6 activation. This inhibition could be caused by blocking STAT3 p300-binding sites (Bienvenu et al., 2001).

In a study, Adnane and colleagues (1999) described that cyclin D1 represses Sp1-mediated transcription thanks to its interaction, both *in vivo* and *in vitro*, with a component of the transcription machinery, TAFII250(Adnane et al., 1999). It is one of the 10-12 TATA-binding protein (TBP) -associated factors that interacts with TBP and the TFIID complex on the promoter (Wassarman and Sauer, 2001). Binding of TFIID to a promoter is a prerequisite for the formation of the transcription initiation complex and the recruitment of RNA polymerase II. This TBP protein is the largest subunit of the TFIID complex and it is a component of the basal transcription machinery. Moreover, TAFII250 have kinase and histone acetyltransferase activities. This factor interacts with other TFs at the pre-initiation complex. Interestingly cyclin D1 has homology with one of TAFII250 interactors, the transcription factor, TFIIB. Although this binding is independent of CDKs, surprisingly overexpression of other cyclin D1 interactor proteins, like RB or CDK4, reduced these cyclin D1 transcription effects, giving strong evidence to the idea that the amount of free cyclin D1 is important in order to understand its non-catalytic roles.

Recent studies have preferentially evaluated the genome-wide transcription function of cyclin D1 rather than their specific function in a subset of promoters. To date, three chromatin immunoprecipitation studies (ChIP) have shown that cyclin D1 can bind an important number of promoters (Bienvenu et al., 2010; Casimiro et al., 2012; Casimiro et al., 2015). One of these reports showed that this chromatin binding was CDK-independent (Casimiro et al., 2015). These studies were conducted in genetically modified mice overexpressing cyclin D1, so there is a lack of studies investigating the binding pattern of endogenous cyclin D1 in human cells. A very recent study, the first in human stem cells, showed that cyclin D1 binds development genes, and cyclin D1 chromatin location change depending on the cell cycle phase (Pauklin et al., 2016).

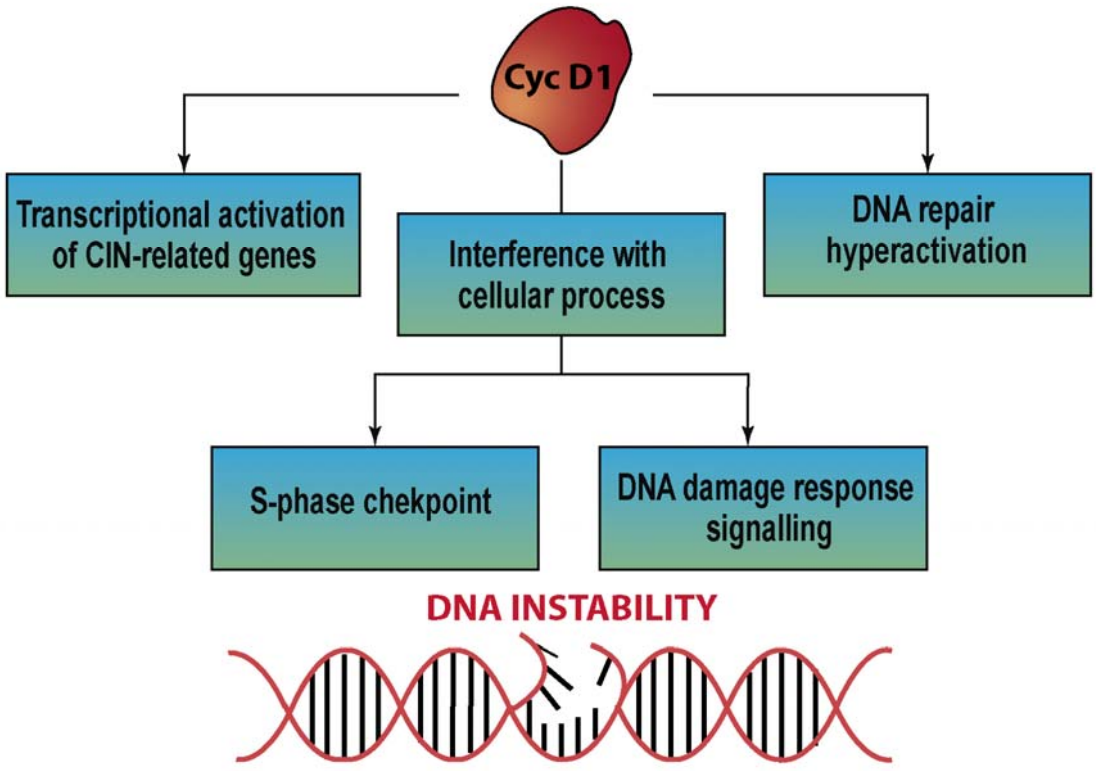


Figure 12. Main mechanisms described for cyclin D1-mediated genomic instability.

2.3.2 Cyclin D1 and chromosomal instability

Cyclin D1 and its relation with the DNA damage response (DDR) and chromosomal instability (CIN) have been investigated in several studies, although it remains unclear the contribution of cyclin D1 to these processes. Many manuscripts have reported that cyclin D1 can participate in the DDR and CIN (Figure 12). To interprete the experiments addressing the role of cyclin D1 in DDR and CIN should take into account the genetic background of the cell models, such as *TP53* mutational status.

First of all, Cylin D1 may regulate the transcription of CIN-related genes (Casimiro et al., 2012; Casimiro et al., 2015). In these studies, cyclin D1 directly dysregulate the expression of genes related to DNA damage and chromosomal instability, interfering in

the assembly of the mitotic spindle. The association between cyclin D1 and mitotic problems has been reported in other studies. (Nelsen et al., 2005)

Cyclin D1 may also cause genomic instability interfering into the normal progression of cell cycle. High cyclin D1 levels at G1 enables the transition through the G1/S checkpoint. On the other hand, cyclin D1 concentration increases during G2 and it is maximum in M phase in proliferating cells (Stacey, 2003) . Low mitogenic signals fail to preserve enough cyclin D1 concentration in G2, so cells exit cell cycle and become arrested. Nevertheless, multiple studies have shown cyclin D1 must be eliminated just after the entry to S-phase, in order to maintain chromosomal integrity (Figure 13) (Aggarwal et al., 2007) (Pontano et al., 2008). Constitutive expression of non-degradable cyclin D1 in mice splenocytes led to genetic abnormalities caused by rereplication, since Cdt1 was re-positioned on the chromatin restarting origin licensing before finishing S phase (Aggarwal et al., 2007). p53 pathway mediated the apoptosis of pre-malignant cells undergoing replication, but its abrogation in tumor cells highly increased DDR and tumor instability.

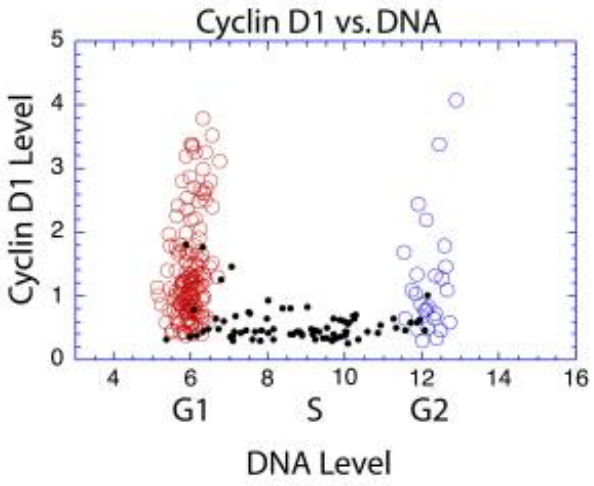


Figure 13. Cell cycle pattern representing Cyclin D1 levels through cell cycle. Cyclin D1 levels increase late G1 and G2/M phases. However, cyclin D1 is degradated during S-phase (Yang et al., 2006).

Different studies have reported that cyclin D1 overexpression causes DDR hyperactivation, in many studies as a consequence of inefficient/impaired response to damage. Strikingly, cyclin D1 downregulation upon DNA damage seems quite specific of cyclin D1, as cyclin D2 remains unperturbed after DNA damage or replication stress. (Pontano et al., 2008). It is not clear that cyclin D1 wt expression is able to activate basally DDR, although an incorrect maintenance of the pre-origin complexes upon cyclin D1 overexpression could be an explanation. Nevertheless, it is well known that cyclin D1 must be degraded in order to preserve genomic integrity. Inhibiting cyclin D1 degradation increases the number of chromosomal abnormalities and enhances DDR (Pontano et al., 2008). Consequently, non-degradable cyclin D1 causes pChk2 and γH2ax phosphorylation in pre-malignant and malignant lesions (Aggarwal et al., 2007) and chromosome breaks were detected in mouse splenocytes overexpressing cyclin D1 T286A and treated with genotoxic agents (Pontano et al., 2008). Cyclin D1 degradation occurred in impaired ATM or pChk2 signaling, so the ATM-Chk2 pathway

was not essential for the degradation of cyclin D1 upon DNA injuries (Pontano et al., 2008). These results are controversial with different reports showing cyclin D1 T286A colaboration with ATM and nuclear cyclin D1 in tumor formation (Vaites et al., 2014), as well as other reports that show both ATR and ATM can phosphorylate cyclin D1 to mark it for export (Hitomi et al., 2008). Further studies must clarify how cyclin D1 activates the double strand breaks DDR pathway and how it is regulated by ATM.

Some data supports the idea cyclin D1 may cause DDR activation through interfering with DNA replication, what may induce replication stress and, in turn, activate DDR. Pontano and colleagues (2008) proved that cyclin D1 degradation was not induced after aphidicolin-mediated DNA replication stress (Pontano et al., 2008). Supporting this idea, Shimura and colleagues (2013), using hepatocarcinoma and Hela cells, demonstrated cyclin D1 stabilization in long term irradiation-resistant cell lines led to fork collapse and basal DDR signaling (Shimura et al., 2013). Controversially, other studies silencing cyclin D1 in MCL cell lines showed that cyclin D1 removal also activates DDR signaling, suggesting a protective role of cyclin D1 in replication and DDR in MCL cell lines. However, these last results could reflect side effects of the oncogenic-addiction of MCL cells to cyclin D1 (Mohanty et al., 2017).In any case, further studies must be conducted in order to shed light on cyclin D1 role in normal and cancer B cells.

Cyclin D1 has laso an important role in DNA repair, although controversial results have been published. Cell lines overexpressing cyclin D1 showed enhanced apoptosis in response to γ irradiation (Coco Martin et al., 1999; Zhou et al., 2000) but cells resistance to ionizaing irradiation increases cyclin D1 concentrations as a protective mechanism (Shimura et al,2012; Shimura et al,2011). In 2011, a proteomic-profilling study found an interesting network of interactors centered on cyclin D1 and revealed an important role of cyclin D1 in DNA repair (Jirawatnotai et al., 2011). Cyclin D1 interacts with Rad51 and BRCA2 proteins, members of the homologous recombination (HR) pathway and enables effective DNA repair. This could be an explanation why cyclin D1 negative cell lines may be more sensitive to double strand breaks.

Recent publications have looked into further detail the cyclin D1 activation of DNA repair. For example, short cyclin D1 b isoform cannot be recruited effectively to DNA damaged foci, so isoform a elicit higher DNA damage signaling and enhances repair mechanisms (Li et al., 2010). Moreover, studies in prostate and breast cancer have shown that cyclin D1 DNA repair mechanisms can by activated by its interaction with AR and ER. Casimiro et colleagues (2016) claim DNA repair induced upon dihidrotestosterone incubation is mediated by cyclin D1. Moreover, binding cyclin D1 to chromatin is sufficient to induce DDR and increases DNA repair. However, their results also show how cyclin D1 negative prostate tumors have higher expression of DNA damage markers, indicating again the different effects cyclin D1 can induce in different tissues (Casimiro et al., 2016). Cyclin D1 binds both BRCA1 and ER preventing the inhibitory effect of BRCA1 on ER transcription in breast cancer cells (Wang et al., 2005). In addition, estradiol enhances DDR and DNA repair by cyclin D1 recruitment of RAD51 to damaged foci (Li et al., 2014).

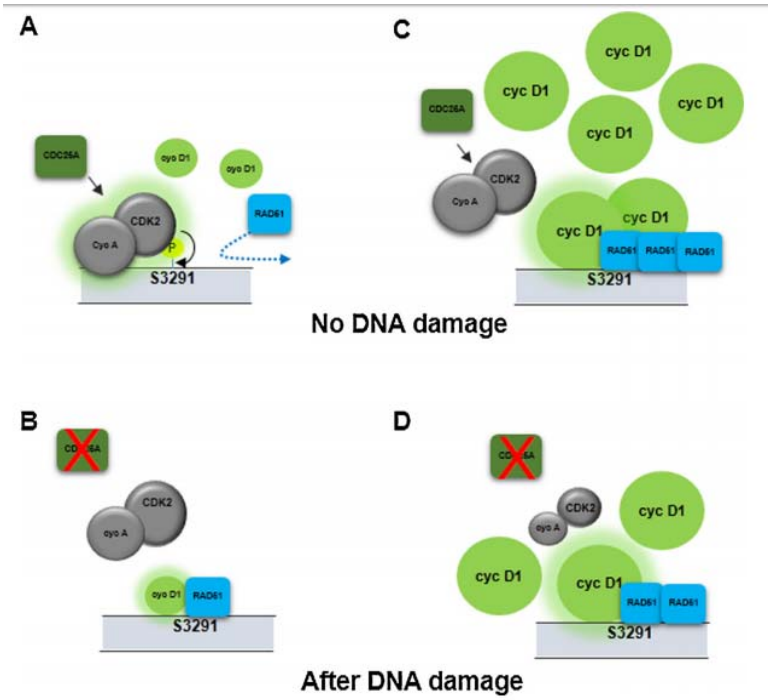


Figure 14. Cyclin D1 differential regulation of HR in normal (A,B) and cancer (C,D) cells. Normal cells prevents RAD51 binding to BRCA2 by the phosphorylation of serine 3291 by CDK2(A). However, DNA damage inactivates CDC25 and, subsequently, CDK2 kinase activity, so RAD51 can bind free unphosphorylated BRCA2, helped by low levels of cyclin D1 (B). However, overexpressing cyclin D1 activate basal recruitment of RAD51 to BRCA2 and HR even in the absence of DNA damage (C). Damaged cancer cells can present a hyperactivated response caused by cyclin D1 overexpression (D) (Jirawatnotai and Sittithumcharee, 2016)

Altogether, these controversial results may rise from an oversimplification of the model. Recent theories hypothesize cyclin D1 cellular concentration and type of genotoxyc agent may have important nuances (Figure 14). For example, high doses of DNA damage cause sharply decreased of cyclin D1 concentration. However, low DNA damage hardly decreases its concentration (Shimura et al., 2013). In fact, very low doses of DNA damage enhanced anti-apoptotic activities dependent on cyclin D1 (Ahmed et al., 2008). Nuclear low levels of cyclin D1 are related to increased DNA repair, especially HR. The regulation of the DDR and repair can be, thus, a balance controlled by the quantity of free cyclin D1.

2.3.3 Other biological functions

Cyclin D1 can modulate other pathways through directly binding different proteins. The apoptotic-direct effect of cyclin D1 has been observed in MCL cell lines and primary tumors, but it is poorly studied in other cancers. Cyclin D1 can sequester BAX, an anti-apoptotic protein, inhibiting its functions (Beltran et al., 2011). Cyclin D1 may also has a role in cell migration. Cyclin D1 may induce migration through its binds to Rho-GTP proteins. This non-canonical role, seems to be mediated through p27 and CDK4/6 complexes (Li et al., 2006). Similarly, other cyclins D have also been implicated in cell motility (Bendris et al., 2015).

The effect of cyclin D1 on mitochondrial size and activity is something well-established. However, only recently some of the main pathways and interacting proteins have been described (Pestell, 2013). Cyclin D1, associated with CDK4 complexes, regulates the transcription of NRF1, a crucial TFs in mitochondrial regulation and biogenesis. CDK4 - cyclin D1 complexes also bind NRF1 and inactivate its functions (Wang et al., 2006). Consequently, cyclin D1 positive cells present impaired mitochondrial biogenesis and, thus, they progress from oxidative metabolism to cytosolic metabolism. Moreover, cyclin D1 inhibits the supply of metabolites to perform correct electronic transport inside the mitochondrion since it can bind to a voltage-dependent anion channel competing with hexokinase 2, both in normal and cancer cells (Tchakarska et al., 2011).

2.4. Cyclin D1 and cancer

Among all the three D-type cyclins cyclin D1 is the only one frequently upregulated in cancer. For decades, researchers have considered the frequent cyclin D1 dysregulation in cancer as evidence that cyclin D1 can provide specific growth advantages to cancer cells that cannot be supplied by cyclin D2 and D3. Interestingly, cyclin D1 overexpression in cancer do not always correlate with increased proliferation or increased E2F-target genes expression (Ertel et al., 2010), suggesting that cyclin D1 may perform other roles in cancer biology different to increasing proliferation.

Indeed, decades of intense research have not led to a clear position about cyclin D1 and whether it does regulate proliferation in cancer. First of all, ectopic expression usually results in only modest accumulation of the cyclin D1 protein(Russell et al., 1999). In addition, cyclin D1 overexpression may induce apoptosis and senescence of several cell types (Han et al., 1996; Han et al., 1995). As indicated above, cyclin D1 functions as a weak oncogene that enhances cancer progression in combination with other oncogenes or after TSG removal in cultured cells (Bodrug et al., 1994; Opitz et al., 2002). Just few examples in the literature show that cyclin D1 overexpression could lead to cancer development. In these experiments, long latency and incomplete penetrance was observed (Wang et al., 1994).

Nevertheless,molecular and clinical data strongly evidence that cyclin D1 plays an essential role in different cancers, such as non-small lung carcinoma (Jin et al., 2001), breast carcinoma (Barnes and Gillett, 1998; Dickson et al., 1995) head and neck (Callender et al., 1994; Jares et al., 1994) and esophagus (Shamma et al., 2000). MCL and MM are paradigmatic examples of cyclin D1 overexpression caused by genomic translocations. In many of these tumors, cyclin D1 is frequently amplified and correlates with worse survival, In addition, in most cancer types including lung, breast, sarcoma, and colon cancer, cyclin D1 overexpression results from induction by oncogenic signals (Fu et al., 2004).These apparently controversial scenario about cyclin D1 role in cancer biology present compairing molecular and clinical evidence could be understood in the lights of its weak oncogenic characteristics.

2.4.1 Cyclin D1 and hematologic tumors

Cyclin D1, unlike D2 and D3, is negative or lowly expressed in normal hematological cells but it can be upregulated in different non-Hodgkin lymphomas, such as mantle cell

lymphoma, multiple myeloma (MM), chronic lymphocytic leukemia, diffuse large B-cell lymphoma, hairy cell leukemia and splenic marginal zone lymphoma (Cao et al., 2012; Pruneri et al., 2000). Among all these hematological neoplasms, the cyclin D1 dysregulation reaches high expression levels in MCL and MM, where it is overexpresed in the means of the translocation of CCND1 and the Ig gene, t(11;14).

Strikingly, MCL cells have additional genetic mechanisms to further increase cyclin D1 levels (Figure 15). For example,, secondary rearrangements can lead to amplification of the translocated allele in MCL cases (Bea et al., 2009). In addition, some MCL cases express shorter transcripts of cyclin D1, caused by secondary rearrangements occurring at 3' of the *CCND1* locus (Bosch et al., 1994; de Boer et al., 1995)or by genomic deletions and point mutations at the 3′UTR (Wiestner et al., 2007).These genetic alterations correlate with the presence of higher transcript levels. This is consistent with the fact that these shorter transcripts lack miR-16-1 binding sites in the 3′ UTR, resulting in escape of the miR-16-1-mediated down-regulation (Chen et al., 2008). Very recently, cyclin D1 mutations in the first exon have been related to a major stability of the protein (Mohanty et al., 2016). However, other mechanisms used by solid tumors to increase cyclin D1 are not detected in MCL cases, like the expression of the highly oncogenic b isoform that it is absent in primary MCL cases (Carrere et al., 2005), being cyclin D1a the isoform mostly expressed (Marzec et al., 2006).The relevance of these secondary genetic events that increase cyclin D1 quantity in LCM cells is reinforced by the fact that cyclin D1 mRNA leveles correlates with worse outcome and higher proliferation in LCM patients (Rosenwald et al., 2003). In thise sense, MCL cases with short cyclin D1 transcripts, with higher RNA stability, are more proliferating, more aggressive and accumulate *TP53* mutations (Rosenwald et al., 2003).
Cyclin D1 t(11;14) translocation is present in 15% of MM cases, although cyclin D1 overexpression is detected up to 40-50% of MM cases (Lesage et al., 2005). The other half of MM display increased cyclin D2 , so it is thought cyclins D are essential in MM pathogenesis. Initial studies described cyclin D1 expression in MM as a bad prognostic marker (Fonseca et al., 1998; Hoechtlen-Vollmar et al., 2000; Sonoki et al., 1999). However, some more recent claim the contrary: better prognostic and response to treatment (Fonseca et al., 2003; Moreau et al., 2002; Soverini et al., 2003). In contrast to MCL, the proliferative index correlates inversely to cyclin D1 expression (Fonseca et al., 2002; San Miguel et al., 1995). Therefore, the role of cyclin D1 in multiple myeloma pathogenesis or how it impacts on B-cell proliferation is something that remains unclear (Lesage et al., 2005).

These different results in MCL and MM question the role of cyclin D1 in enhancing B cell proliferation. In fact, it is still not clarified weather cyclin D1 overexpression in B cells leads to an increase in proliferation (Lesage et al., 2005). Cyclin D1-CDK4/6 can drive cell proliferation but cannot render cells competent for proliferation, so if cells have lost their replication potential, cyclin D1 will not be able to induce proliferation. MCL tumors usually present a broad range of concomitant abnormalities targeting CDK4/6-ciclin D1 pathway, such as *CDKN2A* deletion or *CDK4* amplification (Jares et al., 2007).These mutations may increase proliferative signals in tumor cells. Consequently, RB1 appears frequently hyperphosphorylated in MCL cases (Zukerberg et al., 1995), particularly in highly proliferative blastic variants (Jares

et al., 1996). Other hypotheses suggest these alterations target other cellular pathways different to the canonical axis. However, we also can imagine a scenario where cyclin D1 is not only affecting proliferation, but also other non-canonical functions. In this regard, concomitant alterations in cell cycle pathways may enhance proliferation in MCL progression. Up to date, these new functions of cyclin D1 have not been further elucidated in MCL.

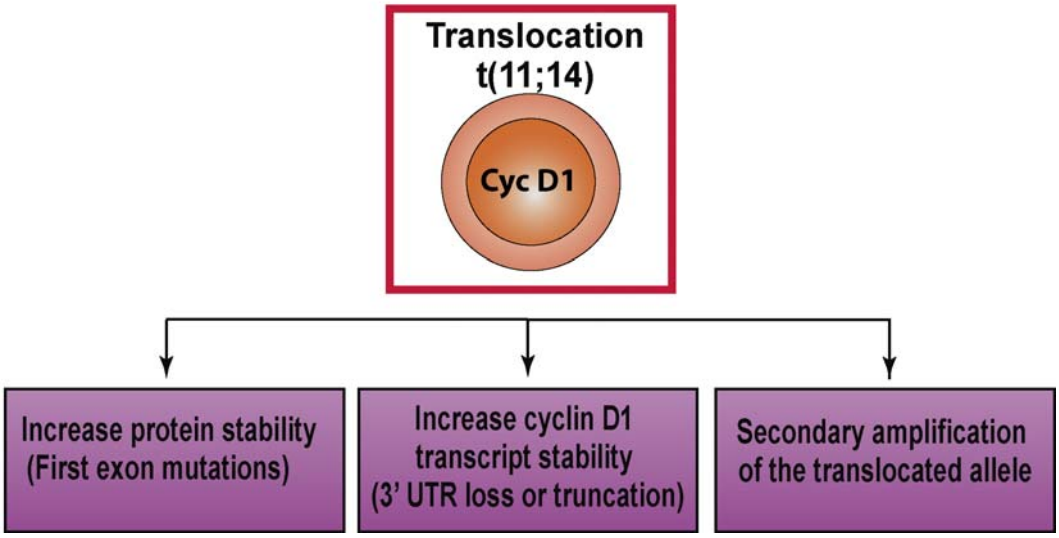


Figure 15. Genetic mechanisms enhancing Cyclin D1 levels in MCL beyond the upregulation produced by the translocation t(11;14).

2.4.2 Cyclin D1 dysregulation in solid tumors

Cyclin D1 is frequently dysregulated in different solid tumors through different mechanisms. To note, cyclin D1 is the most common oncogene amplified in human cancers (ref de las mutaciones), especially in head and neck carcinoma, pituitary tumors, esophaegal squamous cell carcinoma and breast cancer. (Dickson et al,1995). Impairment of post-translational mechanisms controlling cyclin D1 nuclear activity are also frequently present in a high number of solid tumors (Bartkova et al., 1994a; Bartkova et al., 1994b; Dickson et al., 1995). In this regard, the most important alterations occur in the degradation pathway, mediated by GSK3β. This kinase phoshophorylates the treonin 286 in the PEST domain, enabling cyclin D1 nuclear export and cytoplasmic ubiquitinization. Esopharyngeal and endometrial cancers present mutations in the PEST sequences, increasing nuclear cyclin D1 (Benzeno et al., 2006). Mutations in ubiquitin ligases participiating in cyclin D1 degradation have also been reported in some tumors (Barbash et al., 2008).Eventually, all these mechanisms target cyclin D1 nuclear export and degradation, incrementing stability of the protein and its nuclear localization.

In addition to genetic alterations, several pathways dysregulated in cancer lead to cyclin D1 overexpression/upregulation, notably RAS-MEK-ERK and PI3K-AKT-mTOR pathways (Musgrove, 2006) but also NF-kB and WNT pathways (Musgrove et al., 2011).Some examples of tumors in which dysregulated pathways give rise to cyclin D1 overexpression are head and neck, non-small-cell lung, endometrial, melanoma,

pancreatic, breast and colorectal cancers (Musgrove et al., 2011).Some microRNAs that participate in cyclin D1 mRNA instability, are often downregulated in prostate and lung cancer. Indeed, the genomic location where they are encoded appears recurrently lost in different tumors (Bandi et al., 2009; Bonci et al., 2008)

In addition, some polimorphisms contribute to differential splicing between cyclin D1a and cyclin D1b isoforms. The G/A polymorphisms occurring at the nucleotide position 870 hinders normal splicing and increase cyclin D1b (Betticher et al., 1995).This truncated form of cyclin D1 has been linked to higher oncogenity (Lu et al., 2003) and, consequently, this polymorphism is associated with an increased risk of tumor onset including lung cancer, colon cancer, and other cancer types (Kiyohara et al., 2002; Yaylim-Eraltan et al., 2010). As depicted before, isoform b lack the residue involved in nuclear export , increasing cyclin D1 stability.

3. Stress response pathways: enabling characteristics of human oncogenesis

Nearly two decades ago, Hanahan and Weinberg published a seminal paper proposing that cancer cells share six common "hallmarks" or biological attributes that govern the transformation of normal cells into cancerous cells. These attributes include the capacity to produce their own mitogenic signals, to be refractory to exogenous antiproliferative signals, to elude apoptosis, to foster angiogenesis, to promote invasion and metastasis and enable replication immortality (Hanahan and Weinberg, 2000).This work has been cited more than 15700 times and has marked a milestone in the comprehension of cancer. The "hallmarks of cancer" are acquired during the multistep development process of human tumors due to the accumulation of genetic and epigenetic) alterations that occurs during tumors progression.

A decade after, the authors revisited the concept of "hallmarks of cancer" in a new publication. Interestingly, this paper stressed the idea that cancer cells require some enabling characteristics or phenotypes that facilitate the acquisition of these capabilities or hallmarks (Hanahan and Weinberg, 2011), (Figure 16). One of the most important enabling characteristic suggested by the authors was the “Genome instability and mutation acquisitions” (Hanahan and Weinberg, 2000, 2011). The current model of genotype selection and clonal expansion places genome instability as a critical force for cancer progression (Gorgoulis et al., 2005; Halazonetis et al., 2008). Although genome alterations can vary dramatically between cancers, genomic instability appears inherent to the great majority of tumors. Indeed, cancers often develop mechanisms to increase mutability and/or,DNA damage tolerance in order to cancer progression.

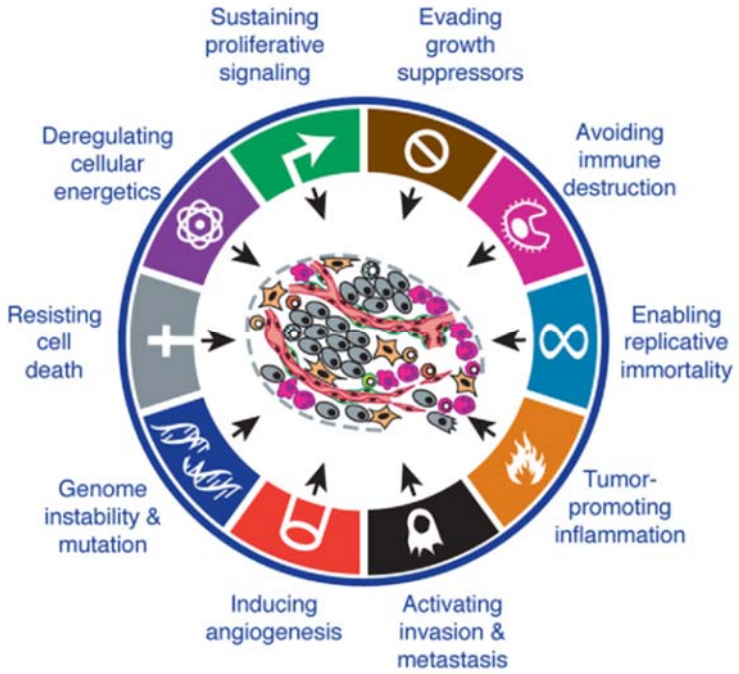


Figure 16. Hallmarks and enabling characteristics of human oncogenesis (Hanahan and Weinberg, 2011)

This chapter aims to give a general overview of the most remarkable circuits that cope with genomic instability and protect DNA integrity. In addition, we highlight the basis of replication stress and transcription stress. Failure in the regulation of these mechanisms or in the response to these stresses may induce DNA damage and promote genomic instability.

3.1 DNA damage response

The preservation of genomic integrity is crucial for the development, homeostasis, and survival of all organisms, acting also as a barrier against tumorigenesis. Genomic insults are, however, continuously inflicted on cells. DNA lesions may affect crucial physiological processes such as transcription and replication, be cytotoxic or result in gene mutations and genomic instability.(Ciccia and Elledge, 2010) (Jackson and Bartek, 2009)

Cells are endowed with a complex signaling pathway known as the DNA damage response (DDR) that helps them to cope with and respond to DNA lesions and thereby maintain genomic stability(Ciccia and Elledge, 2010; Giglia-Mari et al., 2011; Jackson and Bartek, 2009) .For this purpose, DDR encompasses different essential steps. Firstly, DDR may arrest cell cycle progression(Jackson and Bartek, 2009). Secondly, it activates the DNA repair mechanisms, which mediate the removal of specific DNA injuries (Giglia-Mari et al., 2011) . Thirdly, DDR can activate DNA damage tolerance processes, which allow cells to overcome persisting lesions in the absence of their repair(Lehmann, 2006). Lastly, DDR can regulate cell death and cell senescence, which selectively depletes or arrests damaged cells when severe DNA damage is not effectively repaired (Bernstein et al., 2002). Cancer cells often inactivate caretaker genes involved in DDR, enhancing mutability and enabling cancer progression

(Jackson and Bartek, 2009; Kastan, 2008; Sigal and Rotter, 2000)(Ciccia and Elledge, 2010; Harper and Elledge, 2007; Jackson and Bartek, 2009).

3.1.1 Sources of DNA damage

DNA damaging agents tend to be classified according to their origin: exogenously-derived or endogenously-generated. Exogenous agents may come from physical or chemical sources. Ionizing radiation (IR) or ultraviolet light (UV) are the most remarkable physical genotoxycs. Chemical agents that damage the DNA of proliferating cells are frequently used in cancer chemotherapy. This vast group consists of a myriad of compounds that can alkylate DNA, crosslink DNA helix or impair DNA replication by inhibiting topoisomerases, for instance (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). The damaging nature caused by the agent over the DNA double helix may correspond to single strand breaks (SSBs) or double strand breaks (DSBs). Human conditions characterized by defective response to DNA damage frequently exhibit congenital defects even in the absence of exposure to exogenous sources of DNA damage (Kerzendorfer and O'Driscoll, 2009). This indicates that the load of endogenously generated DNA damage, arised from normal cellular processes, is significant and, with non appropriate removal, it can adversely impair healthy physiology.
Endogenous-generated DNA damage is often split up into two major groups : metabolic-derived or stress-derived. Metabolic-derived DNA damage comes from the reactivity of the biomolecules that form living organisms. First, enzymes can make mistakes like dNTP misincorporation during DNA replication. Secondly, normal oxidative reactions like the ones taking place in peroxysomes can generate reactive oxygen species that can react with DNA and cause injuries. Moreover, nucleotide metabolism can introduce mutations after deamination of DNA bases or loss of DNA bases following DNA depurination and nucleotide alkylation (Ciccia and Elledge, 2010; Lindahl and Barnes, 2000).

Stress-derived DNA damage is produced when cells fail to resolve abnormal situations that compromise the integrity of the cell. Stresses may come from either external (like inhibitors of proteasome) or internal (like oncogene overexpression) sources. Cells have developed a complex network of proteins in order to overcome these potentially harmful situations, assuring DNA integrity (Luo et al., 2009). As stresses are collateral effects of deregulated process, tumor cells are under more stressful conditions compared to normal (Luo et al., 2009) .Although stresses do not initiate tumorogenesis, they participates in cancer progression. Oncogenic deregulation may induce re-wiring of many processes together with the loss of normal gene regulation, what in turn leads to augmented cellular stress (Nagel et al., 2016). Consequently, cancer cells rely on extensive adaptations to this “stress phenotype” in different cellular processes that are themselves not oncogenic. In particular, cancer cells can over-activate normal pathways to reduce certain stresses or to overcome cycle checkpoints, sometimes displaying strong dependence on these mechanisms.

3.1.2 Components of the DNA damage response

Last decade has shed light on many different pathways activated by DNA lesions. Based on the fact that repair mechanisms must be adapted to the nature of the genetic insult, a complex network of proteins play different roles, interconnecting DDR with cell cycle, apoptosis, senescence and transcription. A simple view of the DDR would distinguish two main axes: Mre11-Rad50-Nbs1 (MRN) /ATM/CHK2 and RPA/ATR/CHK1 (Figure 17). These groups represent a conceptual, rather than real, separation: both have important crosstalks and they share mediators and final effectors (Bartek and Lukas, 2003; Stracker et al., 2009; Zaugg et al., 2007). Generally, MRN/ATM/CHK2 axis is associated with DSB detection, while RPA/ATR/CHK1 senses mainly SSBs. Both axes have proteins that act as DNA damage sensors, activating specific effectors adapted to the nature of the injury. DDR mediators amplify the initial signal and send the signal to final effectors.

3.1.2.1 DDR sensors and apical kinases ATM and ATR: when the alarm goes off

The initiation of the signaling in the DDR cascade depends on two initial types of proteins: sensors and apical kinases. DNA damage sensors are proteins that bind to a particular DNA injury and activate the apical kinases. Apical kinases are proteins upstream the signaling process whose kinase activity begins the activation of different DDR components. Upon DSBs, the sensor complex MRN is recruited to the lesion. This complex helps to the correct localization of the apical kinase ATM to DSBs and its activation (Lee and Paull, 2005; Uziel et al., 2003) Before being recruited to DSBs, ATM forms inactive dimmers, but it undergoes autophosphorylation and dimmer dissociation upon DNA damage signals (Bakkenist and Kastan, 2003). MRN complex is more than a scaffold for ATM assembly, is also a substrate of ATM whose phosphorylation is important for downstream signaling, participating in the recruitment of other substrates. Although ATM is not essential for cell survival, individuals with ATM axis defects have severe conditions and increased genomic instability (Brown and Baltimore, 2003; Cortez et al., 2001).

Although less dramatic for cell survival than DSBs, SSBs can also compromise genome integrity. These lesions leave free ssDNA that can be recognized by specific sensors in order to deal with the injury. Cells experience these lesions more frequently than DSBs, because intrinsic replication defects during unperturbed S phase may block polymerases and cause SSBs lesions . Problems during normal replication causing ssDNA exposition are sensed by the RPA/ATR/CHK1 axis. Consequently, these proteins are not only components of DDR, but also important controllers of cell cycle progression and DNA replication.

ssDNA is recognized and coated by RPA protein, so this protein is generally considered the DDR sensor in this pathway. RPA-coated ssDNA is also important for the localization of the apical kinase ATR to sites of DNA damage (Zou and Elledge, 2003). ATR recognition of RPA–ssDNA depends on another protein, called ATRIP (Cortez et al., 2001)·Biochemical studies indicate that ATRIP binds RPA directly (Ball et al., 2007) and that it is a obligatory subunit for ATR activity(Cimprich and Cortez, 2008). In this regard, ATR needs a multiprotein complex including different proteins

such TopBP1, RAD17 or RAD9 in order to be fully active. As this pathway mediates with endogenous DNA injuries generated during unperturbed replication, RPA/ATR axis is essential for cell proliferation and defects on this pathway lead to early embryonic lethality in mouse and cell lethality in human cells (Brown and Baltimore, 2003; Cortez et al., 2001).

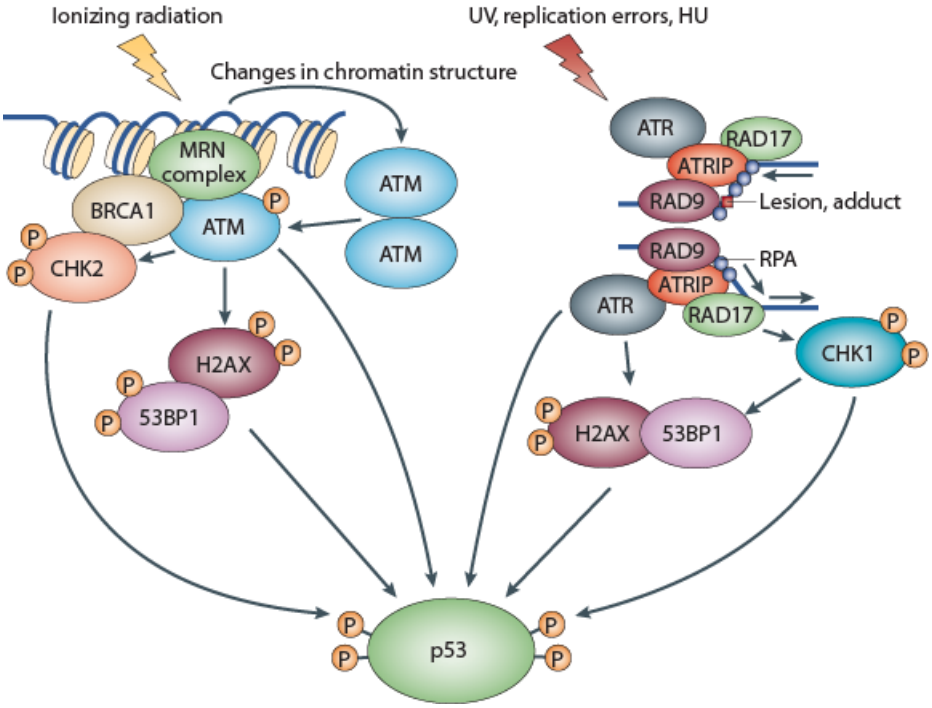


Figure 17. Schematic representation of the ATM and ATR signaling axis in the DNA damage response converging in p53 activation(Modified from (Sengupta and Harris, 2005))

Crosstalks are quite common in DDR signaling. Although ssDNA appear to be the key structure that elicits the ATR response (Zhou and Elledge, 2000), DSBs activate both ATM and ATR kinases. This is because some repair mechanisms activated by ATM, like homologous recombination, require ssDNA ends to search for homologous sequences. In this regard, there are many evidences that not repaired SSBs also generate DSBs (Casper et al., 2002; McNees et al., 2010).Although the mechanism is not fully understood, it is thought that SSBs cause replication fork stalling and it may collapse and provoke DSBs. Consequently, ATR and ATM axes are often activated together independently of the kind of lesion that initiates the DDR.

3.1.2.2 Mediators and secondary kinases: interconnecting DDR to cell physiology

Activation of the apical kinases leads to the phosphorylation of a set of proteins that participate in amplifying the DDR and spreading intracellular signaling, connecting different pathways (Zhou and Elledge, 2000).Some proteins participate in orchestrating DNA repair response, like BRCA1 (Tibbetts et al., 2000), while others are recruited to the lesion and regulate the assembling of DDR factors. As a consequence of their relocalization, mediators usually are seen as foci inside the nucleus after DNA damage induction. In addition, the phosphorylation of secondary kinases by apical kinases enables the eventual activation of the final effectors of the DDR. The principal effector

kinases of ATM and ATR are the checkpoint kinases CHK2 (Smith et al., 2010) and CHK1, respectively (Bartek and Lukas, 2003).

Upon activation by ATR or ATM , secondary kinases CHK1 and CHK2 are recruited to chromatin (Sancar et al., 2004; Smith et al., 2010)..Contrarily to other DDR components, secondary kinases are mobile proteins that transmits DDR signals to the whole cell, so its release from DNA lesions after activation is crucial (Bartek and Lukas, 2003). Although structurally unrelated, both kinases perform partially redundant roles, since they share plenty of final effector substrates. Some targets of these kinases are E2F1, MDM2, p53, Cdc25A or Cdc25B, and they regulate pathways such diverse as apoptosis, transcription, chromatin remodeling and, remarkably, cell cycle checkpoints (Bartek et al., 2001; Bartek and Lukas, 2001; McGowan, 2002). Apart from its role in signaling, they can also mediate effector functions. For example, CHK1 contributes to DNA repair by recruiting the protein BRCA2 and RAD51 to DNA damage foci (Bahassi et al., 2008; Sorensen et al., 2005) or phosphorylating proteins of Fanconi anemia pathway (Wang et al., 2007).

It is interesting to note that alterations of the ATM–CHK2 and ATR–CHK1 pathways are asymmetrically distributed in cancers. On the one hand, frequent epigenetic or genetic defects on ATM or CHK2 lead to downregulation of these proteins in many different cancers (Ai et al., 2004; Haidar et al., 2000; Kim et al., 2009; Ripolles et al., 2006; Salimi et al., 2012; Seshagiri et al., 2012) . Alterations on a single element of the ATM-CHK2 axis cause little oncogenic transformation, but they have synergetic effects when secondary mutations appear (Barlow et al., 1996; Maclean et al., 2007; Vaites et al., 2014). Strikingly, some cell lines have increased levels of ATM or CHK2, suggesting more complex roles in stress release and DNA damage tolerance (Zoppoli et al., 2012) . On the other hand, ATR/CHK1 mutations in cancer cells are rare. Indeed, they are frequently upregulated in human neoplasms (Albiges et al., 2014; Sarmento et al., 2015; Verlinden et al., 2007; Wallace et al., 2014; Xu et al., 2013) and their expression often positively correlates with tumor grade and disease recurrence(Verlinden et al., 2007) . In fact, a transgenic mouse line carrying amplification of *CHK1* facilitates cell transformation (Wallace et al., 2014). In this sense, *ATR* suppression in adult mice decreased the development of MYC-induced lymphomas (Murga et al., 2011). Altogether, these experiments suggest that ATR-CHK1 axis plays an essential role preserving proliferation in cancer, probably enhancing the ability of cancer cells to deal with replication stress (Greenow et al., 2014; Kawasumi et al., 2011; Lam et al., 2004) . However, the importance of the ATR/CHK1 axis is far from being completely understood. For example, heterozygotic losses of ATR or CDHK1 had no effect on tumorogenesis. However, perturbations in the DDR such as *CHK2* deletion showed increased tumor susceptibility in ATR haploinsufficient mice (Niida et al., 2010). It is well-known that reduction of ATR/CHK1 expression leads to genomic instability by provoking rereplication and failure to inhibit mitotic entry (Lam et al., 2004). These results suggest that ATR and CHK1 may act as haploinsufficient tumor suppressors in specific genetic backgrounds (Bartek et al., 2012).Therefore, the genetic context of each cancer may determine the oncogenic/tumour suppressor role of ATR-CHK1 network.

Among other mediators activated by apical kinases, H2AX and 53BP1 play a crucial role locally at sites of DNA lesions. They are rapidly activated by ATM and ATR, although their roles are mostly studied upon ATM activation by DSBs. (Jowsey et al., 2007). Presumably, H2AX foci specifically attract repair factors, leading to higher concentration of repair proteins surrounding a DSB site (Kouzarides, 2007; Taverna et al., 2007). These suggest that H2AX acts as a molecular scaffold and an amplifier of the signal. However, H2AX also participates in chromatin remodeling, preventing physical dissociation of break ends. Besides, it may directly be a signal for recruitment of recombination and repair factors, like cohesins (Podhorecka et al., 2010).

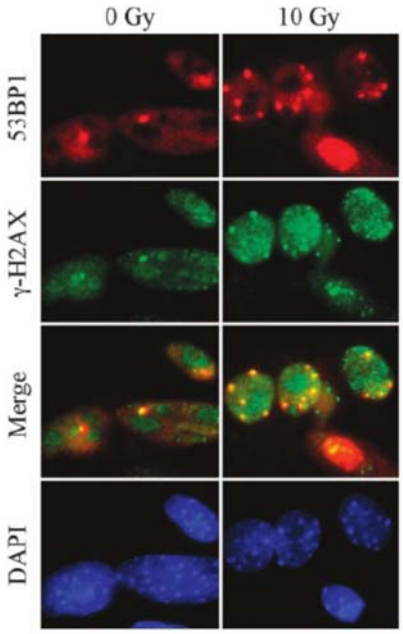


Figure 18. 53BP1 and yH2AX foci colocalize, in cell nucleous and they increase upon DNA damage causing DSBs. Foci quantification is a typical way to analyze DNA damage and repair efficiency (Holcomb et al,2008).

53BP1 role upon activation by ATR or ATM, on the contrary, remains less characterized (Jowsey et al., 2007). Cells lacking 53BP1 can initiate DDR after some genotoxic agents, but it seems important in the amplification of the ATM signal after exposure to lower genotoxic doses. However, strong DNA damage leaves 53BP1 as a dispensable factor. In addition, other studies show that 53BP1 deletion may increase genomic instability (Fernandez-Capetillo et al., 2002; Wang et al., 2002). Some studies provide strong evidence that H2AX and 53BP1 are not only downstream elements of apical kinases, but also essential components of the active ATM complex and participate in the phosphorylation of ATM-dependent cell cycle checkpoints. This would not contradict the chronological activation of the DDR proteins studied above; rather it exemplifies the different crosstalks between sensors and mediators in DDR signaling (Zgheib et al., 2005). In fact, DDR may rely on incrementing the concentration of activated proteins in foci, in order to let the signal reach a certain threshold. This has led to the broadly use of 53BP1 and γH2AX foci as surrogate of DSB and DDR activation (Figure 18)(Shibata et al., 2010).

3.1.2.3 The effectors: decision makers of the DDR

Many different proteins can act as DDR effectors and be activated by the DDR signaling pathways. Effectors not only control cell cycle checkpoints, DNA replication, DNA repair and transcription, but also they connect DDR with insulin signaling, RNA splicing, nonsense mediated decay or the mitotic spindle assembly and its checkpoint (Harper and Elledge, 2007).

As depicted before, CHK1 can be activated by ATR upon replication stress during perturbed or unperturbed S phase. Consequently, CHK1 have a crucial role intercommunicating DDR and cell cycle, participating in the replication checkpoint and the G2-M checkpoint. It develops these functions mainly targeting the CDC25 family of phosphatases: CDC25A (G1/S and S transition) and CDC25B and CDC25C (G2/M transition) ((Mailand et al., 2000; Peng et al., 1997). CDC25 phosphatases are responsible for CDKs activation by removing the inhibitory phosphorylations at T14 and Y15 residues produced by Wee-1 protein kinase. Following DDR activation, CHK1 and CHK2 inactivate CDC25 by phosphorylation that will label the phosphatase for degradation or nuclear export (Lopez-Girona et al., 1999). As consequence the CDKs would remain inactive due to the T14/Y15 phosphorylation. Furthermore, CHK1 may phosphorylate and increase activity of Wee-1. This cascade of phosphorylation events determine a blockage of the cell cycle. (Figure 19)(Swaffer et al., 2016).

TP53 is a TSG with a unequivocal importance in oncogenesis. It is inactivated in more than half of all sporadic tumors, and individuals with a germinal mutation of *TP53* show increased susceptibility to cancer , and mice with homozygote deletion of the gene develop tumors more frequently1, 2. During tumor development, a *TP53* mutation, either sporadic or inherited, is typically followed by loss of heterozygosity, which results in complete p53 deficiency (Bieging et al., 2014). In addition, tumors lacking p53 are commonly characterized by more malignant characteristics, such as a lack of cellular differentiation, genetic instability, and increased invasiveness and metastatic potential (Fearon and Vogelstein, 1990; Malkin et al., 1990; Mizuno et al., 2010). It coordinates such different pathways like angiogenesis, invasion, apoptosis, cell cycle, autophagy, chemoresistance and DNA repair (Sengupta and Harris, 2005). At the crossroads of different pathways, its regulation is very complex and not fully understood. p53 also plays a pivotal role in the DDR model as a main effector. DSBs activate the ATM/CHK2 axis, causing the downstream activation p53 protein via CHK2 phosphorylation. p53 controls the transcription of the CDK inhibitor p21, promoting cell cycle arrest. Besides, MDM2, a protein implicated in p53 degradation, becomes phosphorylated and inactivated by direct action of ATM and ATR (Cheng and Chen, 2010).This would promote p53 stabilization following ATR/CHK1 and ATM/CHK2 activation. Consequently, p53 accumulates upon different genotoxyc agents and endogenous stresses (Bieging et al., 2014). In addition, different levels of DDR signaling may lead to different effector functions of *TP53*. For example, low levels of DNA damage could provoke direct interaction of p53 with the repair machinery without p21 transcription. However, higher activation may overcome p53's ability to stimulate repair, so p53 begins the transactivation of specific genes that will counteract the effects of the genotoxic agent (Sengupta and Harris, 2005). Altogether, current evidences seem to indicate that p53 response seems to depend on subcelullar localization, cell phase

and/or the type, but also the duration and intensity of the damaging agent (Sengupta and Harris, 2005).

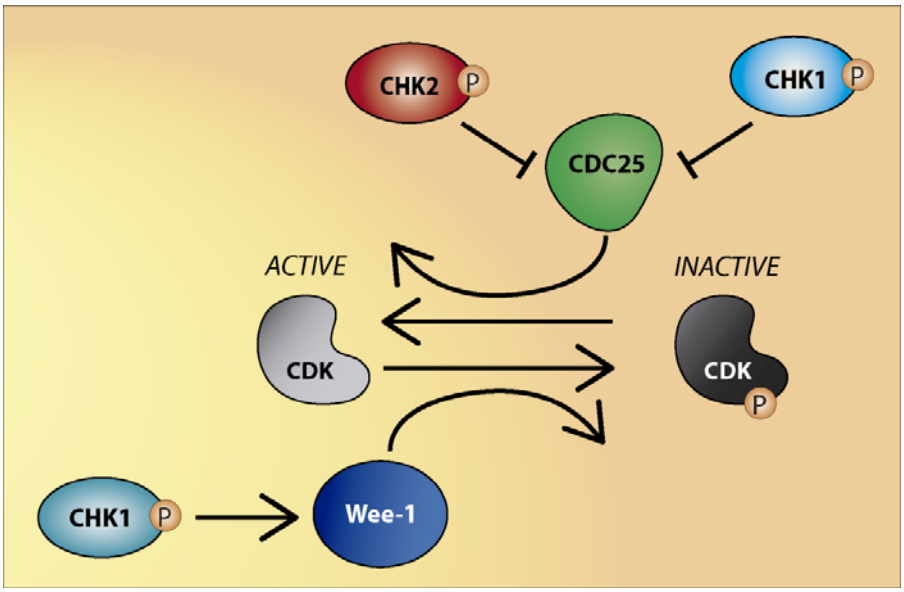


Figure 19. CDK proteins are phosphorylated by wee-1 in T14 and Y15 residues, inducing reversible inactivation. Cdc25 proteins activate CDK dephosphorylating these residues. Upon DDR, CHK1 and CHK2 proteins inhibit Cdc25 proteins. In addition, CHK1 activates Wee-1 kinase.

3.2 DNA Replication stress

The discovery of the mechanisms underlying DNA replication represented an important milestone in the XX century. Replication is more than copying during the S-phase more than 3200 million of base pairs that represent the human genome. DNA must be copied exactly only once and be assembled into chromatin ensuring inheritance of methylation and other epigenomic features. During this process, many different external and internal agents can hamper DNA replication determining a slow or stall of the replication forks producing DNA replication stress. This stress is a physiological challenging condition for the cell, since unresolved replication stress can lead to genomic instability (Carr and Lambert, 2013; Gaillard et al., 2015; Zeman and Cimprich, 2014).

3.2.1 Molecular basis of DNA replication initiation

In eukaryotes, the large size of the genome makes necessary the assembly of thousands of replication complexes through chromosomes in order to duplicate the whole genome during the duration of the S-phase. Even though time differs widely between cell types, a rapid rapidly proliferating human cell with a duplication time of 24 hours, S-phase lasts about 8 hours (GM., 2000). DNA replication is tightly regulated to ensure that genomic DNA is replicated once, and only once, in each cell cycle. This is achieved by partioning DNA replication into two temporally separated steps: the assembly of a double hexameric minichromosome maintenance (MCM) complex at replication origins (origin licensing) in the absence of CDK activity and then converting

these complexes to the active helicase in a CDK dependent way, which lead to open the DNA helix and start DNA replication (origin firing) (Machida et al., 2005; Takeda and Dutta, 2005) (Figure 20).

Replication origins are the chromosomic regions from where DNA replication initiates. In eukaryotes, they are not defined by a particular sequence, but DNA structures seem to determine their position (Machida et al., 2005; Takeda and Dutta, 2005). After mitosis, prereplication complexes are assembled onto replication origins. The main proteins forming the prereplication complex are ORC1-6 proteins, which recognize the origins and recruit Cdc6 and Cdt1 to end up with the licensing of origins by the loading of the hexamere Mcm2-7. Origin licensing is restricted to G1 phase because of the low CDK activity (since CDK activity degrades and/or inactivates Cdc6, Cdt1 and ORC proteins) and the absence or low expression of geminin, a licensing inhibitor normally expressed in S and G2 phases that sequesters and inactivates Cdt1(Machida et al., 2005; Takeda and Dutta, 2005).When CDK and DDK levels arise, the prereplication complex are converted to an active helicase complex by recruiting different proteins including CDC45 and GINS. Not all licensing origins will be triggered: it is estimated that only between one third and one tenth of them will be used in unperturbed S-phase. Those origins that will not participate in DNA replication are known as “dormant origins” (Blow et al., 2011). Dormant origins are passively replicated by oncoming forks and are not essential for normal S-phase progression (Woodward et al., 2006). However, they can be activated depending on the circumstances, so they are an effective way to complete replication under stressful conditions or when replication forks are stalled (McIntosh and Blow, 2012).

In addition, origin firing follows a controlled time-space pattern. The spacial pattern arises as a consequence of the fact that close replication origins tend to be fired together. As a result, eukaryotic DNA replication is characterized by the generation of replicon clusters, large chromosomic domains with 2-10 active origins replicating synchronically. These clusters are activated at different moments during S-phase, so we distinguish early origins and late origins. This replication timing is highly conserved in each cell type, changing during cell differentiation and in cancer cells. How this pattern is regulated and transmitted is still not known, although chromatin states (methylation patterns and histone codes) are thought to be closely related to this time-space coordination. In this sense, euchromatin is early replicated, while heterochromatin replicates in the last parts of S-phase. Some evidences even suggest the importance of replicating timing in the heritance of epigenetic marks (Lande-Diner et al., 2009).

3.2.2 Basic aspects of the replication stress response

DNA replication stress and its consequences/repercussions in cell homeostasis are still under intense study. Cells have developed surveillance systems in S-phase which cope with replication stress, interconnecting cell cycle, DNA replication and DDR: the intra S-phase checkpoint .Nowadays, the correct control of DNA replication and the integrity of the replication stress response (RSR) are considered one of the most important processes to prevent genetic instability (Myung et al., 2001; Myung and Kolodner, 2002).

3.2.2.1 Sources of DNA replication stress

Many different mechanisms can lead to DNA replication stres during normal S phase. (Figure 21). For example, conflicts between transcription and replication are also a main souce of replication stress, caused as a consequence of transcription stress (discussed below). Other less frequent situations such as misincorporation of ribonuclelotides, changes in nucleotide concentrations or the production of reactive oxygen species can also contribute to activate RSR. (Gaillard et al., 2015). Furthermore, particular DNA sequences and structural variations can lead to a local increment of replication stress, due to the fact that they are sequences that are difficult to replicate. Structural unrepaired lesions can be both the cause and the consequence of replication stress (ssDNA, DSBs, gaps…), since replication forks unavoidably stop over those lesions. In addition, unusual DNA secondary structures like Z DNA can result in lower replication speeds, increased formation of DSBs and deletions (Paeschke et al., 2013).Besides, some regions of the genome like fragile sites are more prone to suffer replication stress (Debatisse et al., 2012). Some recent studies have even found more DSB in heterochromatin regions, hypothesizing replication-stress may occur more severely in these areas (Zeman and Cimprich, 2014).

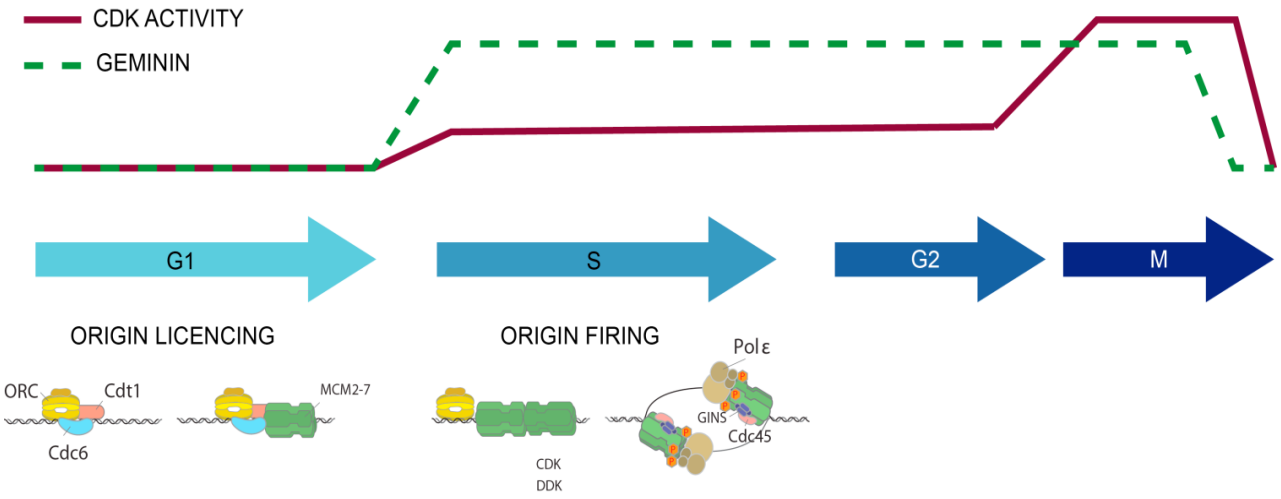


Figure 20. CDK and geminin activities control origin firing and origin licencing through the cell cycle, avoiding rereplication. (Modified from Hills and Diffley,2014)

However, one of the main causes of replication stress is the oncogene activation, what is known as oncogene-induced replication stress (OIRS). In fact, oncogene overexpression even in pre-malignant tissues often provokes RSR and DDR activation(Bartkova et al., 2005; Gorgoulis et al., 2005). Many evidences show that OIRS is not a passenger event, but has a role to play in early progression of many tumours (Hills and Diffley, 2014). Even single oncogenes can induce replication stress by different mechanisms depending on context. Thus, it is unlikely that a single type of replication stress defines any cancer and causes of replication stress might be quite dynamic during tumorigenesis (Hills and Diffley, 2014). Oncogenes often accelerate cell cycle and enable S phase entry with incomplete licensing and, as a consequence, OIRS is produced. Although not fully understood, the licensing checkpoint is overcome

by two main ways. First, CDK2 overactivation or Rb inactivation lead to an increase in E2F release and premature S phase entry. Secondly, p53 inactivation causes a failure in the inactivation of S phase entry upon low number of licensed origins.

First of all, increased CDK2 activity, in addition to drive uncontrolled proliferation, limits the low CDK "window" during G1 phase. As a result, cells enters to S-phase with a lower number of origins. Cyclin E overexpression is commonly found in different tumors (Strohmaier et al., 2001) and its overexpression causes a reduction in origin firing (Ekholm-Reed et al., 2004; Spruck et al., 1999) and increases fork stalling. Some evidences suggest that oncogenes such as HPV E7, KRAS and Myc also generate OIRS through licensing deregulation (Liu et al., 2009; Steckel et al., 2012; Zimmerman et al., 2013). Other situations that lead to low licensing are the reduction in pre-RC factors or increasing the levels of licensing inhibitors, as evidenced by mice displaying low levels of Mcm2 and Mcm4 (Pruitt et al., 2007; Shima et al., 2007).How reduced licensing generates DNA damage is still not fully understood, there are some theories. When cells have less licensed origins, replication forks have to cover greater distances and the probability to get stalled is increased. When it stalls, there are less dormant origins capable to rescue it, increasing the likelihood of fork collapsing. This may led cells to finish S phase without complete replication, what would cause chromosome breakage during the following mitosis (Burrell et al., 2013).

CDK2 hyperactivation also increases OIRS accumulation increasing the number of fired origins during S-phase, what is known as origin over-usage. Increased origin firing may exhaust the limited susbtrates required for replication, such as nucleotides or RPA protein (Hills and Diffley, 2014). In turn, this leads to reduced fork speed and an increased chance of fork stalling and collapsing. In addition, increased origin firing may augment the number of collisions between the transcription and replication machineries. Increased CDK activity, thus, causes late origins to fire early. Therefore, oncogenes suggestive to increase CDK activity would not only contribute to less licensed origins, but also to more origin firing. In this regard, Myc, Ras, HPV E6 and E7 contribute to increase replication stress via this mechanism. Interestingly, cyclin E is a good example of oncogene that induces OIRS on different ways. As depicted before, it decreases origin licensing, but it also increases the CDK activity during S-phase aberrantly, activating origin firing. As a third way to induce OIRS, current reports proven that cyclin E increased the number of collisions between transcription and replication machineries (Jones et al., 2013).

To finish, p53 inactivation or RB/E2F defects not only impairs the inhibition on G1/S transition in suboptimal conditions, but also favors inappropriate re-licensing of origins and, therefore, a origin re-usage that leads to rereplication. Overexpression of cdt1 can cause rereplication, but cdc6 and ORC overexpression are not producing this phenomenon. Interestingly, this is only seen in cancer cells, indicating that normal cells have redundant mechanisms to block rereplication that are lost in cancer cells (Tatsumi et al., 2006; Vaziri et al., 2003). These works show that rereplication causes chromosomal damage, although it is not explain the exact mechanism. It is though that rereplication increases the head to tail collisions of replisomes, deregulate origin firing and increase fork collapse. In addition, it can increase the number of replication forks during S –phase in a similar way that origin over-usage. Although rereplication is not in

the strict sense replication stress, it may sensitize the cell to other the sources of replication stress(Zeman and Cimprich, 2014). Taking everything into account, licensing factors are usually upregulated in tumors and proteins such as cdc6 or cdt1 are considered as oncogenes (Pinyol et al., 2006; Xouri et al., 2004). As many pre-RC factor encoding genes are E2F targets (Tsantoulis and Gorgoulis, 2005), a dysregulation of RB/E2F pathway may lead to rereplication. Some oncogenes causing rereplication are MYC, cyclin D1 or cyclin E (Fujita, 2006; Srinivasan et al., 2013).

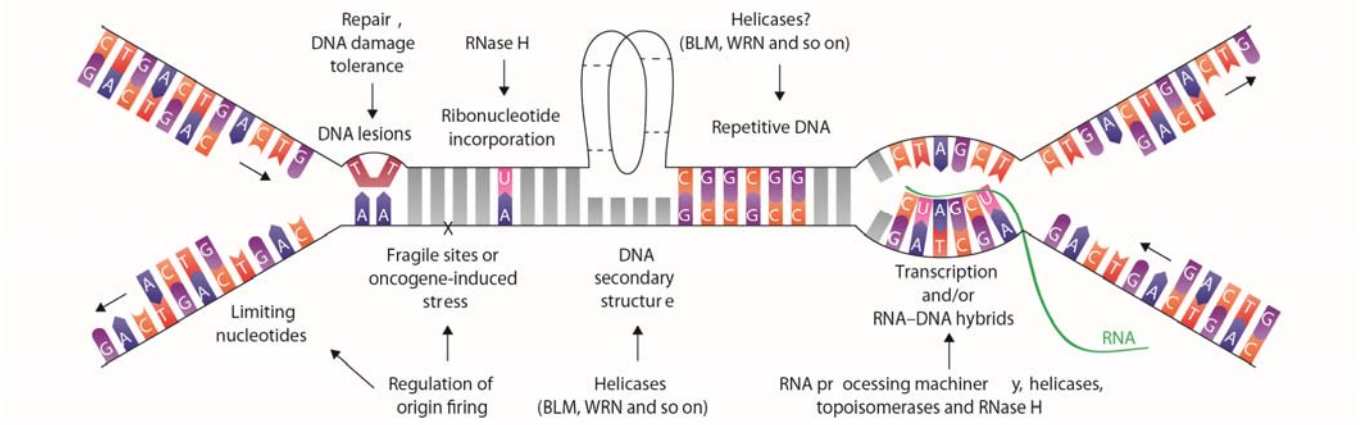


Figure 21. Replication stress can be generated from many different sources. Oncogenic-replication stress participates in some of them, such as dysregulation of origin firing, or increasing DNA damage tolerance (Zeman and Cimprich, 2014)

Finally, other mechanisms that are not related to origin dynamics have been studied in OIRS generation. Replication fork stalling can be mediated by direct interaction of oncogenic proteins with proteins participating in replication, like the inhibition of BCL-2 oncogene on the nucleotide metabolism (Xie et al., 2014). Alternatively, oncogenes may deregulate metabolism and increase the accumulation of reactive oxygen species, which in turn provockes DNA damage with the potential to impair replication. MYC overexpression, for example, causes an increase in DNA injuries through this mechanism (Maya-Mendoza et al., 2015).

3.2.2.2 Replication stress response: the intra S-phase checkpoints

The cells in order to cope with the constant challenge of replication stress have evolved the intra S-phase checkpoint (also called replication checkpoint). This checkpoint is activated when replisomes become stalled, independently the cause of its blocking. This replication checkpoint aims to protect the integrity of stalled forks, avoiding aberrant recombination (Alabert et al., 2009; Boddy et al., 2003), removing chromosomal torsional stress (Bermejo et al., 2011) and allowing the recovery from the stress condition. Moreover, the intra-S checkpoint reduce the rate of DNA replication by inhibiting the origin firing (Santocanale and Diffley, 1998; Shirahige et al., 1998; Zegerman and Diffley, 2010). Although mainly studied in yeast, in mammals the suggested models enrolls ATR and its partners directly in inhibiting origin firing regulating CDK and Dbf4-dependent kinase (DDK) activities. ATR helps stabilize and restart the stalled fork, and suppress recombination. Nevertheless, severe stresses can

reconvert this blockage into SSB, which in turn activates DDR through the classical RPA/ATR/CHK1 axis and final activation of p53(Nyberg et al., 2002).

Genotoxic stresses during DNA replication only delay progression through S phase in a transient manner. The presence of not repaired damaged DNA would cause cell arrest later on, in G2 checkpoint. During S-phase, three different checkpoints can be activated: replication checkpoint, S/M checkpoint and the replication-independent checkpoint(Bartek et al., 2004). When problems arise in replication forks are detected such as reduced speed fork or exhausting of replication components, ATR/CHK1 pathway is activated and starts a signaling cascade ending up in the inhibition of late origin firing. When replication forks cannot be rescued and reactivated or intense replication stress is affecting the cell, cells activate dormant origins that would allow replication of DNA fragments located between stalled forks. More extreme replication stress forces replication machinery to restart replication downstream of a DNA lesion leaving behind an ssDNA gap. These gaps, detected by the DDR, have to be repaired using specialized polymerases. Despite all mechanisms enumerated before, RSR can fail stabilizing and restarting stalled forks, especially if replication stress persists. It is then when we talk about “fork collapse”: the loss of replication proteins attached to the DNA. Last studies point out that some proteins can remain, but they are not fully functional or properly positioned. The main problem of fork collapse is that they likely evolve to DSB at the stalled fork, although it is not clear how stalled forks end up generating DSBs.

A second checkpoint that can be activated in S-phase is the S-M chekpoint. ATR and cyclin B-CDK1 complex are beneath the molecular control of this checkpoint, although some results are controversial (Eykelenboom et al., 2013) . It aims to preserve the genomic integrity, avoiding mitosis before completing replication. (Eykelenboom et al., 2013) Contrarily to G2/M checkpoint, S-M checkpoint is activated by ATR, is not sensing DNA lesions and does not halt cell cycle progression, but it decreases S-phase speed up to completion of DNA duplication. To finish, there is a third mechanism called replication-independent intra-S-phase checkpoint. This mechanism is on charge of sensing DSBs occurring during S-phase outside the active replicons. ATM essentially regulates this pathway, delaying S-phase progression and decreasing origin firing through the activation of ATM/CHK2 axis. Contrarily to the other checkpoints, this is only induced by DSBs and, besides, it does not require active replication forks for its activation (Bartek et al., 2004).

To conclude, the activation of these mechanisms has been implied in genome instability. For example, ssDNA gaps caused as a consequence of fork rescuing through DNA injuries give rise to SSBs (Petermann and Helleday, 2010).DSBs coming from fork collapse can be detected by ATM pathway. In any case, the main mechanism repairing these injuries is HR. As noted, HR can use the correct homologous region as template, but it can provoke genetic aberrations by failure in recombination. ATM deficiency or components of HR repair pathway also increase DNA instability. However, the loss of RSR proteins also leads to increase DNA instability and higher replication stress. As it seems, it must exist a balance between RSR response, DDR mechanisms and DNA repair that, if dysregulated, may be a key factor in understanding human tumorogenesis (Karakaidos et al., 2004; Shima et al., 2007).

3.3 Transcription stress

Transcription is a cellular process that encompasses the copy of the DNA to RNA by enzymes called RNA polymerases (Pol) (Cai et al., 2004; Lee et al., 2004; Wild and Cramer, 2012). Eukaryotic genome is transcribed by thee RNA Pol enzymes. Pol I transcribes the ribosomic RNA precursor, while Pol III is specialized in the transcription of small-non coding RNAs like transference RNAs. Pol II is in charge of the transcription of all protein coding genes and multiple long and small non coding RNAs. Transcription is a highly regulated process, so a vast number of different proteins participates in the regulation of PolI activities. Transcription regulation is a process not fully characterized and, due to its importance for transcriptomics, efforts have been focused on understanding Pol II regulatory pathways. Although different stages of transcription are susceptible to be regulated, regulation of the initiation process is a key mechanism for the control of gene expression. Due to its biological importance, and the study conducted in this thesis, we will develop the essential aspects regarding transcription initiation, as well as the main modifications on Pol II during the transcription cycle.

As other cellular processes, transcription may suffer interferences by external or endogenous agents.especially, elongation and initiation processes are prone to be affected, decreasing the pool of active polymerases and provoking pausing or even complete blockage of transcription. Transcription stress is a deleterious condition tha can suffer cells as a cause of impaired transcription, that leads to Pol stalling and/or blockage. This phenomenon has been recently found and is not fully understood yet, regarding its causes, consequences, and regulatory pathways.

3.3.1 Molecular basis of Pol II regulation

During transcription initiation, Pol II is recruited to promoters and forms the pre-initiation complex together with the proteins TFIIB, TFIID, TFIIE, TFIIF and TFII. They are general initiator TFs that participate in opening the promoter and helping Pol II to begin RNA synthesis. Importantly, TFIID contains the TATA box-binding protein (TBP) and several TBP-associated factors (TAFs) that may perform specific functions in regulation Pol II processivity (Sainsbury et al., 2015). The classical model of pre-initiation complex assembly begins with Pol II–TFIIF complex binding to a pre-formed TFIIB–TBP–DNA promoter complex. TFIIE and TFIIH factors binds afterwards, enabling the DNA melting in the promoter. However, the basal levels achieved with these general TFs are very low. For a correct processivity of the enzyme, other complexes participate during the transcription cycle. One of the most important factors recruited to the pre-initiation complex is the Mediator complex (Sikorski and Buratowski, 2009) . Although it is not fully understood how it increases Pol processivity, many evidences suggest that it is crutial for Pol II release for promoter. Apart from the general machinery, recent high throughput analyses of active promoters implicate multiple factors that can both positively and negatively regulate Pol II entry to promoters. All the proteins interacting with the initiation complexes not only collaborate in the recruitment of Pol II, but also they allow nucleosome disassembling, effective elongation and control pausing or/and release of these polymerases from the TSS (Sainsbury et al., 2015).

Until recently, it was generally believed that expression of most protein coding genes was regulated at the level of holoenzyme recruitment to promoter regions .(Margaritis and Holstege, 2008; Ptashne and Gann, 1997; Roeder, 2005). However, following Pol II entrance into TSS, it usually stops and accumulates at very high levels on 30-60 nucleotides downstream the TSS. Different genome-wide works revealed that this accumulation occurs in the vast majority of transcriptionly active promoters(Core et al., 2008; Kim et al., 2005; Rahl et al., 2010; Zeitlinger et al., 2007), suggesting a functional role of this pausing across the genome. This process, known as pause-release, is thought to be an important, general mechanism to regulate gene transcription together with the pol II loading to promoters (Bernstein et al., 2002; Guenther et al., 2007; Lee et al., 2006). In fact, recently, global analysis of Pol II pausing naïve B indicated that around 90% of promoters that will be activated are bound by Pol II activation but unmelted and with low levels of basal transcription. This Pol II poising regulates rapid activation of gene transcription (Roeder, 2005). Different TFs and nucleosomes contribute to Pol II pausing. For instance, proteins such as M1BP may regulate Pol II release (Li and Gilmour, 2013) and non-canonical forms of H2A histone like H2A.Z increases Pol II pausing (Weber et al., 2014).

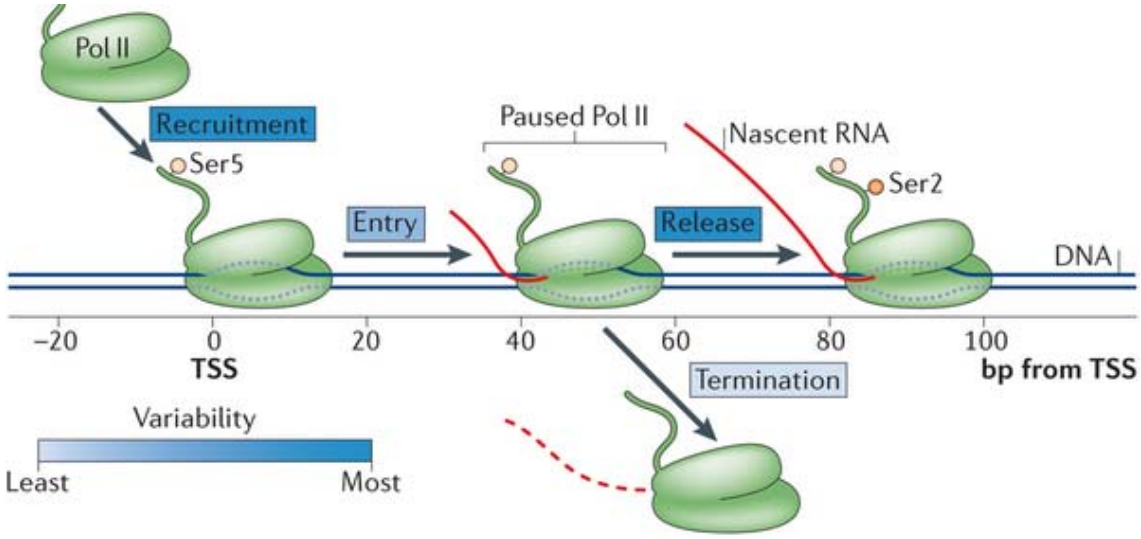


Figure 22. Pol II enzyme destinies during the initiation phase. After Pol II recruitment, it enters TSS +30-60 bp. Pause Pol II stay there until further stymuli are detected, which wil cause productive elongation or low processivity early in gene (processivity gap)(Jonkers and Lis, 2015).

After being released from the proximal promoters, polymerases may start productive elongation or, on the other hand, transcription can terminate and Pol II molecules evict from the DNA (Figure 22). Consequently, after pause-release, a molecule of Pol II may generate improductive termination or begin processive elongation. These two processes are less subject to regulation and they occur to similar rates (Jonkers and Lis, 2015). In this thesis, we will focus on transcription initiation, but it is not surprising the fact that multiple factors regulate elongation and termination processes in the transcription cycle. Elongation rates may vary between and within genes(Alexander et al., 2010; Saponaro et al., 2014; Veloso et al., 2014) and seem to play a part in co-transcription processes such as splicing and transcription termination, as well as in the

maintenance of genome stability(Alexander et al., 2010; Descostes et al., 2014; Saponaro et al., 2014; Skourti-Stathaki et al., 2014).

During the Pol II travelling across the transcribed gene it suffer post-traductional modifications by transcription regulators during the whole process. The main functional modifications occur at the carboxy-terminal domain (CTD) of Pol II domain, where regulatory phosphorylations correlates/occur together with the advance of the Pol II across the gene(Descostes et al., 2014; Hintermair et al., 2012). The CTD represents the largest domain of the Pol II and consists of multiple repeats of the consensus motif Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7. Modifications in CTD stimulate the recruitment of specific factors required for the progression through the different transcription steps. For instance, capping enzymes usually detect modifications from "early transcription", while termination factors recognize modifications in this domain specific from ·"late transcription". Consequently, CTD modifications allow us to distinguish different transition points during the transcription process (Figure 23 A).

When Pol II is loaded into promoters, CTD remain unphosphorylated (Figure 23 B). Although more than 10 modifications have been described, phosphorylation on Ser2 and Ser5 are the most relevant modifications. A generalized model of CTD phosphorylation during transcription depicts that at the beginning of genes, the CTD is phosphorylated on Ser5 by the complex CDK7-Cyc H-MAT1 bound to TFIIH. To note, Proximal-paused Pol II, consequently, present Ser5 phosphorylation. However, some evidences claim that other CDKs may be involved in the initial modifications of non-phosphorylated CTD, such as CDK8-Cyc C, subunits of the Mediator complex (Liao et al., 1995; Sun et al., 1998) , that have been ported to be able to phosphorylate Ser2 and Ser5 *in vitro.* (Galbraith et al., 2010)

P-TEFb is a key factor that was initially found to overcome pausing of Pol II, stimulate transcription and increase elongation (Marshall et al., 1996; Marshall and Price, 1995).This factor is formed by CDK9 and cyclin T1 or cyclin T2 and it is the main source of Ser2 phosphorylation *in vivo*(Greifenberg et al., 2016; Laitem et al., 2015; Schuller et al., 2016) P-TEFb is recruited as an inactive complex to promoters. Different transcription factors participate in the recruitment and activation of P-TEFb complex to promoters(Zhou et al., 2012) . Dysregulated activation of P-TEFb has been reported in cancers, since MYC oncogene interacts with P-TEFb causing an icnrease in pause-relase and global transcription (Rahl et al., 2010). Activated P-TEFb can phosphorylate a myriad of proteins, such as negative and positive elongation factors, histones and, especially, the CTD tail at Ser2P. This last modification enables to set Pol II free and to begin active elongation (Kim and Sharp, 2001; O'Hagan and Ljungman, 2004; Sanso et al., 2016; Shchebet et al., 2012). In addition, P-TEFb not only regulates pause-release, but also contributes to continued elongation and even termination (Laitem et al., 2015; Sanso et al., 2016). Due to its pivotal role in different phases of the transcription cycle, defects on CDK9 activation could provoke pol II blockage and premature termination. On the other hand, some studies suggest that other other CDKs may have a role in the elongation process, like CDK12-Cyc K complexes. However, their role is not fully understood(Bartkowiak et al., 2010; Blazek et al., 2011; Bowman et al., 2013; Cheng et al., 2012).To sum up, CDKs have an essential role in transcription, a process that can be more complex that initially was

thought with the participation of transcription CDKs, such as CDK7,8,9,11,12 or 13 (Kohoutek and Blazek, 2012)(Figure 23).

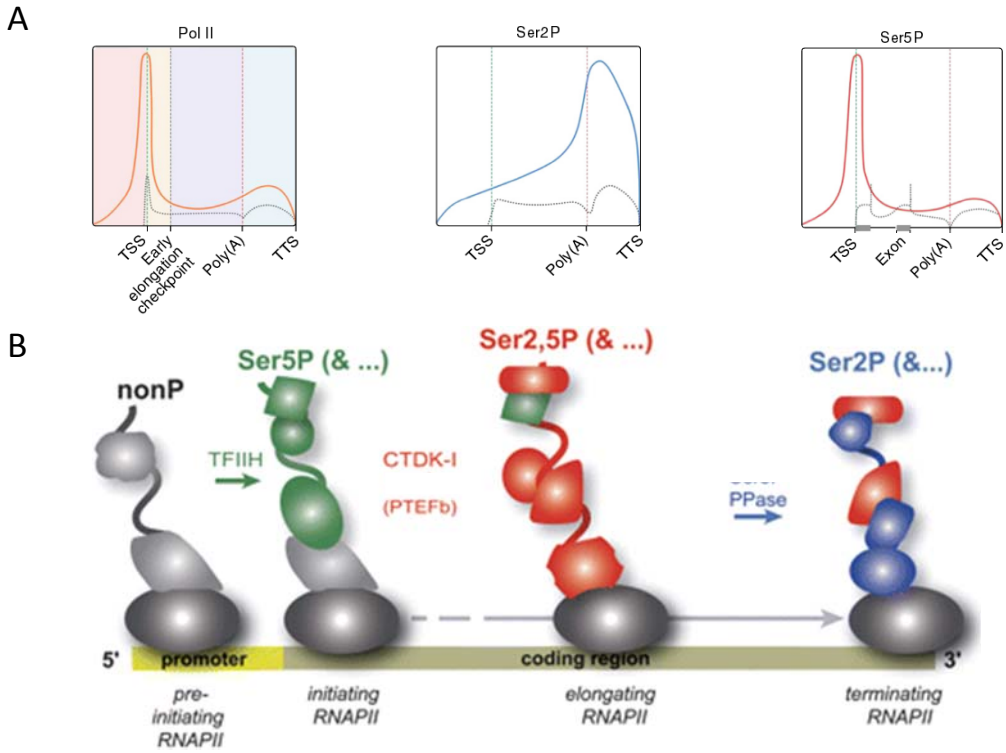


Figure 23. CTD modification is a dynamic process acrros transcription cycle. A) Schematic representation of transcriptiona patterns of total Pol II, Ser2P and Ser5P across meta-genes. B) CTD modifications during transcription. Firstly, CTD remains unphosphorylated when RNA pol II binds a promoter. Enzymes like CDK7 participate in Ser5 phosphorylation, which activates Pol II and stops 50 nucleotides after the TSS. Activation of CDK9 phosphorylates Ser 2 to start effective elongation, while Ser5 is mainly lost across gene body.(Modified from (Phatnani and Greenleaf, 2006) and (Zaborowska et al., 2016).

3.3.2 Transcription stress response

Pol II elongation in human cells occurs at a variable speed of 0.5–5 kb/sec, although it depends on the sequence of the gene and the region of the gene being transcribed (Fuchs et al., 2014; Jonkers and Lis, 2015; Singh and Padgett, 2009). RNA polymerases frequently face different challenging conditions, such as transcribing particular sequences, clashes with the replisome or transcribing imperfect templates. Blocked or slowing down polymerases decreases RNA synthesis and cells activate intracellular signaling to cope with these deleterious problems, globally known as transcription stress response (TSR).

Among the different situations inducing Pol II blockage, structural chromatin features are known to be able to slow down elongation. Non-B DNA conformations, for instance Z-DNA, hairpins and G-quadruplexes (Ditlevson et al., 2008; Fuchs et al., 2014; Nojima et al., 2015; Tornaletti et al., 2008) can block transcription elongation. High GC content or exon density also stalls Pol II progression.(Kwak et al., 2013; Veloso et al., 2014) Noteworthy, promoters enriched in CpG sequences causes RNA polymerase blockage and helps to the generation of unusual stable RNA/DNA hybrid structures, known as R-

loops (Belotserkovskii et al., 2010). In addition, collisions of replication machinery and transcription are an important cause of transcription blockage. To avoid that, replication and transcription are temporally and spatially separated (Meryet-Figuiere et al., 2014; Wansink et al., 1994).Consequently, DNA damaging agents that arrest replication machinery affect transcription and, consequently, some genotoxyc agents increases apoptosis in proliferating cells through this mechanism (McKay et al., 2002). DNA mutagenic lesions or helix distortions also provoke Pol II blockage, 35 and 40 nucleotides centered symmetrically over the lesion in the transcribed strand (Francis and Rainbow, 1999; Protic-Sabljic and Kraemer, 1985; Sauerbier and Hercules, 1978). DNA lesions caused by UV light lead to a dose-dependent decrease in the synthesis of RNA (Kantor and Hull, 1979; Ljungman and Paulsen, 2001). Other DNA-damaging agents are also inductors of Pol II blocking such as cisplatin (Damsma et al., 2007),carcinogens(Schinecker et al., 2003; You et al., 2015) or reactive oxigene species (Cline et al., 2004).

Transcription is capable to cause mutations and genetic alterations on DNA template. Among them, transcription--associated mutagenesis (TAM) and transcription-associated recombination (TAR) are two mechanisms leading to genomic instability and mtuations. Collisions between transcription and replication forksespecially increases TAR and generate chromosomal instability (Gaillard et al., 2013).Although not fully understood the whole mechanism, collisions between transcription and replication increases DSBs, so cells rely on specific helicases with a protective role (Brambati et al., 2015).Inhibiting replication in mammal cells increases TAR, but transcription inhibition seems to supress TAR (Gottipati et al., 2008),suggesting that transcription is necessary for this recombination. However, the increase in TAR is suppressed by concomitant treatment with transcription and replisome inhibitors. Altogether, these results indicate that transcription-replication conflicts induce replication stress in mammalian cells that can be alleviated by diminishing transcription activity. We have to keep in mind that OIRS can be generated by an increase in conflicts between replication forks and polymerases. Cyclin E-mediated OIRS promotes TAR and it is partially reversed by transcription inhibition 203. The greater number of DSBs may lead to the activation of DDR. Transcription amplification, thus, may lead to higher genomic instability due to an increment of an increase in collisions and replication stress (Kotsantis et al., 2016; Stoimenov et al., 2011). On the other hand, CDK9 silencing also increases genomic instability, indicating that reduced transcription may also cause replication stress (Yu et al., 2010). In this regard, TBP silencing can also lead to an increase in replication stress (Kotsantis et al., 2016).

As already indicated, one of the effects of slowing down transcription is thought to be the generation of R-loops, which have also been related to genomic instability .How they generate this instability is not fully understood, but some theories suggest that R-loops formed behind the elongating Pol II may restrict transcription and increase the collisions between transcription and replication in the way we have explained before. Other authors suggest that the displaced ssDNA of the R-loops can act as a substrate to DNA-damaging agents (Aguilera and Garcia-Muse, 2012). To finish, G4 structures formed on the rich G sequences, typically found in R-loops, can generate sites susceptible to be attacked by nucleases (Duquette et al., 2004). In any case, an increase in R-loops is deleterious for the cell, so active pathways exist to resolve them

and to avoid replication-transcription collisions (Santos-Pereira and Aguilera, 2015; Skourti-Stathaki et al., 2014).

The blocking of the transcription process leads to the activation of the TSR. However, whether proliferation is needed for activating these responses remains controversial. Pol II inhibition may cause apoptosis even in non proliferating cells (Ljungman et al., 1999; McKay et al., 2001; te Poele et al., 1999). However, other studies detected UV-associated apoptosis was increased in S-phase and require cell proliferation. (Dunkern and Kaina, 2002; McKay et al., 2002) In the first case, blockage or slowing down of Pol II would suggest DDR direct activation, whereas the second study may be detecting the interference of stalled transcription complexes collapsing with replication forks (Helmrich et al., 2011). Further studies are needed to characterize what triggers TSR activation.

One of the first cell responses upon transcription blockage is the activation of the transcription-coupled repair (TCR) pathway, what helps polymerases to evict from the blockage. This mechanism uses similar complexes participating in the nucleotide-excision repair pathway. One of the main differences is the sensor protein, which uses CS proteins instead of XPC or DDB proteins (Hanawalt, 2002; Leadon and Lawrence, 1991; Mellon et al., 1987). Defects in CS family proteins cause severe clinical manifestations involving high mutational rates and tumorogenesis, suggesting that inefficient TCR may play a central role in these syndromes. It is thought that the activation of this pathway is pivotal for DNA repair in lesions caused by UV light and explains the fact that transcribed regions repaired faster than non-transcribed areas (Mellon et al., 1987).

One phenomenon associated to the TSR response comes from the fact that when transcription is inhibited nuclear mRNA export is also stopped. Some proteins containing nuclear export sequences depend on these mRNA in order to efficient be transported from nucleus to cytoplasm (O'Hagan and Ljungman, 2004).Consequently, reduced transcription leads to an increase of this proteins to nucleus. More than 75 proteins have been detected to accumulate by this mechanism upon transcription inhibition (Derheimer et al., 2005).Noteworthy, p53 is one of this proteins, although other examples are VHL, HIF-a, or survivin (Ljungman, 2007). Although not totally understood, this could be a mechanism of gradual internal measure of the level of transcription stress in order to trigger more severe reactions to transcription blockage. Alternatively, proteins can be performing specific pro-apoptotic actions in nucleus, so inhibition of nuclear export to cytoplasm may end up in cell death (Stauber et al., 2007).. Besides, the balance between survival/apoptotic proteins can be broken. Pro-survival proteins have short live mRNA half-lives, so cells may rapidly lose them upon transcription inhibition (Chaturvedi et al., 2005).In addition, Pol II blockage leads to DDR activation in proliferating cells due to the collapsing between transcription and replication process, so it can lead to apoptosis and/or cell cycle arrest (McKay et al., 2002; Rudolph et al., 2007). To sum up, apoptosis is one of well characterized reactions activated by the TSR by different mechanisms.

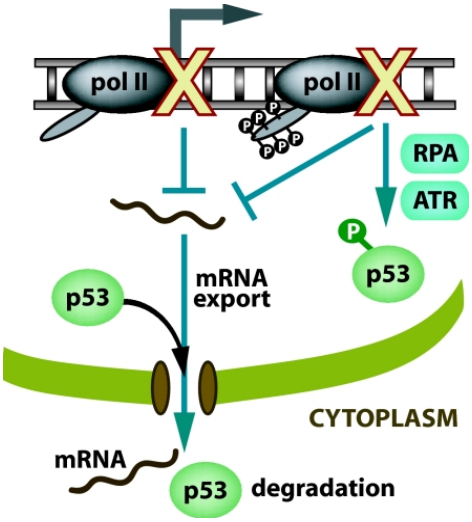


Figure 24. TSR activation leads to differential p53 functions. First, Pol II blockage can be sensed by RPA and RPA/ATR and stabilize p53 protein mediating its phosphorylation in Ser15. Second, loss of mRNA synthesis results in diminished amounts of mRNA available to be exported out of the nucleus, leading to attenuated coupled nuclear export of p53 and accumulation in nucleus.(Derheimer et al., 2007)

However, may be one of the most critical consequences of transcription inhibition is the accumulation and activation of P53 in nucleous(Derheimer et al., 2007; Ljungman et al., 2001; O'Hagan and Ljungman, 2004), although controversial results also report its accumulation in mitocondria (Arima et al., 2005).RPA/ATR signaling pathway is involved in the activation of p53 by the phosphorylation Ser15 of p53. Even though p53 can be phosphorylated by multiple proteins, Ser15 phosphorylation a focal point that causes the activation and triggers the phoshphorylation of other residues (Ser9, Ser20, Ser46 and Thr18). This phosphorylation stabilizes the protein (Shieh et al., 1997) prevents p53 nuclear export and increases p53 transcription activity (Loughery et al., 2014) .Many DDR proteins activate this residue, such as ATM or ATR (Banin et al., 1998; Tibbetts et al., 1999).Thus, ATR or RPA inactivation abrogated Ser15 phosphorylation. Furthermore, this modification happens even in the absence of proliferation when transcription elongation was impaired, implying ATR also in TSR. How can RPA/ATR detect these lesions? One hypothesis would suggest that blocked Pol II may cause DNA unwinding and a subsequent RPA binding to ssDNA, due to non balanced negative supercoiling normally induced by nucleosome dissembling. As in replication stress, this may provoke RPA/ATR activation and an analogous response to RSR. (Figure 24). Recently the role of ATR in stress managing has been clarified.(Kemp and Sancar, 2016), suggesting that ATR activation may have different effects depending on the stress. Moreover, it seems that ATR may trigger release of the transcribed gene from the nuclear pore to prevent fork collapse. As we said before, p53 can also accumulate in the nucleus without Ser15 phosphorylation by inhibiting its nuclear export. Interestingly, it seems that the transcription phase influences the phosphorylation of p53 in a way not fully understood. For instance, promoter proximal defects lead to p53 accumulation without Ser15 modification, but defects on elongation led to p53 phosphorylation. (Derheimer et al., 2007; Ljungman, 2007; Ljungman et al., 2001; O'Hagan and Ljungman, 2004) . Taking these results together, maybe RPA/ATR detection of Pol II blockage and p53 phosphorylation only occurs during Pol II elongation. Concordantly with all these evidences, pharmacological elongation inhibitors induce similar responses than genotoxyc agents activating

RPA/ATR (Derheimer et al., 2007; Ljungman et al., 2001; Yamaizumi and Sugano, 1994). It is a general assumption that cancer cells require high levels of transcription for proliferation and survival (Adhikary and Eilers, 2005; Cabarcas and Schramm, 2011). Pol II activity increases the global transcription of the majority of genes expressed in the cell through P-TEFb, and this activation may be directly mediated by oncogenes such as MYC (Lin et al., 2012; Luo et al., 2012)Tumors can harbor mutations or genetic backgrounds that enhance transcription, such as the ones detected for P-TEFb in leukemia (Morris et al., 2008).Besides, CDK8 activity correlates with the Wnt/β-catenin pathway in some colon cancers (Morris et al., 2008).

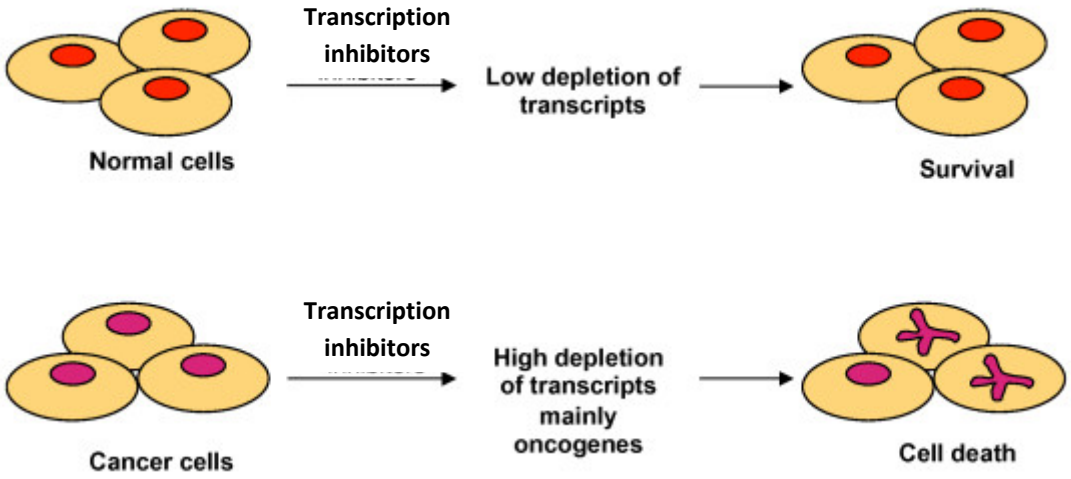


Figure 25. Hypothesis of mechanistic sensitization of cancer cells to transcription inhibitors. Transcription inhibitors lead to mRNA decrease, especially from overexpressed oncogenes. As cancer cells depend on oncogene-dependent for survival, transcription downmodulation triggers cell death in cancer cells, while preserving normal cells (Adapted from (Villicana et al., 2014)).

All these data explain the fact the tumor cells are more sensitive to apoptosis upon transcription inhibition than normal cells (Bywater et al., 2012; Koumenis and Giaccia, 1997; Radhakrishnan and Gartel, 2006). Consequently, there is a strong therapeutic interest to find molecules capable of directly target transcription. Genotoxic agents used in general chemotherapy affect both cancer and normal cells, but transcription inhibition specifically affects tumor cells without killing normal cells. Transcription inhibition downregulate oncogenic transcription programs affecting key oncogenes , what may lead to dysregulating favorable growth conditions contribute to tumor proliferation and survival (Figure 25) .Besides, transcription inhibition may sensitizes to different cancer clones in the same tumor. Since it decreases oncogene-mediated transcription, policlonal tumors with different activated oncogenes can be killed by the same inhibitory drug (Villicana et al., 2014).In addition, cancer stem cell resistant to normal chemotherapy would be affected by these inhibitors (Adler et al., 2012; Kaichi et al., 2011). However, some reports are quite controversial in this field and suggest that global inhibition of transcription in certain tumors, like breast cancer cells, may increase invasiveness (Villicana et al., 2014). Authors associate these sticking results to the differential ability a protein can display depending on the context. Thus, transcription inhibition may be better considered as a mechanism to sensitize cells to other drugs rather than a single agent in the majority of cases (Villicana et al., 2014).

Nevertheless, just a few inhibitors of transcription have been described. The majority of transcription inhibitors currently under research are CDK inhibitors, targeting eminently CDK9. In addition, other elements of the basal transcription machinery are under consideration (Andrade-Lima et al., 2015; Demidenko and Blagosklonny, 2004; Derheimer et al., 2005; Ljungman et al., 1999; McKay et al., 2002; Villicana et al., 2014) .The majority of CDK inhibitors not only affect transcription CDKs, but also cell cycle CDKs (Stellrecht and Chen, 2011). Among them, flavopiridol is a potent pan-CDK inhibitor that strongly affects CDK8 and CDK9 and that has been tested in many hematological neoplasms (Chen et al., 2005; Lin et al., 2010). A novel compounds, CDKI-71, activates apoptosis in several tumor cell lines, even in *TP53* negative tumors (Liu et al., 2012).Ibulocydine induces apoptosis without toxic side effects in mouse xenografts with promising results (Cho et al., 2011).

Pol targeting is a less extended strategy, since only a few drugs have been reported to directly affect these enzymes. To date, just TAS-106,a cytidine analog, have been studied for cancer treatment due to the fact that other compounds (like a-amanintin) display strong toxicity. Recently, a DNA intercalator called BMH-21, induces the degradation of the RPA194 subunit of RNPI (Peltonen et al., 2014). This effect is independent of p53 and opens the possibility that this drug may be used in cancer treatment. Finally, targeting transcription complex compon ents can be also useful for cancer treatment In this regard, triptolide covalently binds to TFIIH, inhibiting its ATPase activity and, consequently, transcription (Vispe et al., 2009). Triptolide has been widely used for the treatment of various cancers with promising outcomes (Villicana et al., 2014). Interestingly, apoptosis is enhanced in p53-deficient cells by JNK pathway activation (Villicana et al., 2013). Other compounds targeting bassal transcription machinery are currently under research, such as the BRD4 inhibitors JQ1 (Filippakopoulos et al., 2010) and I-BET151 (Dawson et al., 2011). In summary, transcription inhibition can interrupt transcription programs directed by key oncogenes or disrupt favorable growth conditions associated with the overexpression of non-oncogenes that contribute to survival and tumor progression.

AIMS AND OBIECTIVES

Mantle cell lymphoma is a lymphoid neoplasm characterized by the translocation (t(11;14) that lead to cyclin D1 overexpression. This primary oncogenic event is usually followed by secondary molecular alterations targeting genes involved in cell proliferation, DNA damage response and cell survival pathways. Cyclin D1 is thought to contribute to lymphomagenesis through its canonical function as the cyclin regulatory subunit of CDK4/6 complexes inducing the G1/S phase transition. However, previous works from our laboratory and others call into question this simplistic model. Comprehensive studies evaluating the lymphomagenesis role of these new cyclin D1 functions are still missing.

The global aiming of this thesis was to investigate new oncogenic functions of cyclin D1 during MCL lymphomagenesis, focusing on the role of cyclin D1 as potential transcription regulator and its capacity to induce DNA replication stress, and evaluate in primary MCL samples evidence of these oncogenic effects.

From this general goal, specific objectives are derived:

1. To characterize the potential role of cyclin D1 as transcription regulator in MCL lymphomagenesis:
    1.1 Study the chromatin binding profile of endogenous cyclin D1 in MCL cells.
    1.2 Analyze the chromatin binding pattern of cyclin D1 and its relation with the epigenetic landscape of MCL cells.
    1.3 Correlate the chromatin binding pattern with the transcription output of MCL cells and characterize the effect of cyclin D1 on gene expression

2. To study the capacity of cyclin D1 to induce DNA replication stress, that could represent a chronic stress in MCL cells and that might drive MCL lymphomagenesis.

3. To investigate the activation of the DDR following cyclin D1 overexpression, and determine if primary MCL cases display constitutive activation of the DDR pathway.

4. To correlate the expression of DDR markers with the clinicopathological characteristics of primary MCL tumors.

5. To investigate the CpG methylation profile of MCL cells defining the relevance of uncontrolled proliferation in the epigenetic dysregulation and the correlation with the clinicopathological characteristics of the tumors.

MATERIALS AND METHODS

1. Biological samples

1.1 Cell lines

Cell lines used in this thesis are listed below, with selected biological characteristics important to this study (Table 2).

Table 2. Cell lines and growth conditions of all cell lines used in this work.

Cell line	Tissue/Disease	*TP53* status	Cyclin D1 (protein)	*MEDIA
Z-138	MCL	wt	Very high	RPMI, 10%FBS
UPN-1	MCL	mutated	High	RPMI, 10%FBS
JeKo-1	MCL	mutated	Moderate	RPMI, 10%FBS
GRANTA-519	MCL	mutated	High	DMEM,10% FBS
JVM-2	MCL	wt	Low	RPMI, 10%FBS
Hbl-2	MCL	mutated	Non-relevant	RPMI, 10%FBS
Mino	MCL	mutated	Non-relevant	RPMI, 10%FBS
JVM-13	Prolymphocytic leukemia	wt	Negative	RPMI, 10%FBS
HEK 293	Human embryonic kidney	Non-relevant	Non-relevant	DMEM,10%FBS
JJN-3	MM	Non-relevant	Negative	RPMI, 15%FBS
RPMI 8866	MM	Non-relevant	Negative	RPMI, 15%FBS
NCI-H929	MM	Non-relevant	Very low	RPMI, 15%FBS
U266	MM	Non-relevant	Moderate	RPMI, 15%FBS
KMS-12-PE	MM	Non-relevant	Low	RPMI, 15%FBS
KMM-1	MM	Non-relevant	Negative	RPMI, 15%FBS
ARP-1	MM	Non-relevant	Negative	RPMI, 15%FBS

*All cell lines are supplemented with 2mM of L-glutamin, 50units/mL of penicilin and 50uM/mL of streptoMYCin. All cell lines grow at 37°C and 5% CO2.

1.2 Formalin-fixed paraffin-embedded MCL primary tumors

A series of 37 patients diagnosed with MCL by the Haematopathology Unit, Pathology Department, Hospital Clinic of Barcelona were included in our study. The MCL diagnosis was established according to the classification criteria of the 2008 World Health Organization and all cases had the t(11;14)(q13;q32) and/or cyclin D1

overexpression. The study was approved by the Institutional Review Board of the respective institutions. All patients gave informed consent to participate in the study according to the guidelines of the local Ethic Committees.

The primary MCL samples included 30 classic MCL (81%) and 7 were blastoid/pleomorphic (19%). 27/35 (77%) cases showed nuclear SOX11 positivity and 8/35 (22.9%) were negative for SOX11. We had 2/37 (5.4%) cases with unknown SOX11 status. The SOX11 positive cases consisted of 20 (74.1%) lymph nodes, 4 (14.8%) spleens, 1 (3.7%) tonsil, 1 (3.7%) lung and1 (3.7%) eyelid. The SOX11 negative cases consisted of 2 (25%) lymph nodes and 6 (75%) spleens. Among our 37 cases, we studied 22 cases (59.5%) with TMAs and 15 cases (40.5%) with whole slide sections from formalin-fixed paraffin-embedded samples of mantle cell lymphoma. The median age of patients was 66years (range, 30-89 years).

1.3 Frozen MCL primary tumors and normal control samples

Tumor tissue specimens from 132 MCL patients were obtained from the Tumor Bank of the Department of Pathology of the Hospital Clínic/IDIBAPS of Barcelona, the Institutes of Pathology of the University of Würzburg and the Robert-Bosch Krankenhaus in Stuttgart, the Institute of Human Genetics/Pathology Department of Kiel, and from the Lymphoma/Leukemia Molecular Profiling Project (LLMPP) consortium. The MCL diagnosis was established according to the classification criteria of the 2008 World Health Organization and all cases had the t(11;14)(q13;q32) and/or cyclin D1 overexpression (Swerdlow et al., 2016). The study was approved by the Institutional Review Board of the respective institutions. All patients gave informed consent to participate in the study according to the guidelines of the local Ethic Committees.

The primary MCL samples studied included 112 classic (cMCL) (85%) and 20 blastoid/pleomorphic (bMCL) (15%) variants. In four cases, two sequential samples with a median interval period of five years were available. All tumor samples included in the study had at least 80% tumor cells. Clinical and follow-up information was available in 127 MCL patients. We also studied 31 control samples corresponding to different normal lymphoid cells and tissues. These control samples included CD19-positive cells purified from peripheral blood (n=6) or tonsils (n=3), peripheral blood naïve B-cells (n=4), reactive lymph nodes (n=10) and spleen samples (n=8). The mononuclear cell fraction from tonsil or peripheral blood was isolated using Ficoll (Boehringer Mannheim, Germany). Normal CD19+ B and naïve B lymphocytes were obtained by magnetic cell sorting using CD19 human microbeads and Naïve B cell isolation Kit II, respectively, and autoMACS separator following the manufacturer's protocol (MACS, Miltenyi Biotec). The median age of patients with the primary MCL cases was 64 years (range, 37-92 years), and the median age of the individuals from whom the normal samples was 54 years (range, 23 to 83 years). Comparative genomic hybridization (CGH) data had been published previously for 83 cases(Bea et al., 1999; Salaverria et al., 2007). Microarray expression data generated with the Affymetrix GeneChip Human Genome U133 Plus 2.0 array was available for 79 primary MCL (Gene Expression Omnibus (GEO) GSE21452 & GSE36000).

2. Cell culture

2.1 Cell cryopreservation and thawing

Pellet 5 million exponential growing cells centrifuging for 5mins, 300g, at room temperature (RT), and wash pellet with 5 mL of cold PBS. Centrifuge again and put tubes on ice. Resuspend cells in 1mL of FBS and add, little by little, 1mL of cold freezing media (40mL of media+10mL of DMSO). Pass the 2 mL to a labeled cryovial. Place immediately them on dry ice for 5 mins. Store them at –80°C overnight. For long-term storage, transfer cryovials to liquid nitrogen (-196°C).

For cell thawing, remove the cryovial from liquid nitrogen storage on dry ice and place it into a 37°C water bath. Before complete defrosting, add 8mL of warm media (37°C) and immediately centrifuge samples for 8 mins, 400g ,at RT. Wash cells twice with RT PBS (for 5mins, 300g, at RT) and resuspend cells in 5mL of warm media. Add 1mL of warm media for the following two days. After, centrifuge again and change media.

2.2 Cells passaging

Subculturing suspension cells was performed as follows. Firstly, cell number was counted automatically with cell counting slides (TC10, Biorad). Cells were diluted adding completed media at 37°C up to a concentration of 0.5million cells/mL. Cells were fed three times a week. At least once a week cells were fed by centrifuging for 5 mins, 300g and RT, and replacing used media with new media. Cells were always fed the day before cell biology experiment to assure exponential growth.

Adherent growing cells (in this thesis, only HEK 293) were passed when they reached 80-90% of confluence. After media removal, cells were washed once with 5mL of RT PBS.5mL of trypsin at 37°C was added and cells were incubated for 3-5 mins at RT. Adherent cells will detached from the plate. Same volume of completed media was added to the plate and 10mL were recovered to centrifuge for 5 mins, 300g, RT. Cell pellets were resuspended in completed media and cells were split with the desired ratio. For maintenance, usually passaging ratios went between 1:10 and 1:5.

Constitutively overexpressing models were maintained with 0.3ug/mL of PuroMYCin, while inducible models were maintained with 0.3ug/mL of PuroMYCin and 500ug/mL of G418. Experiments were performed without puroMYCin or G418 addition. In order to trigger cyclin D1 expression in the inducible cell lines, doxycyclineup to 0.1ng/mL concentration was added, usually 24 hours before carrying out the experiment.

2.3 Growth curves and Trypan Blue counting and doubling time calculation

In duplicate, we seeded 0.5 million cells in 1mL of media. At day 2, living cells were counted with trypan blue and centrifuge them 300g, for 5 mins, RT. Adjust cell concentration to 0.5million cells/mL. Perform exactly the same at day 4 and day7. Represent the total number of living cells in each point (viable cell concentration x suspension volume), as the mean of the duplicate. Experiments were doing in triplicate. Trypan Blue was performed under the manufacturer's guidelines (0.4% Trypan Blue solution, Sigma). Briefly, mix 50uL of cell suspension and 50uL of Trypan blue solution. Fill both sides of the neubauher chamber with cell suspension (approximately 10uL)

and view under an inverted phase contrast microscope using x20 magnification. Count the number of viable (seen as bright cells) and non-viable cells (stained blue). Calculate viable cell concentration using the following equation: Live cells/mL=Number of live cells x 10000 x 2/ Number of large corner squares counted.

Cell doubling time is calculated as the ln(2)/Growth rate, being the Growth rate=$\ln(Q_7/Q_0)/7$ days^{-1}. Q_7 is the million of cells after 7 days of proliferation and Q_0 is the initial number of cells (in our case, 0.5 million cells). Cell doubling time is important to know the percentage of cells that are in a certain phase. During the exponential phase of growth, the duration of G1, for example, can be calculated from the equation: T_{G1}=TC x ln (fG1 + 1)]/ln 2; where Tc is the cell doubling time and fG1 is the fraction of cells in the phase you want to determine, in our case, G1. Usually, the fraction of cells in a particular phase is calculated using PI cell citometry. (Pozarowski and Darzynkiewicz, 2004)

3. Protein analysis: Western blotting

3.1 Protein extraction and quantification

Total protein extraction for western blotting was performed in RIPA buffer (sigma). Five to ten million cells were washed once with RT PBS and were resuspended in cold RIPA buffer with protease inhibitors (Thermo). If further analysis involved phospho-proteins, phosphates inhibitors were also added (Thermo). Extraction buffer volume was approximately the double of the cell pellet. Cells were incubated on ice for 30 mins, with occasional gently mixing. Extracts were centrifuged at maximum speed, at 4°C , for 15 mins to clarify samples.

Proteins were quantified using commercial protein assays (Biorad) in 96-well plates. Briefly, 2uL of extract were mixed with 200uL of 1:5 of the Protein assay dye reagent in water. After 10 mins RT incubation, absorbance was measured at 595nm. To establish protein concentrations, a standard curve of BSA was also performed (0-10ug/uL). Extracts and curve points were quantified always in duplicate and protein concentrations were calculated with the mean value.

3.2 Electrophoresis and Western blotting

Same protein concentrations (usually, around 45ug) were boiled for 5 mins at 95°C . Samples were separated by SDS-PAGE gel electrophoresis, along with molecular weight marker. Electrophoresis was run at 100V constant for 1–2 h at RT. Protein transfer was done into PVDF membranes (pre-activated 5 mins with methanol incubation). Transfer was done at 4°C .Normal size proteins (20-100kDa) were transferred for 90 mins at 350mA constant Amperage. Small proteins (<20kDa) were transferred for 60 mins art 70V constant Voltage. Big proteins (>200kDa) were transferred for 180 min, 300mA constant Amperage, and reducing methanol concentration to 5% instead of usual 20%.

Membranes were blocked one hour at RT with gently mixing with TBS-Tween 0.1%-5% skimmed milk. If phospho-proteins were aimed to be detected, membranes were blocked in TBS-Tween 0.1%-5% BSA. Primary antibodies were incubated overnight,

prepared in the same buffer used for blocking, at appropriate concentrations. Next day, membranes were washed three times with TBS-Tween 0.1%, 7 min each. Incubate the membrane with the recommended dilution of conjugated secondary antibody (DAKO,1/2000; Cell signaling, 1/1000) in TBS-Tween 0.1%-5% skimmed milk at room temperature for 1 h. Wash the membrane in three washes of TBS-Tween 0.1%, 7 min each. For signal development, we follow the kit manufacturer's recommendations (Pierce ECL Western Blotting Substrate). Chemiluminiscence detection and image acquirement was performed with a ImageQuant LAS 4000 (Fujifilm). Bands were visualized and quantified with the *Multi Gauge* software (Fujifilm). Primary antibodies and working dilutions can be found in Appendix 1.

4. Nucleic acid extraction protocols

4.1 RNA extraction

4.1.1 RNA extraction – Qiagen kit

Total RNA extractions were done under manufacturer's guidelines (RNeasy Plus Mini Kit, Qiagen). Briefly, cellular pellets of 0.-1.5 million cells are resuspended in buffer RLT supplemented with β-Mercaptoethanol as suggested in the protocol. Cell homogenization was favored by using a 20G syringe at least for 8 times/sample. Transfer the homogenized lysate to a gDNA Eliminator spin column. Centrifuge to maximum speed and discard the column, saving the flow-through.Add same volume of ethanol to allow binding to RNeasy spin column. Centrifuge again and wash columns once with buffer RW1 and twice with RPE buffer. Elute in 30-50uL of RT RNase-free water. Quantify by Nanodrop N-1000 (Thermo Fisher).

4.1.2 RNA extraction –TRIZOL protocol

Total RNA was isolated using Trizol (Zymo Research), under the manufacturer's guidelines. Briefly, cell pellets (usually, between 1-10 million/sample) are resuspended in TriZOL and homogeneizated using a 20G syringe at least for 8 times/sample. Incubate 5 mins and add 200uL of chloroform. Centrifuge for 15 mins at maximum speed and 4°C and keep aqueous superior phase. Add approximately same volume of isopropanol and store at -20°C overnight. Next day, centrifuge for 25 mins, maximum speed, 4°C. Wash pellet twice with cold ethanol 75% and let it dry at RT at least one hour. Reconstitute pellets with 20-40uL of RNase-free water. Quantify by Nanodrop N-1000 (Thermo Fisher).

4.2 DNA extraction

4.2.1 DNA extraction – Qiagen Kit

Total DNA extractions were done under manufacturer's guidelines (QIAamp DNA Mini Kit). Briefly, cellular pellets of 10 millions cells were treated with Proteinase K for 10 mins. Add ethanol to samples and proceed to bind DNA to columns. Wash columns by consecutive centrifugations and elute DNA with buffer TE (aprox 10-30uL). Quantify by Nanodrop N-1000 (Thermo Fisher).

4.2.2 DNA extraction- Phenol Cloroform

Add 1mL of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Sigma) to sample and incubate in mixing for 25 mins at RT. Centrifuge 15 mins, maximum speed, RT. Pass aqueous phase(approximately, 400-500uL) to a new tube and add 25ug of glycogen (Ambion),25uL of sodium acetate 3M and 1mL of absolute ethanol at -20°C . Incubate samples at -80°C for 30 mins. Centrifuge at maximum speed, 4°C, for 15 mins. Discard supernatant and wash pellet with 1mL of cold ethanol (70%);15 mins, maximum speed, at 4°C . Discard supernatant and let it dry at RT at least one hour. Reconstitute pellets with 10uL-30uL of TE (pH=8). Quantify by Nanodrop N-1000 (Thermo Fisher).

5. Generation of cyclin D1 overexpressing models

5.1 Plasmid Cloning by Restriction Enzyme Digest

Plasmid cloning requires three essential steps: plasmid digestion, insert digestion and plasmid/insert ligation. For digestions (both plasmid and insert) we perform a double digestion protocol.1ug of total plasmidic DNA was incubated with 4uL of Not1 HF and BamH1 enzymes (New England) and 5uL of Buffer digestion of 10X Cut smart (New England) in 50uL of total volume. Incubate for 4 hours at 37°C . Inactivate enzymes for 20 mins at 65°C . Plasmidic DNA was directly purified with NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). For the insert purification, samples were run into a 0.8% agarose gel and band was cut using transilluminator. In gel purification was done under manufacturers' guidelines (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel).

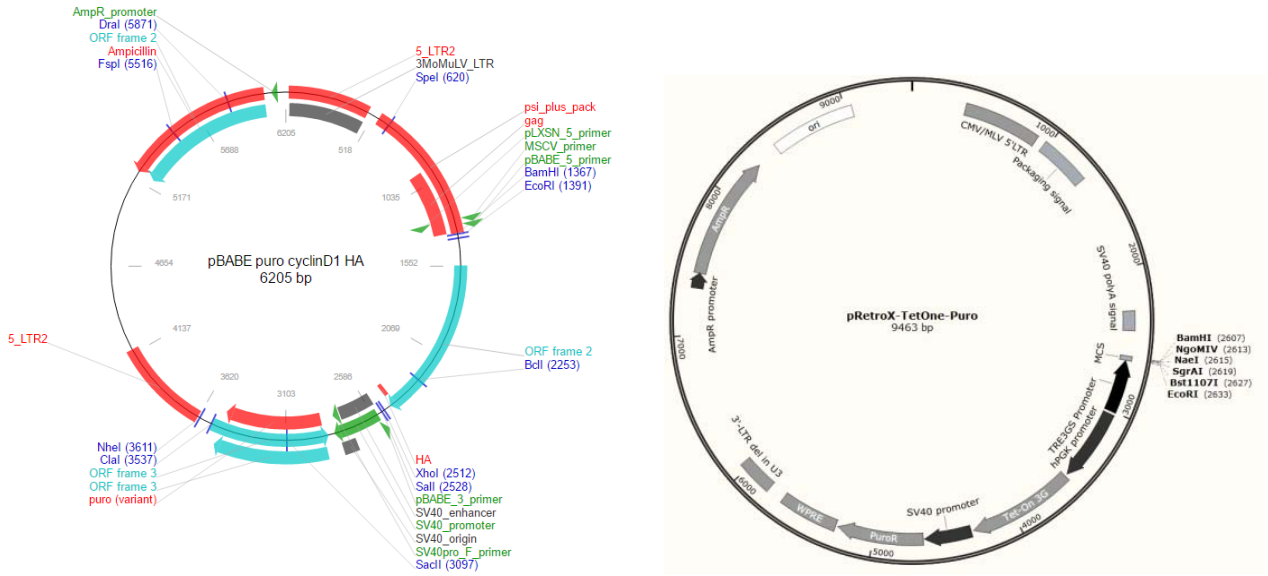


Figure 26. Plasmid used for retroviral vector production of constitutive models (left) and plasmid backbone for cloning cyclin D1 for inducible experiments (right).

Ligation was performed using the following equation: insert(ng)=[vector(ng) x kb insert x molar ratio(insert/vector)] / kb vector. We used a molar ratio of 2:1. Ligation reaction was set up as follows, using T4 DNA ligase (Ambion) : 39ng of vector, 18ng of insert, 1uL of ligase T4, 1uL of ligase buffer (10x) in a final volume of 10uL. Ligation took place for 3 hours at 22°C, and enzyme was inactivated at 70°C for 10 mins. Heat shock protocol was used for bacteria transformation. Briefly, 45uL competent bacteria cells (XL10-Gold) were incubated 30 mins on ice with plasmidic DNA. Heat shock bacteria

for 30-45 secs at 42°C . Quickly, incubate samples on ice for 2 mins. Then, add SOC media at 42°C and incubate samples with gently mixing at 37°C . Put samples in plates of LB agar with ampicilin(100 mg/mL) and incubate at 37°C overnight.

Five colonies of each ligation were chosen, grown in suspension with LB supplemented with ampicilin(100 mg/mL), and plasmidic DNA was extracted using Plasmid Mini Kit (QIAGEN). Samples were run into agarose gel (0.5%) to analyze size. Correct size colonies were grown in suspension with LB supplemented with ampicilin and plasmidic DNA was extracted with Plasmid Maxi Kit (QIAGEN). Both plasmid purifications were carried out following manufacturers' guidelines.

To introduce T2868A stabilizing mutation in the cyclin D1 wt plasmid, we used QuikChange II Site-Directed Mutagenesis Kit (Agilent). We perform directed mutagenesis according to manufacturer's guidelines. Briefly, plasmid was copied using two primers containing the point mutation in the middle of the primer sequence. Non-mutated plasmids are further digested, so point mutated-plasmids can be use to transform competent bacteria. Transformation was performed exactly like previously described. Five colonies of each ligation were chosen, grown in suspension with LB supplemented with ampicilin and plasmidic DNA was extracted using Plasmid Mini Kit (QIAGEN). Samples were run into agarose gel (0.5%) to analyze size. Correct size colonies were grown in suspension with LB supplemented with ampicilin and plasmidic DNA was extracted with Plasmid Maxi Kit (QIAGEN). Both plasmid purifications were carried out following manufacturer's guidelines.

5.2 Antibiotic kill curves set-up

In order to establish the concentration that enables cell selection after retroviral infections, half million cells were incubated in duplicate in different antibiotic concentrations: PuroMYCin (0-2 µg/ml) and G418 (0-1000 µg/ml). Growth curves were performed at days 2,4 and 7 after antibiotic delivery. After one week, selection dose was calculated as the minimum antibiotic concentration that killed all non-infected cells. JVM-13 was selected with 0.3ug/mL of puromyicin, while JVM-2 was selected with 0.4ug/mL. Same procedure was used for G418, although antibiotic selection takes longer (14 days). All cell lines were successfully selected with 1000 µg/ml of G418.

5.3 Retroviral infection for constitutive overexpressing/silencing models

Retroviral-overexpressing cell models is divided in different steps: retroviral production, transduction and antibiotic selection. Retroviral production begins with transfection of HEK 293 T subconfluent cells (around 40-50% of concluence). In 500uL of Opti-MEM media(37°C), add 17.5uL of lipotransfectin (Niborlab) and incubate 5 mins. Add 5Ug of total DNA (2.6ug plasmid of interest, 1.73ug og Gag/pol and 0.66ug of 10A1 capsid) and incubate for 30 mins. Pour all the content into the HEK 293 plate. Incubate for 5-7 hours and change with new media. Plasmids of interest were represented in figure 26.

Transduction begins 40 hours after media removal.1.5 million exponentially-growing cells were centrifuged and resuspended in 1mL of warm media. Add 1mL of filtered, virus-containing media from transfected HEK 293 cells and 8ug of Polybrene. Add new media to HEK 293 transfected cells. Spin cell suspensions in 6-well plates for 90 mins, 27°C , 2700 rpm. Then, incubate cells overnight. Cells undertook a second round of

spin-infection using 64 hours HEK 293T supernatants. Remove virus-containing media and add fresh media the following day. Antibiotic selection began 1-2 days after transduction.

Silencing of cyclin D1 in exponential growing GRANTA-519 cells similarly, but it was performed by lentiviral spin-infection (1000g, 90 min, 32ºC) in presence of polybrene, followed by additional 3 hours incubation; the protocol was repeated for two consecutive days. Cells were selected with 0.5 µg/ml of puroMYCin (Sigma-Aldrich) 24 hours after infection.

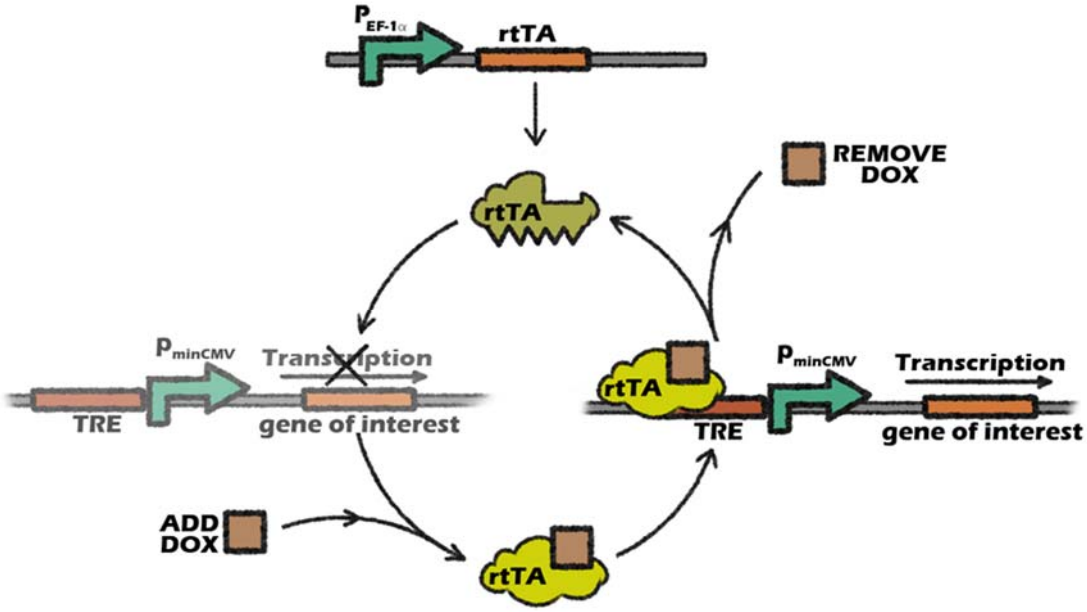


Figure 27 Tet-on inducible system. The bacterial rtTA proteinteracts with doxycycline (DOX) and induces the expression of the gene of interest. This system requires two rounds of transduction. First, introducing *rtTA* gene for constitutive expression and second, the construct containg the gene of interest. Removing doxycycline switches off the expression system (Extracted from http://2013.igem.org/Team:SYSU-China/Project/Design)

5.4 Retroviral infection for inducible overexpressing models

We used tetracyclin-inducible models, which allow tightly activation of transgene upon doxycycline adding to the media. To generate them, we used plasmids supplied in the Retro-X™ Tet-One™ Inducible Expression System Kit (Clonetech). Briefly, two rounds of retroviral infection must be done to generate the cell model. In the first one, TET gene has to be transfected constitutively to the cell line (obtaining the TET cell line). After selection, TET cell lines suffer a second round of infection in order to integrate the desired transgene cloned under the control of TET-inducible promoter. Doxyciline binds to TET protein and allows the expression of this transgene (Figure 27).

Both rounds of retroviral infection were done following the previous infection protocol with small changes. Remarkably, TET cell lines were generated by retroviral plasmids containing neoMYCin resistance, so they were selected for 14 days with G418. Selected TET cell lines were cryopreserved or secondly-infected with a plasmid containing the transgene of interest and puroMYCin resistance. Importantly, normal FBS can contain traces of doxycicine, so it is mandatory to use FBS-Tetracycline free

in order to maintain the system off in all steps. Inducible cells lines must be grown in media containing FBS-Tetracycline free sera (Clonetech).

6. Chromatin immunoprecipitation (ChIP) experiments

6.1 Cyclin D1 ChIP

DNA Fifty million of exponential-growing cells were crosslinked for 10 minutes in 1% (v/v) formaldehyde at room temperature, neutralized with 125mM of glycine and washed twice with cold PBS. Cells were lysed according to the manufacturer's guidelines and chromatin was fragmented with the Covaris S220 instrument for 20 min (5% duty factor, 135w intensity, 200 cycles per burst) using the truChIP™ HighCell Chromatin Shearing Kit with Non-ionic Shearing Buffer (Covaris). Sheared chromatin was clarified by 10 mins of maximum speed centrifugation at 4°C. Sheared DNA was immunoprecipitated overnight at 4ªC with 20 µg of anti-cyclin D1 antibody or equivalent amount of control IgG. Antibody complexes were recovered with 80µL of equilibrated Protein G Dynabeads (Thermo Fisher Scientific) through co-incubation for 90 min at 4°C. After intensive washes in low salt buffer (1% Triton X-100, 150nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), high salt buffer (1% Triton X-100, 500nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), LiCl buffer(1% Nonidet 40, 0.25M LiCl, 10mM TrisHCl pH=8.0, 1% sodium deoxycholate, 1mM EDTA) and TE (10mM Tris HCl pH=8.0, 1mM EDTA), bound chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M $NaHCO_3$). Chromatin was decrosslinked for 4 hours at 65°C in the presence of NaCl and Proteinase K. Lastly, samples were treated with RNAse and Proteinase K before purification. Agencourt AMPure beads (Beckman Coulter) were used to purify DNA according to the manufacturer's guidelines.1% of sheared DNA was used as input control and it was used for qPCR validation and for analyzing sonication efficiency (Figure 28).

6.2 Pol II ChIP

Twenty million of exponential-growing cells were crosslinked for 10 minutes in 1% (v/v) formaldehyde at room temperature, neutralized with 125Mm of glycine and washed twice with cold PBS. Cytoplasmic membranes were lysed in Pol II lysis buffer (1%SDS, 10mM EDTA, 50mM Tris-HCl pH=8) with protease inhibitors (Thermo Fisher Scientific) for 5minuts incubation on ice and nuclei were pelleted by centrifugation (2000g, 5min, 4°C). Pol II lysis buffer was added to obtain a concentration of 25 million nuclei/mL and sonicated with a Bioruptor (Diagenode) for 18 mins (Cycles: 30''on/30''off, High Power, 4°C). In order to precipitate the SDS, sonicates were incubated on ice for 30-60 mins. Following 15 mins 4ºC centrifugation at maximum speed, the supernatants were diluted with ChIP buffer between 8-10 times. ChIP buffer was added to ensure that all samples had the same final volume.30µg of total chromatin were immunoprecipitated overnight at 4ºC with 20 µg of anti-cyclin D1 antibody or equivalent amount of control IgG. Antibody complexes were recovered with 80µL of equilibrated Protein G Dynabeads (Thermo Fisher Scientific) though co-incubation for 90 min at 4°C. Beads were washed for 5 mins at 4ºC in low salt buffer (1% Triton X-100, 150nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), high salt buffer (1% Triton X-100, 500nM NaCl, 20mM Tris-HCl pH=8.0, 0.1% SDS, 2mM EDTA), LiCl buffer(1% Nonidet 40, 0.25M LiCl, 10mM TrisHCl pH=8.0, 1% sodium deoxycholate, 1mM EDTA) and TE (10mM

Tris HCl pH=8.0, 1mM EDTA). Bound chromatin was eluted in ChIP elution buffer (1% SDS, 0.1M $NaHCO_3$). Chromatin was decrosslinked for 4 hours at 65°C in the presence of NaCl and Proteinase K. Lastly, samples were treated with RNAse A (Sigma Aldrich) and Proteinase K before purification. Samples were purified using the phenol-chloroform method.1% of sheared DNA was used as input control, for qPCR validation and for analyzing sonication efficiency.

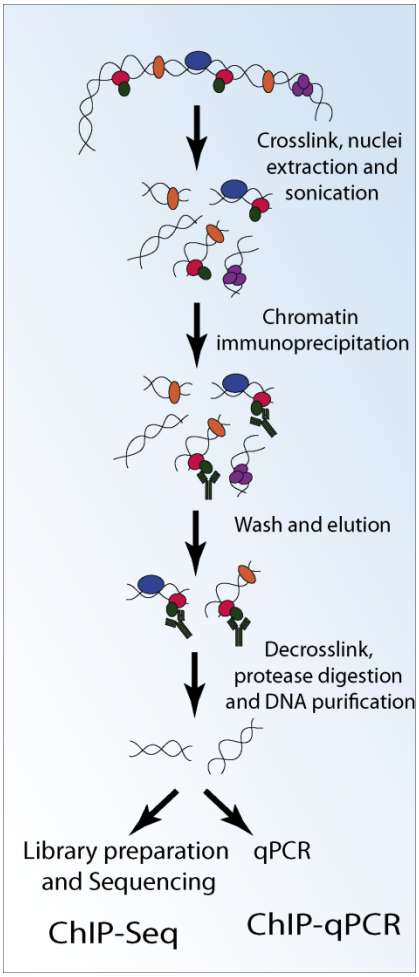


Figure 28. Schematic representation of ChIP-Seq and ChIP-PCR experiments. Briefly, DNA is crosslinked and nuclei are extracted from cell lines. Sonication is used for DNA shearing and generating small chromatin fragments that can be specifically immunoprecipitated with antibodies of interest. After extensive washes, DNA is decrosslinked, proteins and RNA are removed and DNA is purified.

6.3 ChIP-qPCR

ChIP experiments were validated using quantitative PCR. Same volumes of eluted chipped DNA were amplified using SYBR Green PCR Master Mix (Applied Biosystems) under the manufacturer's guidelines. Specific primers were designed on the peaks obtained in the ChIP-Seq analysis using Primer3 program (*bioinfo.ut.ee/primer3-0.4.0/primer3/*) (Appendix 2). Primers for negative regions were also designed to test the specificity of the enrichment. We also performed amplification with the DNA immunoprecipitated IgG and the 1% Input recovered.

7. Cell cytometry

7.1 Cell cyle: EdU incorporation/PI

Exponential growing cells were incubated one hour in cell incubator after adding 20uM of EdU to detect nucleotide incorporation. Between 0.5-1 million cells were centrifuged and washed with PBS RT. Ethanol fixation 70% was performed resuspending cells in 300uL of cold PBS and adding drop by drop and mixing -20°C absolute ethanol while vortexing. Fixed cells were stored at -20 degress for at least one overnight. Fixed cells were centrifugued and washed once in RT PBS. Cells were incubated 10 mins in 500uL of Saponin buffer (50ul FBS+50saponin 1%+400uL PBS) and centrifuged RT. EdU detecting reaction was performed according to Click-iT® Plus EdU Pacific Blue™ Flow Cytometry Assay Kit (ThermoFisher). After 30 mins incubation in Click-It reaction cocktail, cells were centrifugued and incubated for additional 30 mins in 500 uL of PI (Propidium iodide) staining solution (500 ul of PBS+2.5ug of PI+100ug of RNAse A). Edu-Pacific blue was excited by 405 nm laser and emitted at 450 nm. PI was excited by 488 nm laser and signal was registered using the filter 562-585 nm (Figure 29).

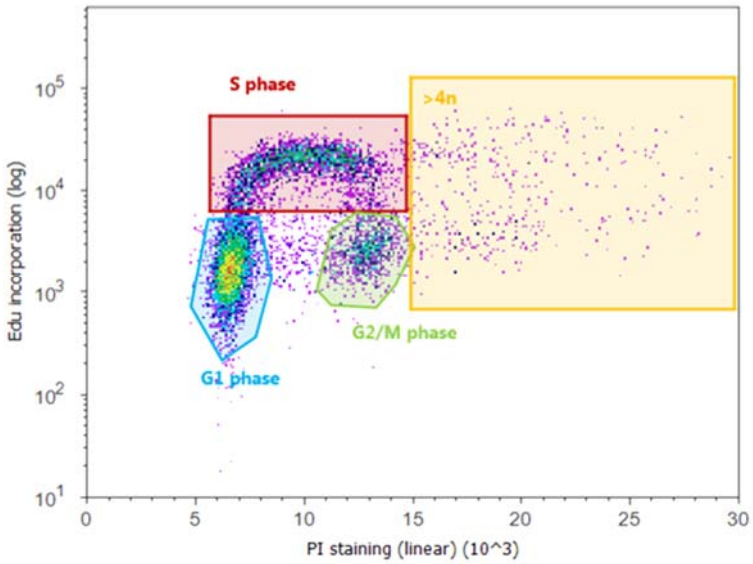


Figure 29. Edu/PI cell cycle analysis. Proliferating cells in S-phase (red gate) incorporate EdU, what can be detected by flow citometry. EdU negative cells G1/G0 cells (blue gate) and G2/M cells (green) gate are separated by different DNA conent (PI staining). Cells having high PI signal are classified as >4n (tetraploidy)

7.2 Apoptosis: Annexin/PI

Apoptosis was analyzed using Annexin V Apoptosis Detection Kit FITC (eBioscience) in a Attune Nxt cytometer (Thermo Fisher) according to manufacturer's guidelines. Briefly, one million cells were centrifuged and washed once with Annexin incubation buffer RT. Cells were incubated in Annexin incubation buffer including Annexin-FITC for 20-30 mins at RT. Cells were centrifuged and 500uL of Annexin incubation buffer including PI was added to all samples. Annexin-FITC and PI were excited by 488nm laser. FITC was analyzed using default filter with a range of 507-537nm and PI signal was registered using the filter >645nm (Figure 30).

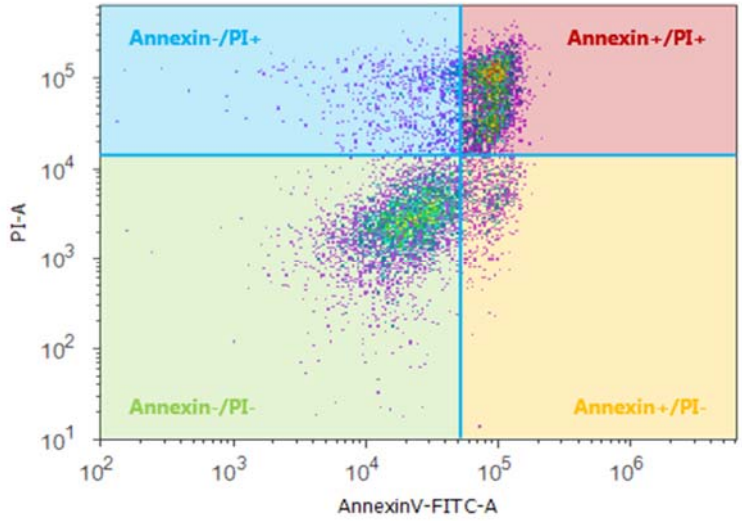


Figure 30. Annexin V/PI staining. Annexin V-FITC binds strongly to phospholipid phosphatidylserine translocated in apoptotic cells (Annexin V positive). Viable cells with intact membranes exclude P (PI negative cells)I, whereas the membranes of dead and damaged cells are permeable to PI (PI positive cells). Living cells, thus, display Annexin V/PI negative signal.

7.3 S-phase replication time calculation: EdU-BrdU incorporation/PI

Exponential JVM-13 growing cells (JVM-13 Ctrl, JVM13-D1^{T286A} and JVM-13-D1) were incubated in the presence of doxycycline(0.1ng/mL) for three hours in the cell incubator. Then, 20uM of EdU was added to mark S-phase cells. One hour after, cells were centrifuged and EdU was removed, supplementing RPMI complete media with 0.1ng/mL of doxyciclin. After 10 hours, 10uM of BrDu was added for 30 mins. One million cells were centrifuged and washed with PBS RT. Ethanol fixation 70% was performed resuspending cells in 300uL of cold PBS and adding drop by drop and mixing -20°C absolute ethanol while vortexing. Fixed cells were stored at -20 degress for at least overnight (Figure 31).

Fixed cells were centrifuged and washed once in RT PBS. To detect BrdU marked cells, DNA denaturation is firstly needed. To perform it, we resuspend pellet in 1ml 2M HCl/0.1%triton.Add 1mL 2M HCl more and incubate for 30 mins at room temperature. Add 4mL of 0.5M Sodium Borate buffer (pH 8) /sample and centrifuge cells. Repeat Sodium Borate wash with 4 mL and incubate cells in blocking solution(1mL of 0.1%Tween-20 and 1% FBS in PBS) for 15 mins. EdU detecting reaction was performed according to Click-iT® Plus EdU Pacific Blue™ Flow Cytometry Assay Kit (ThermoFisher). After 30 mins incubation in Click-It reaction cocktail, cells were centrifuged and incubated one hour at RT with 1/150 of BrDu antibody in blocking solution. After one hour, centrifuge cells and incubate with 1/400 Anti-rat A488 secondary antibody in PBS for additional 45 mins. Cells were centrifuged again and incubated for additional 30 mins in 500 uL of PI (Propidium iodide) staining solution (500 ul of PBS+2.5ug of PI+100ug of RNAse A). Edu-Pacific blue was excited by 405 nm laser and emitted at 450 nm. PI was excited by 488 nm laser and signal was registered using the filter 562-585 nm. A488 secondary antibody was excited by 488 nm laser and signal was analyzed using default filter with a range of 507-537nm.

7.4 Cytometric cell counting

Pellets of exact number of cells for certain experiments were done using cell cytometry counting. Briefly, 100uL of cell suspension were resuspended in 400uL of PBS.250uL of this solution were analyzed in the citometer, calculating the number of events in the gate corresponding to living cells. Cell quantification was calculated as (number of events/50) million cells/mL. Cell concentration was calculated as the mean of duplicates using Attune Nxt (Thermo Fisher).

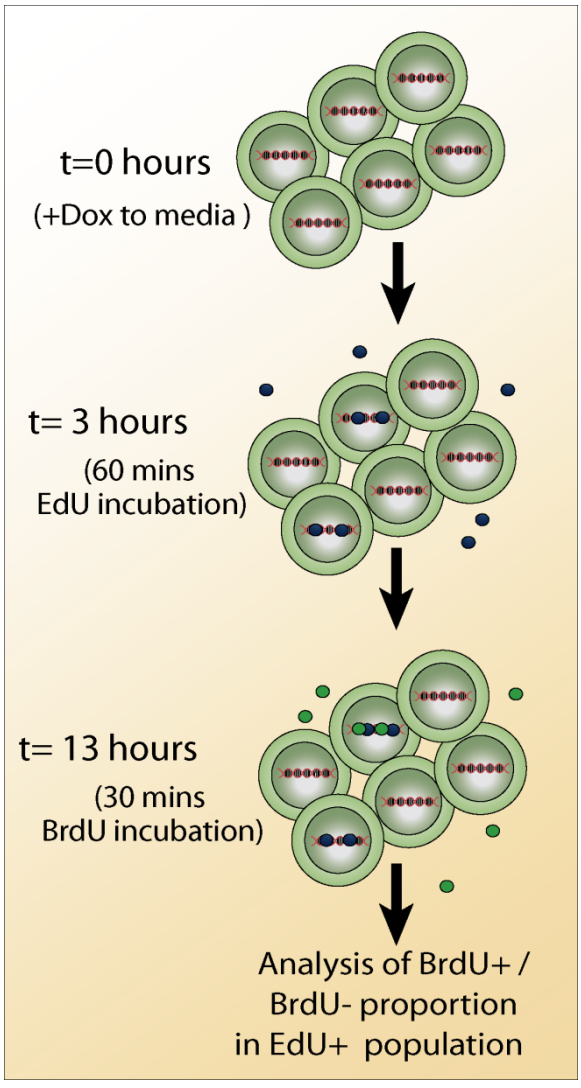


Figure 31. Scheme of the S-phase replication time. Doxycycline is added at time 0. Three hours after, S-phase cells incorporate EdU (blue circles).13 hours after, cells still in S-phase will incoporate also BrdU (green circles). Cell cytometry allow us to calculate the proportion of Brdu+ cell in the EdU+ population.

7.5 Cytometric RNA quantification

$1x10^6$cells were fixed in 0.5% formaldehyde for 15 min on ice. We pelleted cells and washed them with cold PBS. We resuspended them in 300uL of cold PBS and we added 700uL of 100% cold ethanol dropwise while vortexing. We incubated cells overnight at -20°C . Pellets were washed once with PBS at RT and resuspended in 250mL of HBSS medium with calcium and magnesium. This suspension was incubated with a final concentration of 4ng/mL of Hoechst 33342 (sigma) and 8 ng/mL of Pyronin

Y (sigma) for 20 mins at RT. Hoechst 33342 was excited by 355 nm laser and emitted at 450 nm. Pyronin Y was excited by 488 nm laser, emitted at 560 nm.

8. Co-Immunoprecipitation

8.1 Cyclin D1 immunoprecipitation

Exponentially growing cells/IP are washed in PBS RT (300G, 5mins). Prepare pellets of 40million cells and resuspend them in 1mL of SuperB protein extraction buffer (COVARIS), adding protease and phosphatase inhibitors (Thermo). Sonicate using the Covaris S220 instrument for 10 min (10% duty factor, 70w intensity, 200 cycles per burst).Use 450uL of sonicated extract/IP and keep 10uL of sonicated as input.450uL of sonicated extract/IP were incubated overnight at 4°C with gently mixing with 8ug of Cyclin D1 mouse antibody or IgG Mouse control antibody. Then, 50uL of protein G-magnetic beads (Invitrogen) /sample were equilibrated (washed twice with co-IP buffer). Beads were finally resuspended in 25uL of co-IP buffer and added to co-IPS. After 180 mins incubation, supernantats were recovered (Unbound fractions) and beads were washed twice with PBS-Tween-20 (0.2%) at 4°C . To elute, use 36uL of 100mM glycine (pH=2.5) for 10 mins at 65°C in strong mixing. Add 4uL of Tris-HCl (pH=10) and repeat twice more. Finally, 10uL of Sample buffer (5x) were added to each of the three elutions and they were boiled as usual for western blotting. Protein A-HRP conjugate or Anti-Mouse light chain antibody- HRP conjugate were use to detect primary antibodies (Figure 32).

8.2 CDK9 immunoprecipitation

Approximately 15-20 million exponentially growing cells/IP are washed in PBS RT (300G,5mins) twice. Protein extracts are done as indicated before but with CDK9-CoIP buffer (0.5% Nonidet-40, 150nM NaCl, 10mM KCl, 1.5mM MgCL, 10mM Tris-HCl pH=8.0) adding protease and phosphatase inhibitors and final concentration of 1Mm of DTT.1.5mg of total protein/IP were incubated overnight at 4°C with gently mixing with 7.5ug of CDK9 antibody or IgG Mouse control antibody in a final volume of 350uL of CDK9 Co-IP buffer. Then, 50uL of protein G-magnetic beads (Invitrogen) /sample were equilibrated (washed twice with co-IP buffer). Beads were finally resuspended in 25uL of co-IP buffer and added to co-IPS.90 mins after, supernatats were recovered (Unbound fractions) and beads were washed twice for 5 mins with gentle rotating at 4°C with co-IP buffer. To elute, 40uL of sample buffer (x2.5) without DTT were added to beads and hating at 70°C for 10 mins. Second elution was repeated with 40uL of sample buffer (x2.5) and 5uL of DTT 1M. Finally, 5uL of DTT 1M were added to first elution and both sample were boiled as usual for western blotting. Protein A-HRP conjugate or Anti-Mouse light chain antibody- HRP conjugate were use to detect primary antibodies.

9. High-throughput profiling:

9.1 DNA-methylation microarray analysis

The Infinium Assay HumanMethylation27 BeadChip from Illumina was used to quantify the DNA methylation of the samples following the manufacturer's protocols. The Illumina BeadChips were scanned with an Illumina BeadArray Reader and then

preprocessed by the Illumina GenomeStudio software. This array permits the evaluation of the CpG methylation status of 27,578 CpGs islands and methylation hotspots. The output files were processed with the Bioconductor lumi package to get the M-values as a measurement of the methylation levels.[15] A quality control filtering based in the detection p-value was applied. CpG probes that showed a detection p-value >0.05 in more than 10% of the samples were excluded. Afterwards, the array methylation data were color balance adjusted and quantile normalized following the pipeline implemented in the lumi package. Since the biological interpretation of the traditional B-value is more intuitive than the M-values, we generated the final B-values from the M-values following the equation $Beta_i = 2^{Mi} / 2^{Mi} + 1$ for an i^{th} interrogated CpG site. In order to avoid a gender specific methylation bias we removed all probes present on both sex chromosomes.

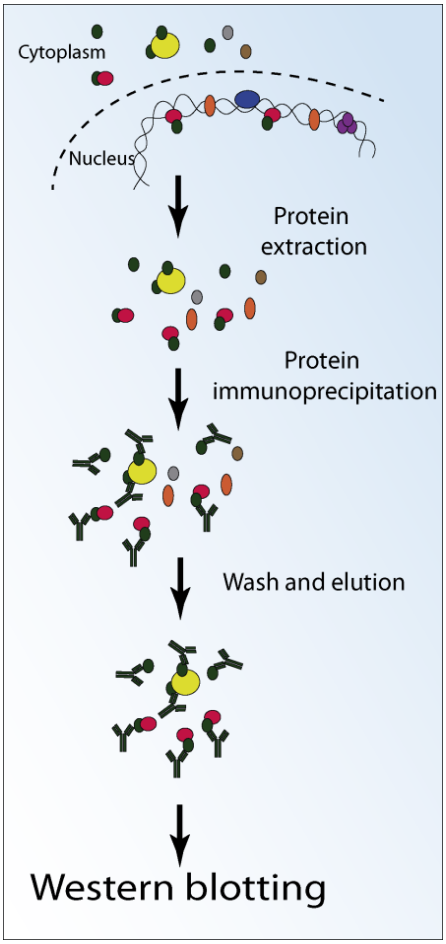


Figure 32. Schematic representation of co-immunoprecpiation experiments. Total cell extracts of exponential gorwing cells are obtained and quantified. Same protein amounts are incubated with specific antibodies or IgG (control). After extensive washes, bound proteins are eluted and Western bloting for detect co-immunoprecipitated proteins is carried out.

9.3 Gene expression microarrays

Total RNA was extracted with the TRIzol reagent following the recommendations of the manufacturer from JVM-13 constituve cyclin D1-overexpression models. RNA integrity was examined with the Agilent 2100 Bioanalyzer (Agilent Technologies) and only high quality RNA samples were hybridized to GeneChip™ Human Transcriptome Array 2.0,

according to Thermo Fisher guidelines. The analysis of the scanned images and the determination of the detection call for each probe set of the array were obtained and summarized expression values were computed using the expression console software.

9.3 Digital quantification of gene expression

Cytometric counted pellets of $4x10^5$ were lysed in 40uL Buffer RLT (Qiagen) for 1 minute at room temperature with strong vortexing and frozen immediately in dry-ice. We followed the manufacturer's guidelines for the nCounter cell lysate hybridization and used two different gene expressions panels: nCounter Human Cancer Reference Kit (GXA-CR1-12) and nCounter CAE Kit (GXA-CAE-12, Nanostring Technologies).

10. Sequencing

10.1 ChIP-Sequencing

Library preparation was performed using the NEBNext Ultra DNA Library Prep kit (New England BioLabs). Briefly, immunoprecipitated DNA (10 ng) was end-repaired, and A-nucleotide overhangs were then added, followed by adapter ligation, PCR enrichment(15 cycles) and purification/size selection (1.25X ratio Agencourt AMPure beads , Beckman-Coulter). The purified DNA library products were evaluated and quantified using Bioanalyzer (Agilent, High Sensitivity DNA Kit) and the KAPA Library Quantification Kit (KapaBiosystems), respectively. Sequencing was performed on the Illumina MiSeq instrument using 50 cycles V2 kit.

10.2 RNA-Sequencing

Total RNA was isolated using Trizol (Zymo Research), under the manufacturer's guidelines. Preparation of RNA-seq libraries was carried out using the TruSeq RNA Sample Preparation Kit according to the manufacturer's standard protocol. Briefly, mRNA molecules were purified from 800 ng of total RNA using poly-T oligo attached magnetic beads. Following purification, mRNA was fragmented using divalent cations at 94°C and copied into first strand cDNA using reverse transcriptase and random primers. Second strand cDNA synthesis was performed using DNA Polymerase I and RNase H. The cDNA fragments were gone through an end repair process, the addition of a single ‘A’ base, and then ligation of the adapters. The products were then purified and enriched with PCR to create the final cDNA library. The samples were then 75 bp paired-end sequenced at ~ 80 million reads per sample with Illumina technology.

11. Bioinformatics

11.1 ChIP-Sequencing analysis

ChIP-Sequenced reads were aligned using Bowtie (http://bowtie-bio.sourceforge.net/index.shtml, v4.3.4) against the human GRCh37/Hhg19 reference genome. Peak calling was performed with the Model-based Analysis for ChIP-Seq (MACS, http://liulab.dfci.harvard.edu/MACS/, v1.4.2). Peaks were visualized on the UCSC browser (https://genome.ucsc.edu/). Annotation and average was achieved using the cis-regulatory element annotation system (CEAS, http://liulab.dfci.harvard.edu/CEAS/ v0.9.9.7). In addition, we used a separate script in

the CEAS package named "sitepro" which draws the average signal in a user-provided list of sites to visualize the average signal. Seqminer v1.3 (http://www.hsph.harvard.edu/cli/complab/dchip/) was used to study genomic clusters. Pausing ratio, also known as travelling ratio, was calculated as indicated before (Rahl et al., 2010). It measures indicated the quantity of stalled Pol II in a group of promoters. Pausing ratio values are calculated for all genes. For a certain gene, it is calculated dividing the number of tags between (-30,+300) around TSS and the number of tags in gene body (from +300 of TSS to TTS). Following perturbation from overexpressing proteins/drugs, pausing ratio can shift either through changes in the density of promoter proximal Pol II or changes in the gene body Pol II density. Statistical changes between correlations were studied using the Kolmogórov-Smirnov test.

11.2 RNA-Sequencing analysis

RNA-seq reads were aligned using Bowtie as described above. To quantify the expression of each gene we used TopHat v2.0.9 with default parameters and the human GRCh37/Hhg19 as a reference genome. Gene expression values (quantified as reads per kilobase of transcript per million reads, RPKMs) were calculated using edgeR Bioconductor package.

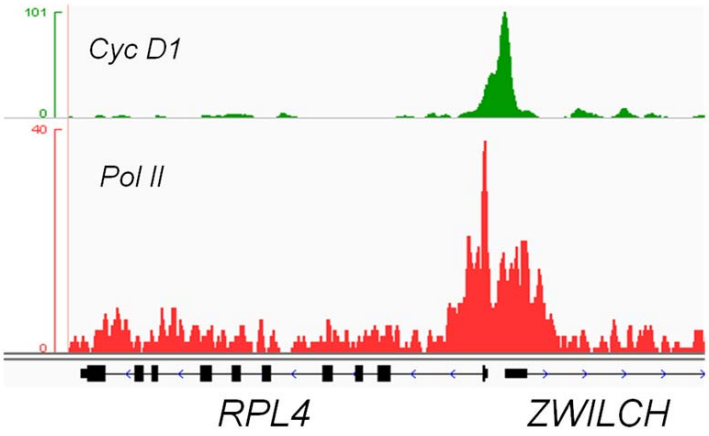


Figure 33. WIG files of ChIP-Seq results in JVM13-D1^{T286A} . Visualiation of the ChIP-Seq tracks of Cyclin D1 and Pol II in the bidirectional promoter of *RPL4/ZWILCH* genes, using the IGV software. Each each is represented with a different scale.

11.3 Transcription factor binding sites analysis

Two different types of transcription factor binding analysis were performed. Firstly, we performed motive analysis with the MEME Suite, a collection of tools for the discovery and analysis of sequence motifs (http://*meme-suite*.org/). We used the AME tool (Analysis of Motive Enrichment) to discover recurrent motives in promoter sequences bound by Cyclin D1. We focused in human motifs present in at least one of the two databases used (JASPAR CORE and JOLMA2013). Factors with adjusted p valor<0.001 were taken into consideration. Secondly, we also carried out co-localization analysis of transcription factor, comparing cyclin D1 bound regions with ChIP-seq data intervals from the ENCODE Project and available through the UCSC Genome Browser. In both cases, we used shuffled input sequences as controls.

11.4 Functional enrichment analysis

The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) v6.7 (https://david-d.ncifcrf.gov/)was used to identify the enriched pathways of genes bound by cyclin D1. We performed hierarchical cluster analysis using two databases (GOTERM_BP_FAT and KEGG_PATHWAY) ,what creates enriched clusters of functions in a certain gene set. Percentage of enrichment was calculated as described before (Casimiro et al., 2012) based on percent enrichment score of the top hits (Enrichment score>5). This measure is a percentage of the enrichment score a certain hit represent of the total sum of all enrichment scores considered.

12. Statistical analysis

12.1 Data visualization and management

Statistics and graphical results were done in R statistical computing language (R v3.1.3) and GraphPad Prism (v4). ChIP-Seq genomic tracks were visualized using the IGV software (version 2.3.81) (Figure 33) and the Genome Browser tool from the UCSC (http://genome-euro.ucsc.edu/cgi-bin/hgGateway). For genomic data management, the TableBrowser tool form the USCS and the Galaxy platform (https://usegalaxy.org/). Venn diagrams were performed using Venny software (http://bioinfogp.cnb.csic.es/tools/venny/)

12.2 DNA methylation array analysis

Hierarchical clusterings with the β-values were performed using Rank Spearman Dissimilarity and the Ward method implemented in the Partek Genomics Suite software (Partek Inc., St Louis, MO). A principal component analysis (PCA) also was performed with Partek. To identify highly significant differential methylation events between groups we established that the average geometric difference of β-value should be at least 0.3 ($\Delta\beta \geq 0.3$) and showed an adjusted p-value<0.001.

12.3 Gene expression arrays analysis

Gene expression profiling of 79 primary MCL for the Study 3 was performed using the Human 133 Plus 2.0 arrays and normalized using the MAS5 algorithm. Clustering analysis with gene expression data was performed with the Pearson correlation metric and the centroid linkage method using the D-CHIP application(Li and Wong, 2001). Differential expression analysis among groups was performed by a multivariate permutation test implemented in the BRB-tool application(Li and Wong, 2001). We used the multivariate permutation test to provide 90% confidence that the false discovery rate was less than 5%. A likelihood ratio-test was performed to test the bimodal distribution of hypermethylated CpG in primary MCL. GeneChip™ Human Transcriptome Array 2.0 were used for JVM-13 constitutive models of cyclin D1 overexpression and analyzed as indicated before.

12.4 Correlation of DNA methylation and expression

To correlate methylation data with gene expression, the probes of the HU133 plus 2.0 array were collapsed to HUGO gene symbol using the probe set that showed the higher median expression level. Functional annotation enrichment and pathway

analysis was performed with the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA) and DAVID Bioinformatics Resources applications.

12.5 Survival analysis

Statistical evaluation of clinical variables was performed using nonparametric tests. Overall survival was estimated using the Kaplan-Meier and compared by means of the log-rank test and Breslow tests. Cox-regression analysis was used to estimate the hazard ratio with 95% confidence interval to determine independent prognostic factors for survival and to correct the confounding effect of differences in prognostic factors. The association between two variables was computed using Pearson correlation. Categorical data were compared using Fisher's exact test for a two-sided p-value, whereas for ordinal data, nonparametric tests were used. Chi-square tests were used to compare percentages in cross tabulations. Paired and non-paired t-test and Wilcox test were used as indicated to calculate statistical changes in mean between conditions/samples. The level of significance was set at 0.05 for positive statistical significance. All calculations were performed with the SPSS software (v.18), R package (v3.1.3) and GraphPad Prism (v4).

13. Other techniques

13.1 Fiber assay

Fiber assay procedures are divided in different steps: *In vivo* nascent DNA labeling, preparation of DNA spreads, immunofluorescence and image acquisition/analysis. Firstly, exponentially growing cells are initially pulse labelled with the first nucleotide analogue (CldU) to a final concentration of 25uM.30 mins after, centrifuge cells (5 mins, 300g,RT) and change media supplemented with IdU (250uM) . Incubate for additional 30 mins. After double labeling, cells are washed with ice cold PBS, harvested and resuspended in ice cold PBS to the 0.6 million cells/mL. Keep labeled cells on ice.2uL of cell suspension are spotted at the end of the microscope slide and air – dried for 5 min. Subsequently, 7 µl of the lysis solution(100mM Tris-HCl pH=7.4,50mM EDTA, 0.5% SDS) are applied on top of the cell suspension, then mixed by gently stirring with a pipette tip and incubated for 2 min. Following cell lysis, slides are tilted to 15° to allow the DNA fibres spreading along the slide. Blocked drops or too fast drops egressing the microscope slide before 2 mins were discarded. Let slides air-dry and fix samples in methanol/acetic acid (3:1) solution in a staining jar and incubated for 10 min. Store slides overnight at 4°C .

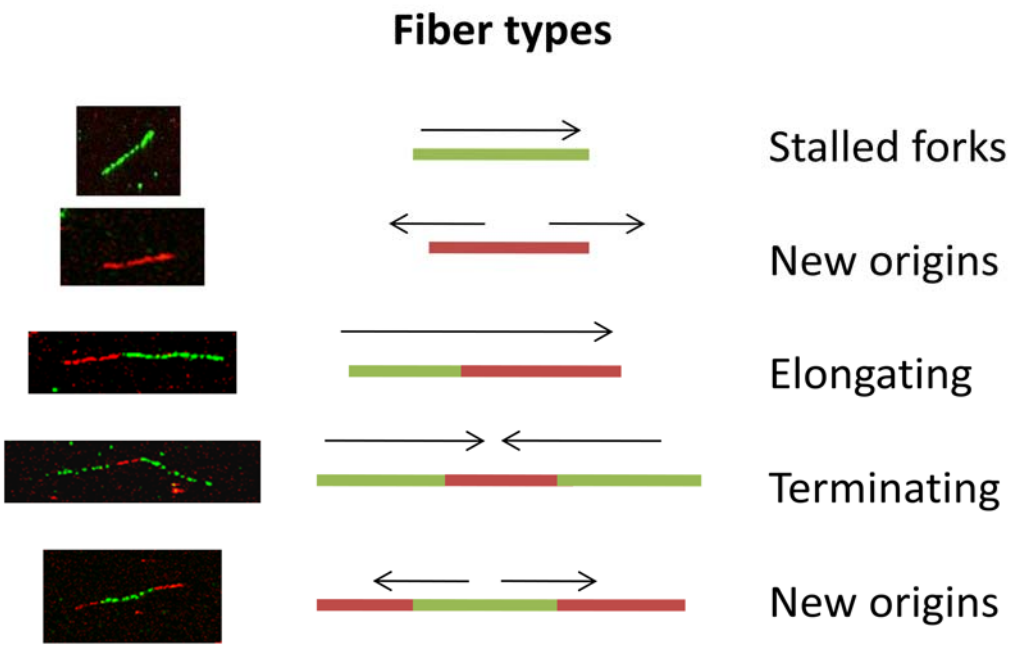


Figure 34. Examples and schematic representations of different labeling variants obtained in fiber experiments. Black arrows represent the direction of replication forks

The following day, slides are washed twice in distilled H2O for 5 mins and immersed in 2.5 M HCl for 75 min. After DNA denaturation, slides are washed twice in PBS. Wash cells once with blocking solution (1% BSA,0.1%Tween in PBS). After, 100uL of blocking solution is applied on each slide and they are gently covered with a coverslip to spread the blocking solution evenly. During the blocking step, the primary antibody solution is prepared (1:200 anti-BrdU (mouse) and 1:1000 anti-BrdU (rat) in blocking solution). After blocking 30-60 mins, the coverslips are removed by gently moving down the slide without applying force. Add 100 µl of the primary antibody solution onto each slide, and cover again with coverslips. Incubate 90 mins at 37°C . Coverslips are removed as before and slides washed three times with PBS. Add 100uL solution of 4%PFA in PBS and fix samples for 10 mins at RT. Wash 3 times with PBS and once with blocking solution. Add 100uL of secondary antibodies diluted in blocking solution onto the slides, covered again with coverslips. Protected from light and incubate 60-90 mins at 37°C . Remove coverslips and wash three-five times with RT PBS. Spot mounting media onto each slide and cover with coverslips gently by pressing down. Slides are sealed with transparent nail polish, let to dry and stored at 4°C .

Stained DNA fibers are visualized using a confocal microscope. To avoid bias only one channel is used to select regions for taking pictures. Pictures were taken just 24-48 hours after finishing the immunofluorescence protocol. Pictures are t analysed using an image analysis software (ImageJ, http://rsbweb.nih.gov/ij/). This software allows to distinguish different patterns of fibers (stalled, elonging,…forks). In addition, replication speed was calculating as the mean of length of IdU signal divided 30mins, in µm/min. Asymmetry was calculated using fibers with the pattern red-green-red, where elonging (red) tracks could be measured. The “asymmetry value” was calculated following the next equation (log2 (Length of the maximum elonging track/length of the minimum elonging track. Samples with “asymmetry value” close to zero represent perfectly

symmetric forks, while samples presenting values far from zero represent forks subpopulation with replication problems (Figure 34).

13.2 Pyrosequencing analysis

Methylation analysis by bisulfite pyrosequencing technology was performed with the PyroMark Q96 ID platform according to standard protocols (Qiagen). Pyrosequencing results were evaluated with the analysis software PyroMark-CpG Software 1.0. Extracted DNAs were bisulfite converted using the EpiTect Plus DNA Bisulfite Kit (Qiagen), according to the manufacturer's instructions. Two amplicons to evaluate 3 and 6 CpG sites in *SOX9* and *SFRP1* genes, respectively, were designed using the PyroMark Assay Design Software 2.0 and amplified using the Pyromark PCR Kit (Qiagen). The forward and reverse primers for the different amplicons appear in the Apendix 1.

13.3 Tissue micro-array (TMA) generation and Immunohistochemistry (IHC)

TMAs were constructed by our tumor bank with double-core representation of each case studied (diameter of cores from 1 to 2mm). Both TMAs and whole slide sections were stained for γH2AX and pCHK2 in the Dako Autostainer machine Link 48 and manually. Specifically, for γH2AX,antigen retrieval was done for 5 min in Envision Flex TRS High pH (pH 9, DAKO) buffer solution at 98 °C. The primary antibody, polyclonal rabbit γH2AX (ser 139, Cell Signaling 2577, dilution 1/25) was incubated for 60 min. A peroxidase based EnVision Flex/HRP system (DAKO) was used for the detection (20min) and 3,3′-diaminobenzidine (DAB) as chromogen (10min). Haematoxylin (10 min) was used as a counterstain. For pCHK2, antigen retrieval was done for 5 min in Envision Flex TRS High pH (pH 9, DAKO) buffer solution at 98 °C. The primary antibody, polyclonal rabbit pCHK2 (thr 68, Cell Signaling 2661, dilution 1/50) was incubated for 60 min followed by amplification (only in the autostainer) with the secondary antibody Envision Flex Rabbit Linker for 15 min. A peroxidase based EnVision Flex/HRP system (DAKO) was used for the detection (20min) and 3,3′-diaminobenzidine as chromogen (10min). Haematoxylin was used as a counterstain.

We evaluated our cases using a semiquantitive score which takes under consideration both the intensity and the number of the positive cells. The cut-off for positivity was ≥10% positive nuclei and we evaluated the intensity as null (grade 0), mild (grade 1), moderate(grade 2) and strong(grade 3). The cases assigned grades 0-1 were grouped as Low and the rest as High. IHC slides were evaluated independently and blindly to the clinical data by three pathologists. Any disagreements were resolved in the multi-head microscope. The images of our cases were captured using an Olympus-BX51 microscope through an attached Olympus-DP70 digital camera, using an Imaging Software for Life Sciences Microscopy, Olympus-Cell/B-3.1.

STUDY I

Cyclin D1 oncogenic overexpression leads to a global transcription downregulation in malignant lymphoid cells

*Manuscript in preparation

1. Introduction

Cyclin D1 is a frequently dysregulated protein in a large variety of neoplasms through different mechanisms, including amplifications in breast (Burandt et al., 2016) and head and neck tumors (Jares et al., 1994) and point mutations disrupting the nuclear export process in esophageal and endometrial carcinomas (Benzeno et al., 2006; Moreno-Bueno et al., 2003). However, the most paradigmatic genetic events causing cyclin D1 overexpression are translocations in multiple myeloma (Avet-Loiseau et al., 1998) and mantle cell lymphoma (Jares et al, 2007). The relevance of cyclin D1 dysregulation in MCL pathogenesis is stressed by the recognition that MCL cells adopt different mechanisms such as amplification of the translocated allele or 3'UTR mutations or secondary rearrangements (Bea et al., 2009; Gruszka-Westwood et al., 2002; Rosenwald et al., 2003) to further increase cyclin D1 levels.

The classical tumorogenesis model considers that cyclin D1 performs its oncogenic effect through the phosphorylation of RB and E2F release promoting G1/S phase transition (Jares et al., 2007). However, during the last decade a growing body of evidence has established that cyclin D1 has additional roles besides its cell cycle canonical function (Casimiro et al., 2014; Coqueret, 2002; Pestell, 2013). The description of cyclin D1 interactions with transcription factors, chromatin-remodeling and histone-modifying enzymes in the context of local chromatin has revealed a potential transcription regulation role of cyclin D1 in different models (Fu et al., 2005b; Fu et al., 2004; Horstmann et al., 2000b; Reutens et al., 2001; Zwijsen et al., 1997). However, whether the oncogenic overexpression of cyclin D1 is responsible of transcription dysregulation in cancer cells remains unknown. The detection of MCL cases that show *RB1* inactivation by mutations and deletions (Pinyol et al., 2007), making cyclin D1 dispensable for cell cycle function, would support the idea that cyclin D1 may play additional oncogenic roles in these tumors beyond cell cycle regulation.

Putting everything together, we proposed a first study investigating the effects of cyclin D1 overexpression on transcription in MCL. We performed ChIP-seq analysis of MCL cell lines to characterize the chromatin genome-wide association of endogenous cyclin D1 in MCL cell lines, integrating this layer of information with gene expression and histone modifications. We also aimed to explore the molecular mechanisms behind cyclin D1 dependent transcription dysregulation and its therapeutic potential utility for MCL treatment.

2. Results

2.1 Cyclin D1 chromatin binding pattern in MCL cells

In order to characterize the chromatin genome-wide association of cyclin D1 we performed chromatin-immunoprecipitation sequencing (ChIP-seq) of endogenous cyclin D1 in four MCL cell lines (Z-138, GRANTA-519, JeKo1, and UPN-1) with variable levels of cyclin D1 protein overexpression (Figure 35A). Cyclin D1 peaks displayed well-defined profiles in all four samples. Signal-to-noise ratios can be evaluated by some representative examples of cyclin D1-bound intervals (Figure 35B). Noteworthy; we found an outstanding number of cyclin D1 DNA associated regions with 19860 peaks common to all four MCL cell lines (Figure 35C). Interestingly, the number of identified peaks showed a strong positive correlation with the amount of cyclin D1 protein (Figure 35D).

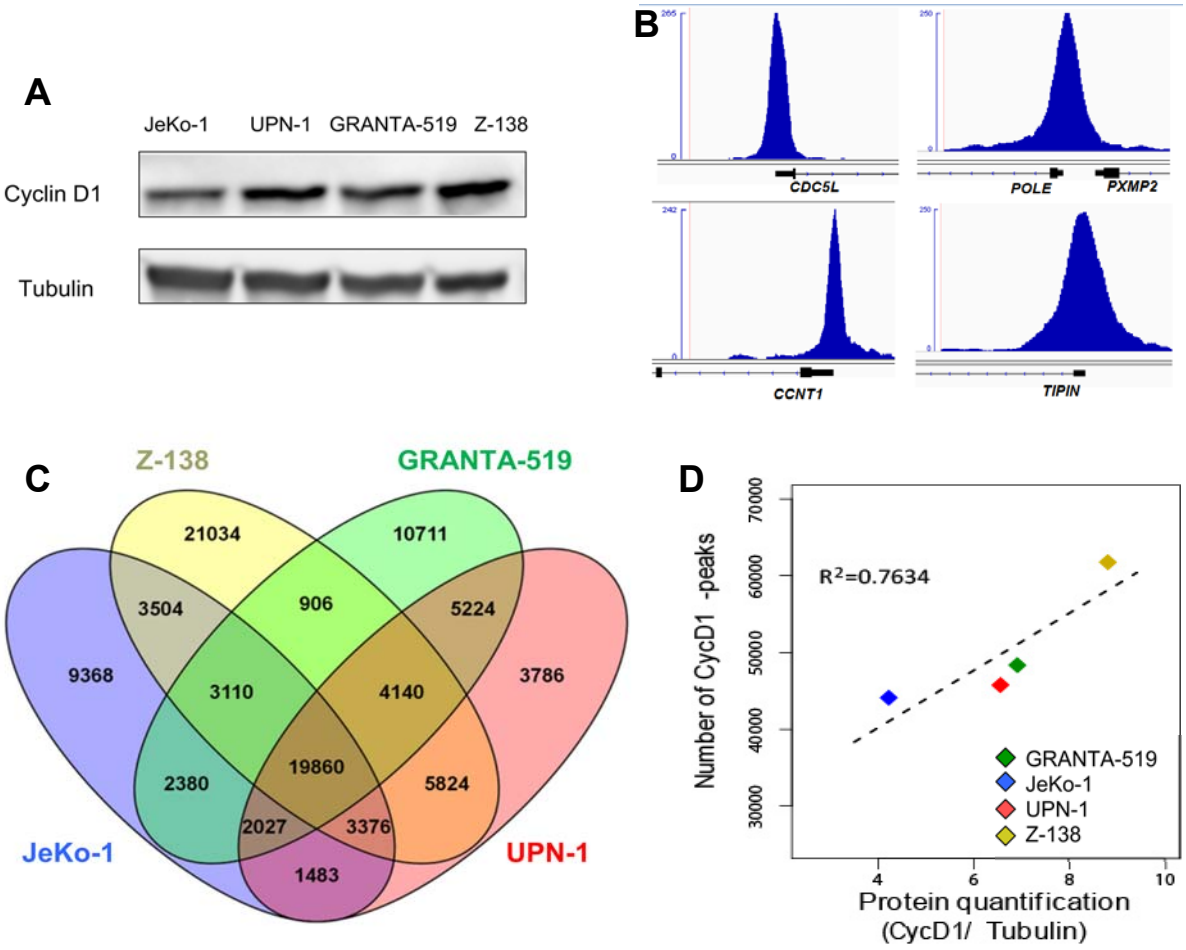


Figure 35. **Cyclin D1 binds genome-wide in MCL cell lines.** A) Western blot analysis of cyclin D1 in MCL cell lines. α-Tubulin was used as loading control. B) Genome browser view of the ChIP-seq tag density plots of four representative cyclin D1 target genes. C) Venn diagram representing cyclin D1 ChIP-seq peaks in four MCL cell lines. D) Linear correlation between cyclin D1 protein amount and the total number of ChIP-seq peaks in MCL cell lines.

Then, we wanted to investigate the genomic distribution of Cyclin D1-bound intervals. The annotation of the identified peaks revealed a highly significant enrichment in promoters and 5'UTR regions (p val<1E-100, Figure 36A). Using the CEAS package, we showed that the prevailing interactions at promoters tend to occur close and centered on the transcription start site (TSS) of the genes (Figure 36B). In total, an average of 11583 coding genes displayed cyclin D1 binding to their promoters and more than 74% of them were commonly found among the four MCL cell lines (n=8638) (Figure 36C). This extensive binding of cyclin D1 observed across the genome is consistent with a potential transcription role of cyclin D1 in MCL cells. Strikingly, cyclin D1 also bound a great number of bidirectional promoters. Bidirectional promoters are short (<2 Kb) intergenic regions of DNA between the 5' ends of two adjacent genes coded on opposite strands, with their 5' ends oriented head to head (Figure 36D). We found that around 68% of bidirectional promoters are bound by cyclin D1 in all four MCL cell lines cell lines (p val <1E-300).

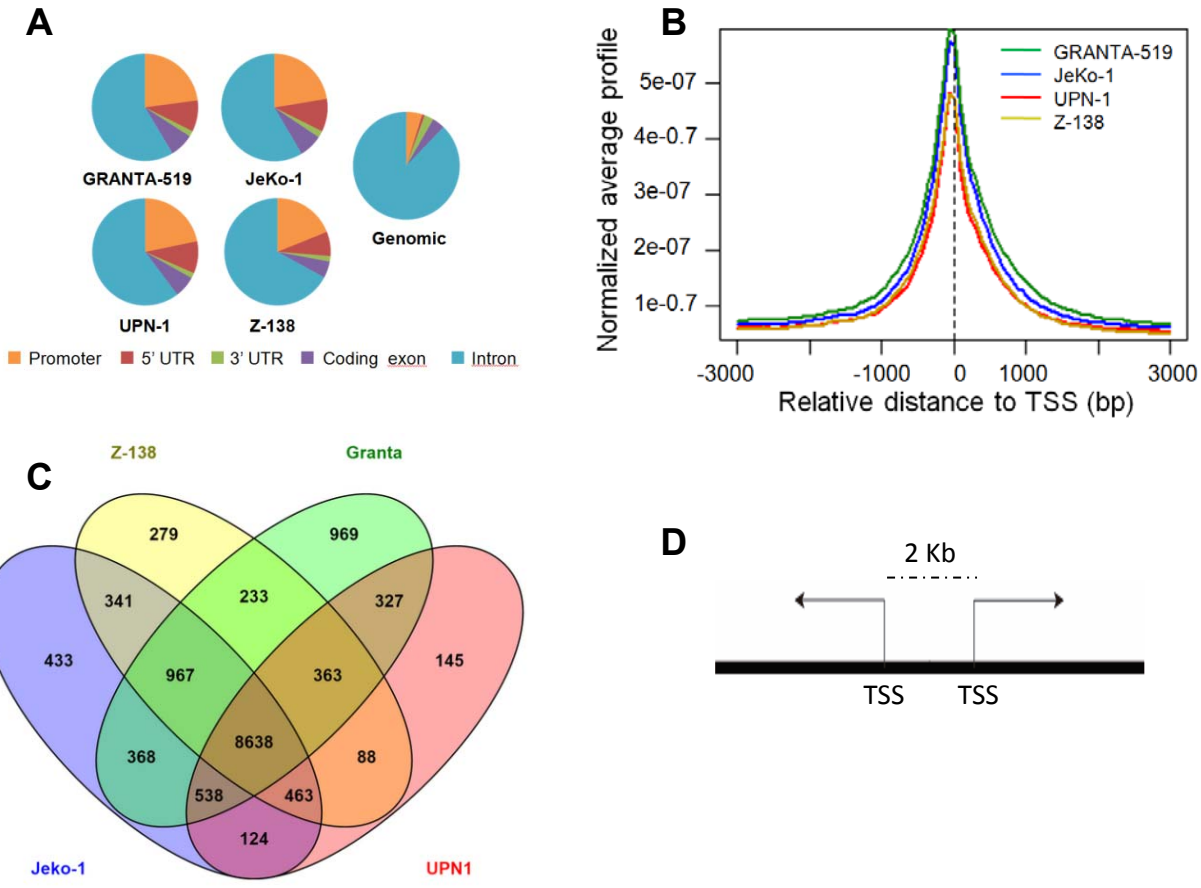


Figure 36. **Cyclin D1 binds to genomic promoters in MCL cell lines.** A) Distribution of cyclin D1 interacting regions in MCL cell lines over specific genomic regions in MCL cell lines. Promoter is defined as -3kb to TSS. The distribution across the human genome is represented as a control. B) Average signal profiling of cyclin D1 around the TSS (+/- 3kb) in MCL cell lines. C) Venn diagram representing cyclin D1 targeted genes identified by ChIP-seq in MCL cell lines. Genes were considered targets when displayed cyclin D1 binding sites located within 1kb upstream of their TSS. D) Schematic representation of the definition used for bidirectional promoter in our study.

Next, we investigated whether cyclin D1 bound genes shared functional characteristics. Functional annotation clustering revealed that these genes govern translation, RNA processing, and cell cycle, among other functions. All these results suggest that cyclin D1 is not binding to chromatin randomly, but showing specific patterns that reinforce the hypothesis of cyclin D1 as a transcription regulator (Figure 37).

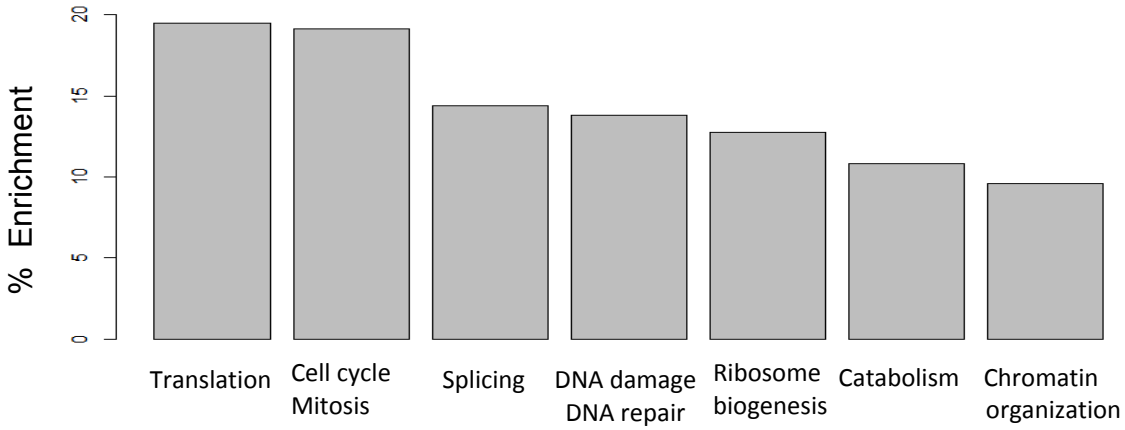


Figure 37. **Top hits of the functional annotation clustering analysis of common cyclin D1 target genes among the four MCL cell lines.** Only the genes with the most significant peaks in their promoters (-log p val >350) were considered for the analysis.

Finally, in order to validate our results we selected eight representative binding sites from the ChIP-seq analysis and performed Cyclin D1 ChIP-qPCR. All the tested genes showed a significant cyclin D1 binding enrichment when compared to a negative region (Figure 38).

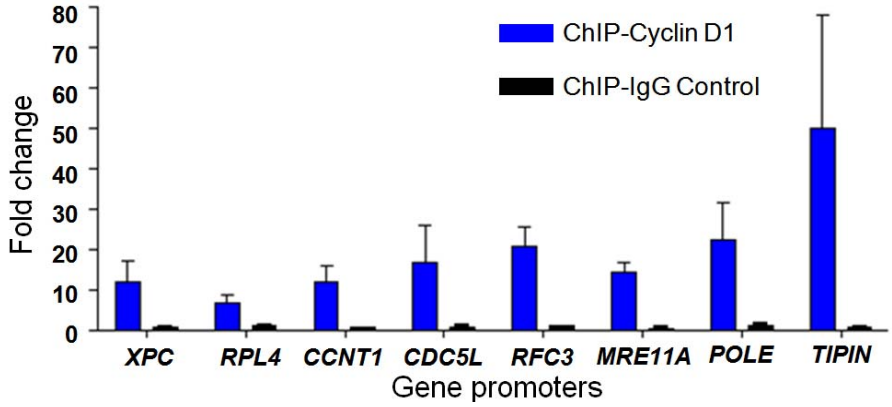


Figure 38. **ChIP-qPCR validation of eight selected cyclin D1 target genes in GRANTA-519.** The fold change enrichments relative to a negative region are represented as means ± SEM of two independent experiments.

2.2 Cyclin D1 binds to chromatin interacting with specific TFs in MCL cells

Cyclin D1 cannot bind directly to DNA, so its genomic binding depends on other proteins bound to DNA. We performed two different bioinformatic approaches to further study cyclin D1 interactions. First of all, we did a motif analysis using AME (MEME tools) involving the common intervals overlapping between the four cell lines. As expected, cyclin D1 binding regions showed a great number of transcription factor motives significantly enriched consistent with the previously described binding of cyclin

D1 to promoters. Among the identified transcription factors, some of them have previously been described to interact with cyclin D1, like NRF1 or AR. As expected, we found some transcription factors typical of bidirectional promoters (ELK1, GABPA) (Appendix 3). We performed functional annotation analysis of these TFs using the KEGG database and we confirmed the relationship between cyclin D1 binding and transcription regulation of certain pathways, such as cell cycle. In addition, we also detected other pathways, for instance "Pathways in cancer" or different signaling pathways related to cancer, such as: WNT or MAPK pathway (Appendix 4).

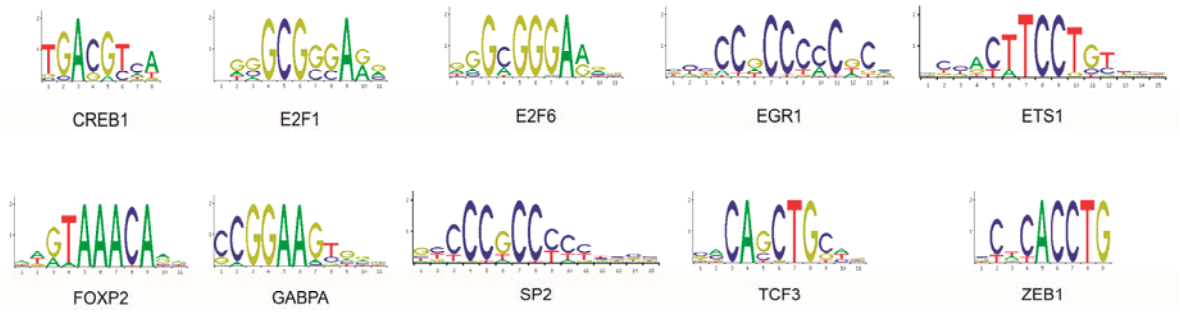


Figure 39. **TF motifs enriched in cyclin D1-bound intervals and confirmed by TF co- localization from ChIP-seq experiments performed by the ENCODE project.**

Secondly, we also analyzed the co-localization of cyclin D1 common peaks with other intervals coming from more than one hundred ChIP-seqs performed as a part of the ENCODE project. We identified also a high number of TFs with chromatin binding regions overlapping with cyclin D1 bound regions, suggesting that they might interact with cyclin D1. From the 25 top results, ten of them were also predicted using the motif analysis (Figure 39, Appendix 5). Moreover, this analysis displayed the likely localization of cyclin D1 with factors that are part of the basal transcription machinery, such as CCNT2, TAF7 or TAF1. This encouraged us to continue focusing on cyclin D1 role in promoters.

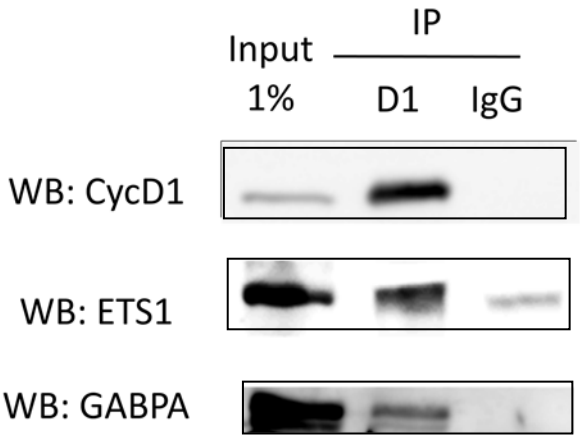


Figure 40. **Co-IP experiments showing cyclin D1 interaction with GABPA and ETS1 in Z-138.**

In order to validate our bioinformatic analysis, we carried out co-IP experiments (Figure 40) immunoprecipitating cyclin D1 and looking into its interaction with some predicted TFs. Cyclin D1 has been described to interact with SP factors and E2F1 and E2F6 in other models (Pauklin et al., 2016). We focused on ETS1 and GABPS because they

are proteins that bind bidirectional promoters. Agreeing with our analysis showing cyclin D1 enrichment in these kind of genomic regions, we observed that both TFs bind to cyclin D1. This result reinforces the idea that cyclin D1 may be performing a specific function in MCL promoters, especially in regulating bidirectional transcription.

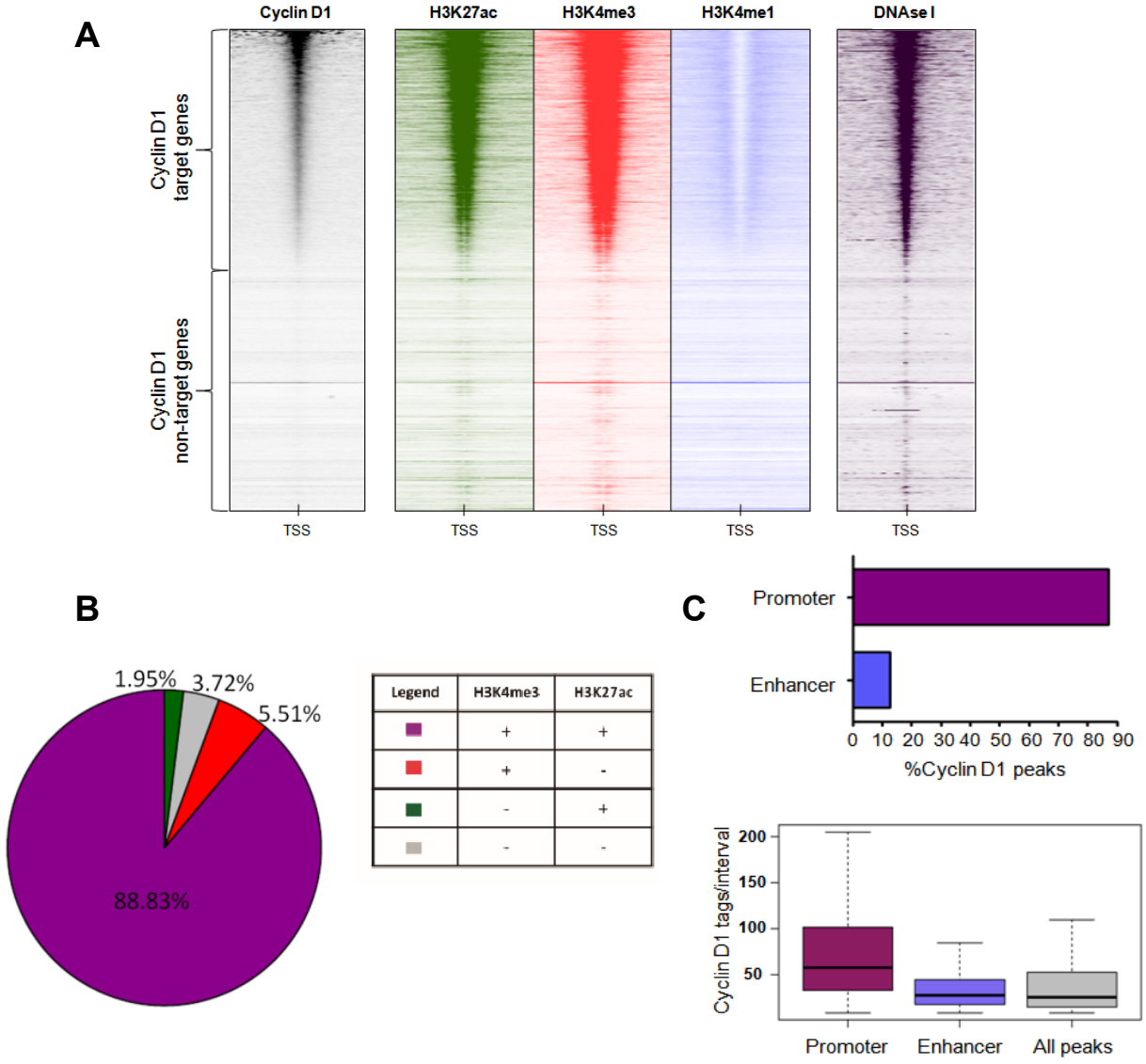


Figure 41. **Cyclin D1 occupancy correlates with active promoter marks and open chromatin conformation.** A) Heatmap showing the ChIP-seq tag density of cyclin D1, H3K27ac, H3K4me3, H3K4me1 and DNAse I cutting sites around all genomic TSS in Z-138. Each row represents a gene centered on the TSS (+/− 5kb). Promoters are sorted by the number of cyclin D1 number of tags. Cyclin D1 bound (top) and unbound (bottom) cyclin D1 genes are shown. B) Pie chart representing common regions bound by cyclin D1, H3K27ac and H3K4me3 marks. Only cyclin D1 peaks at promoters (-5kb TSS) in Z-138 are shown. c) Cyclin D1 occupancy in active promoters and enhancers. Cyclin D1 peaks in active regions defined by H3K27ac presence are shown. Upper panel: Percentage of active promoters (H3K4me3(+)) and active enhancers (H3K4me1(+), H3K4me3(-)) co-localizing with cyclin D1. Bottom panel: Boxplot showing cyclin D1 number of tags in active promoters and active enhancers. The number of all cyclin D1 peaks is represented as control.

2.3 Histone modifications at cyclin D1-bound promoters

To investigate the relationship between cyclin D1 occupancy and chromatin states we compared the cyclin D1 binding pattern to the profiles of several histone marks and DNAse I hypersensitive sites obtained in Z-138 cell line in the context of the Blueprint Epigenome Consortium (Queiros et al., 2016). We observed that promoters occupied by cyclin D1 were enriched in H3K27ac and DNAse I hypersensitivity sites (Figure 41A), indicating the open and activated state of the promoters. Nearly the 90% of cyclin D1-bound intervals close to TSS co-localized with H3K4me3 and H3K27ac peaks (Figure 41B). Conversely, cyclin D1 was not present at promoters lacking active histone marks (Figure 41A, B).

Although cyclin D1 seems to localize preferentially at promoters, we wanted to further investigate whether cyclin D1 also binds to active enhancers regions. For that, we studied the percentage of cyclin D1- H3K27ac co-localizing intervals. In agreement with our previous results, that showed the high occupancy of cyclin D1 at promoters, only 10% of these active regions were enhancers (Figure 41C, upper panel). In addition, the cyclin D1 binding density at active enhancers was significantly lower than the observed in promoters (Figure 41C, bottom panel), although both displayed statistical significance. Taken together, these results indicate that cyclin D1 binds to regions of open chromatin enriched in active histone marks, which include promoters and, to less extent, active enhancers.

2.4 Relationship between Cyclin D1 genomic binding and gene expression

We aimed to study the relationship between the binding of cyclin D1 to promoters and the transcription output, so we performed RNA-sequencing (RNA-seq) of the four MCL cell lines studied. Interestingly, when all coding genes were stratified by the mRNA expression levels, the gene groups with higher gene expression levels displayed larger proportions of cyclin D1-bound genes (Figure 42A). This points out that the more expression level a gene has, the more likely to show cyclin D1 binding to the promoter. Furthermore, cyclin D1 promoter occupancy showed a strong positive correlation with gene expression levels (Spearman correlation, rho= 0.98, $p<2.2E-16$) (Figure 42B). This result indicates that higher expressed genes have greater amounts and/or stronger binding of cyclin D1 in their promoters.

We next studied the binding density around the TSS, regarding its level of expression. As expected, cyclin D1 occupancy of TSS was proportional to the transcription output of the genes (Figure 42C). Supporting the idea that cyclin D1 performs a common effect on all MCL cell lines, all four cell lines showed similar patterns relating cyclin D1 occupancy and gene expression. Altogether, these results demonstrate that cyclin D1 binding occurs predominantly around the TSS of abundantly transcribed genes suggesting that cyclin D1 might regulate transcription in MCL cells.

In order to continue studying the cyclin D1 effects on B-cell transcriptome, we decided to develop constitutive models of overexpressing cyclin D1. To that purpose, we transduced a cyclin D1 negative lymphoblastoid cell line (JVM13) with three different constructs: an empty construct (JVM13-Ctrl), the *CCND1* wt gene (JVM13-cD1) and

the oncogenic cyclin D1 harboring a mutation in the threonine 286 (JVM13-cD1T^{286A}). This mutation increases cyclin D1 nuclear stability impairing the nuclear export of the protein, so overexpressing cells displayed higher amount of cyclin D1. Despite this increased levels, JVM13-cD1T^{286A} still showed lower cyclin D1 levels than MCL cell lines, that represented around 20% of the cyclin amount present in Z138, the highest cyclin D1-expressing MCL cell line (Figure 43A).

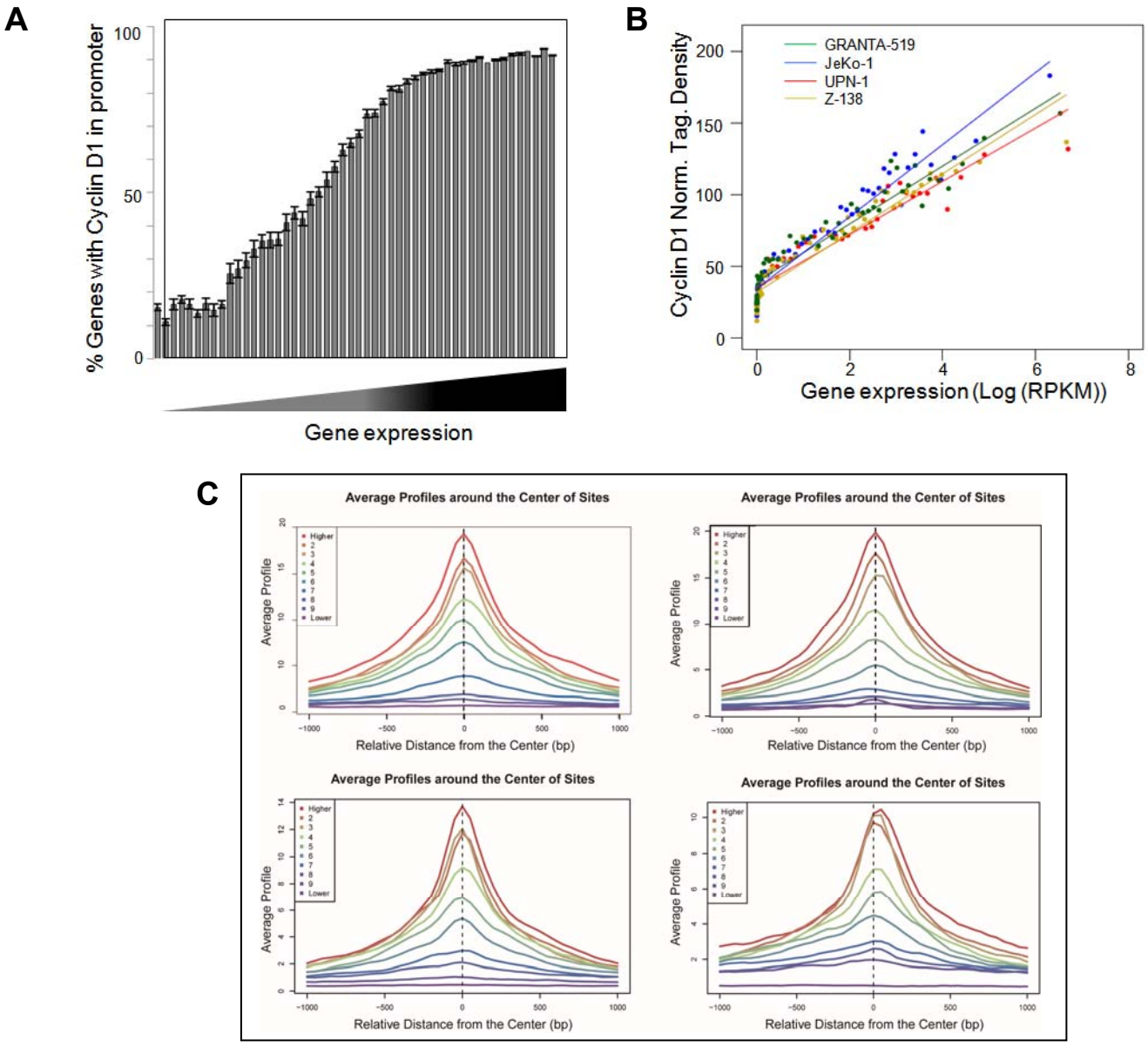


Figure 42. **Cyclin D1 binding correlates with gene expression levels**. A) Distribution of the fraction of genes showing cyclin D1 peaks within their promoter (5Kb upstream of the TSS) according to their respective gene expression levels. All genes were sorted into 50 equal bins based on their expression levels. Results are shown as means ± SEM of all four MCL cell lines. B) Correlation between cyclin D1 binding and transcription output. All genes were sorted into 50 equal bins based on their expression levels. The average of cyclin D1 ChIP-seq normalized tag densities at promoters and the RPKM normalized expression levels are shown for each bin. Solid lines show the linear regression line between expression and cyclin D1 binding. C) Profile of cyclin D1 occupancy around the TSS according to gene expression in Z-138 cell line(Upper, left panel), JeKo-1 (Upper, right panel), GRANTA-519 (Bottom, left panel) and UPN-1 (Bottom, right panel). Genes were divided in ten groups based on their expression levels (from higher to

lower expression). The average cyclin D1 ChIP-seq tag density distribution around the TSS (+/- 1kb) is displayed for each group

We decided to carry out expression arrays in order to study differential expression upon cyclin D1 upregulation. Strikingly, few genes were differentially express between JVM13-cyclin D1 overexpressing cells and normal JVM13 cells. Moreover, differential expressed genes showed low fold changes, indicating cyclin D1 overexpression may not determine significant transcription regulation when measured by standard gene expression profiling techniques (Figure 43B, C).

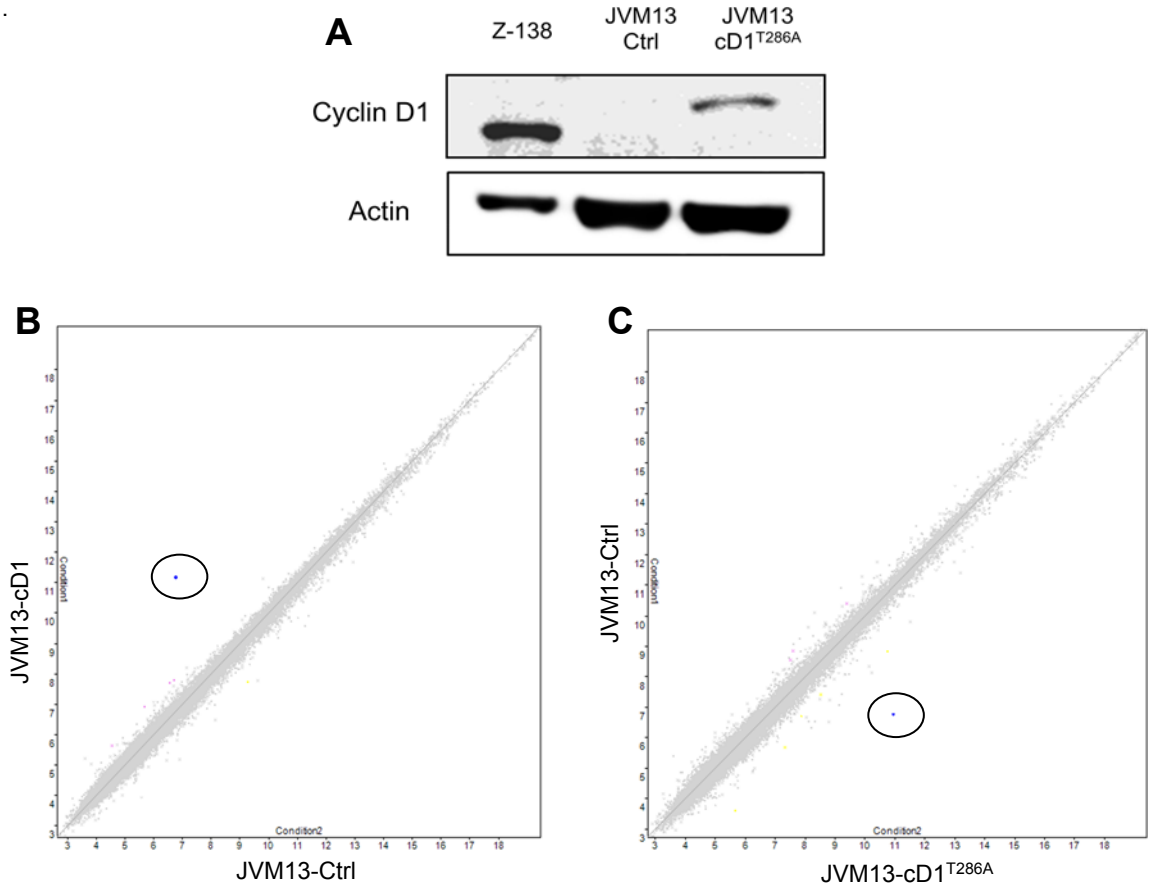


Figure 43. **Cyclin D1 overexpressed constitutively had little changes in global gene expression.** A) Western blot showing cyclin D1 levels in Z-138, JVM13-Ctrl and JVM13-cD1^{T286A}. B) Scatter plot of genes differentially expressed in JVM13-Ctrl and JVM13-cD1. C) Scatter plot of genes differentially expressed in JVM13-Ctrl and JVM13-cD1. Cyclin D1 gene is remarked with a circle.

To further look into these intriguing results, we performed an integrative experiment of ChIP-seq and RNA-seq data sets from JVM13 transduced cell lines (Figure 44A). First, we decided to perform cyclin D1 ChIP-seq of JVM13-cD1^{T286A}, because of its more similar levels of cyclin D1 compared to MCL cell lines. The exogenous cyclin D1 binding profile observed in JVM13-cD1^{T286A} cells was akin to the pattern identified in the MCL cell lines (Figure 44B), although a lower number of cyclin D1 peaks was identified in JVM13-cD1^{T286A} cells. The reduced number of peaks could respond to the lower amount of cyclin D1 protein constitutively expressed by JVM13-cD1^{T286A}, as suggested by the strong association observed previously in MCL cells between the numbers of peaks and the amount of cyclin D1 (Figure 35D). Due to the fact that we

could detect small changes in gene expression induced by cyclin D1 overexpression using microarrays, we wonder if cyclin D1 mainly went to pre-activated promoters rather than regulate specific gene expression. To do so, we determined the basal gene expression levels of all transcripts on JVM13 cells, performing RNA-seq of the cyclin D1-negative JVM13-Ctrl cells. Then, we stratified genes according to the RNA-Seq data in ten different groups (from lower expressed to higher expressed genes) and we represented the distribution of cyclin D1 tags in the JVM13-cD1^{T286A} cell line for each group. We observed a distribution of cyclin D1 around TSS similar to the one observed in MCL cell lines (Figure 44C). This result clearly indicates that cyclin D1 distributes according to the expression levels of the genes before cyclin D1 overexpression. Therefore, we suggest that cyclin D1 is recruited to promoters that are pre-configured in an active state, and the extent of recruitment would be dependent on the transcription levels of the targeted genes. However, the traditional microarrays doesn't elucidate cyclin D1 role in transcription.

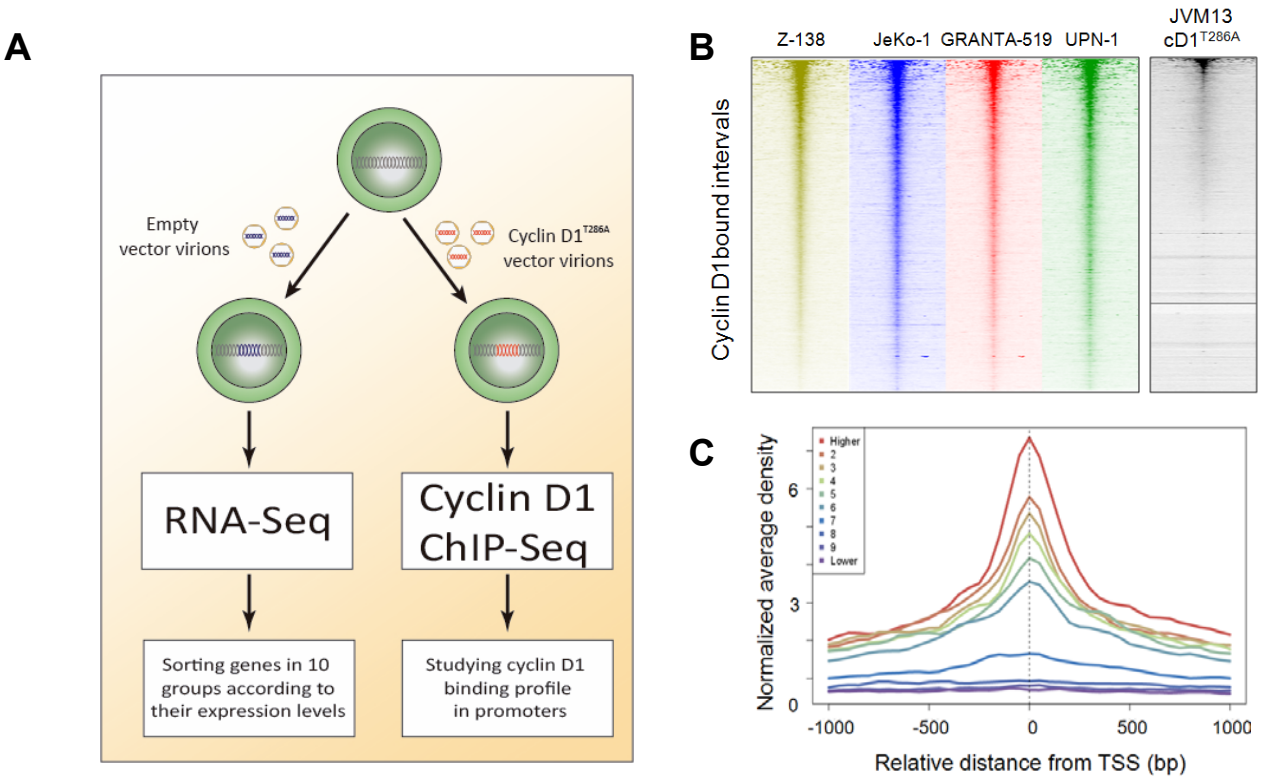


Figure 44. **Exogenous cyclin D1 mimics endogenous cyclin D1 pattern in MCL cell lines and goes to promoters pre-configured in an active state.** A) Experimental diagram for the characterization of cyclin D1 binding after overexpression of the exogenous cyclin D1 T286A construct. B) Heatmap showing the cyclin D1 ChIP-seq tag density within gene promoters of JVM13-cD1^{T286A} and MCL cell lines. Each row represents a gene centered on the TSS (+/− 5kb). Promoters are sorted by the number of cyclin D1 tags in Z-138. C) Profile of cyclin D1 occupancy around the TSS in JVM13-cD1^{T286A} cells. Genes were divided in ten groups regarding gene expression in JVM13 control cells (from higher to lower expression). The average cyclin D1 ChIP-seq tag density distribution around the TSS (+/- 1kb) is displayed for each group.

2.5 Functional effects of cyclin D1 overexpression/silencing on total RNA content in lymphoid and MCL cells

The analysis of ChIP-seq and expression profiling experiments suggested that cyclin D1 could play a complex role on transcriptomics. The cyclin D1 chromatin binding

pattern and the tag distribution identified in MCL cells evidenced important similarities to that recently reported for MYC as global transcription amplifier (Figure 45). Global modulation of transcription is a relatively new concept, thus not many examples can be found in the literature (Lin et al., 2012). In brief, MYC overexpression in cancer causes a transcription burst mediated by its binding profile to a great number of active genomic regions, such as active promoters and enhancers. As we described in our study, cyclin D1 also displays an extensive genome-wide binding to open chromatin, preferentially centered on the TSS of highly expressed genes. In addition, cyclin D1 binding follows an unimodal distribution in a peak-density histogram, (Figure 9), exactly as it was shown for MYC (Lin et al., 2012; Nie et al., 2012; Sabo et al., 2014). To analyze whether cyclin D1 could display a function as a general transcription regulator we explored the relationship between cyclin D1 expression and the total cellular RNA content.

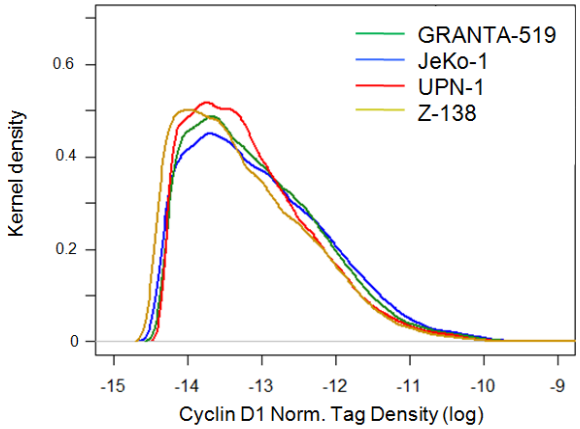


Figure 45. **Kernel distribution of normalized cyclin D1 tag density showing a unimodal distribution in MCL cell lines.**

Since the constitutive models showed significant lower amount of cyclin D1 than MCL cell lines, together with the fact that cyclin D1 chromatin binding was related to cyclin D1 protein levels lead us to develop inducible models, which enable a more controlled and stronger overexpression. We overexpressed in JVM13 cells either the highly stable nuclear form of cyclin D1 (JVM13-D1^{T286A}) or the wild-type form of the protein (JVM13-D1). As expected, JVM13-D1^{T286A} cells showed higher cyclin D1 protein levels than JVM13-D1 cells (Figure 46A). Following cyclin D1 induction we performed a spectrophotometric quantification of the absolute levels of total RNA obtained from a fixed number of cells (Figure 46B).

Unexpectedly, cyclin D1 overexpressing cells showed significant lower amounts of total RNA per cell than JVM13 control cells (Figure 46B). We wanted to validate these results with other technique, so we adapted a total RNA quantification with pyronin to perform the analysis of the total cellular RNA content by flow cytometry. We confirmed that cyclin D1 overexpression resulted in a significant diminution in the absolute levels of RNA content (Figure 46C). Interestingly, this reduction in both experiments was superior in JVM13-D1^{T286A} cells that express higher levels of cyclin D1 protein.

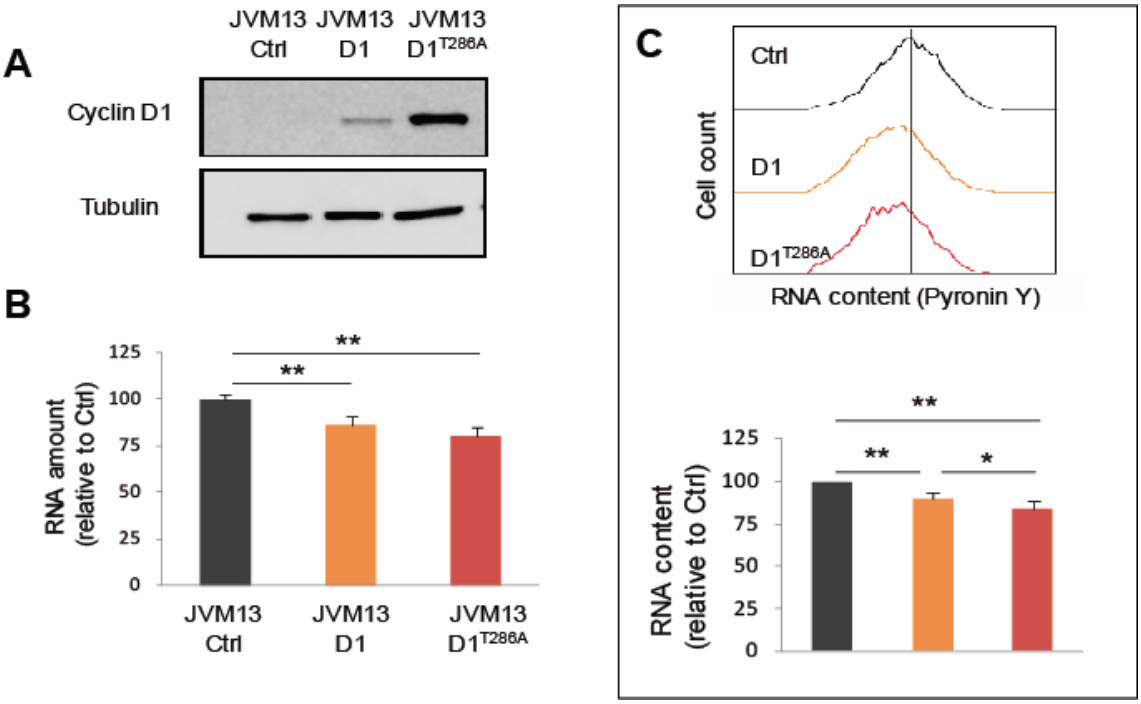


Figure 46. **Cyclin D1 overexpression results in a reduction of the total RNA content in JVM13 cells.** A) Western blot analysis of cyclin D1 in JVM13-Ctrl, JVM13-D1 and JVM13-D1^{T286A}. α-Tubulin was used as loading control. B) Amount of total RNA content extracted from one million cells of JVM13-Ctrl, JVM13-D1 and JVM13-D1^{T286A}. Results are shown relative to the Ctrl as means ± SEM of nine RNA extractions corresponding to three independent experiments in triplicates (**:p val<0.01, two-tailed Student's t-test). C) RNA quantification by pyronin Y staining in JVM13 inducible cell lines; JVM13-Ctrl, JVM13-D1 and JVM13-D1^{T286A}. Only cells in G1 phase were considered for the analysis. Top panel: FACS profile of a representative experiment. Bottom panel: bar graph displaying the pyronin Y mean signal of three independent experiments. The data are shown relative to Ctrl as mean ± SEM (**:p val<0.01, two-tailed Student's t-test).

In order to confirm this result using a model closer to MCL we used JVM2 a MCL cell line with the t(11;14) but low levels of cyclin D1. We successfully developed an inducible model of cyclin D1 in JVM2. JVM2-D1^{T286A} displayed lower total RNA content by Pyronin Y analysis, that could not be detected by spectrophotometric quantification (Figure 47A, B), indicating that the cytometric system could be more sensitive. On the other hand, the low increments in cyclin D1 protein achieved in the JVM2 cells transduced with the wild type cyclin D1 construct were not enough to display a statistically significant downregulation of the transcriptome (Figure 50A).

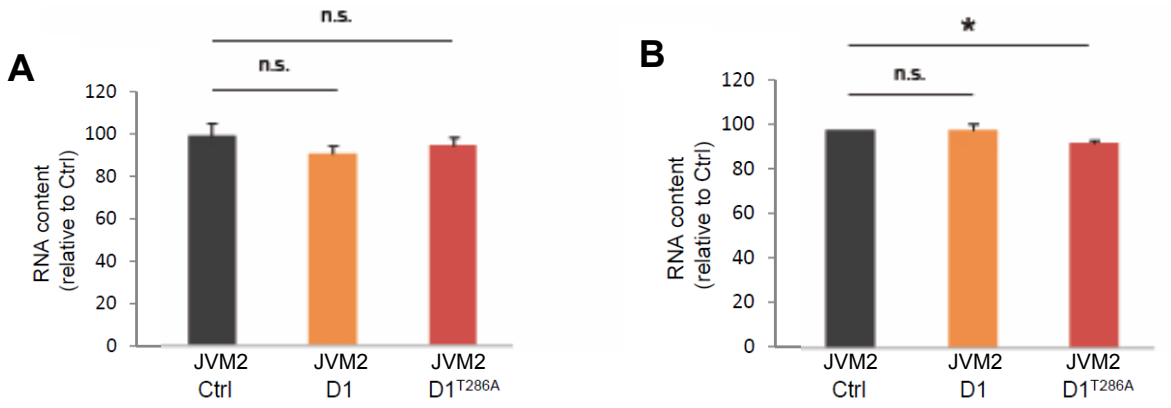


Figure 47. **Cyclin D1 overexpression results in a reduction of the total RNA content in JVM2 cells.** A) Amount of total RNA content extracted from one million cells of JVM2-Ctrl, JVM2-D1 and JVM2-D1^{T286A}. Results are shown relative to the Ctrl as means ± SEM of nine RNA extractions corresponding to three independent experiments in triplicates. B) RNA quantification by pyronin Y staining in JVM2 inducible cell lines; JVM2-Ctrl, JVM2-D1 and JVM2-D1^{T286A}. Only cells in G1 phase were considered for the analysis. The data are shown relative to Ctrl as mean ± SEM (*:p val<0.05, two-tailed Student's t-test).

To further validate these results we analyzed the effect of cyclin D1 silencing in a MCL cell line (GRANTA-519) (Figure 48A) Concordantly with the previous results the reduction of cyclin D1 levels led to an increase in total RNA content measured, by spectrophotometry and Pyronin Y staining (Figure 48B, C). Interestingly, as it happened with the overexpression models, the levels of cyclin D1 correlated to the total RNA content. Consequently, the sh #1 that displayed increased cyclin D1 silencing showed a stronger increase on total RNA than sh #2.

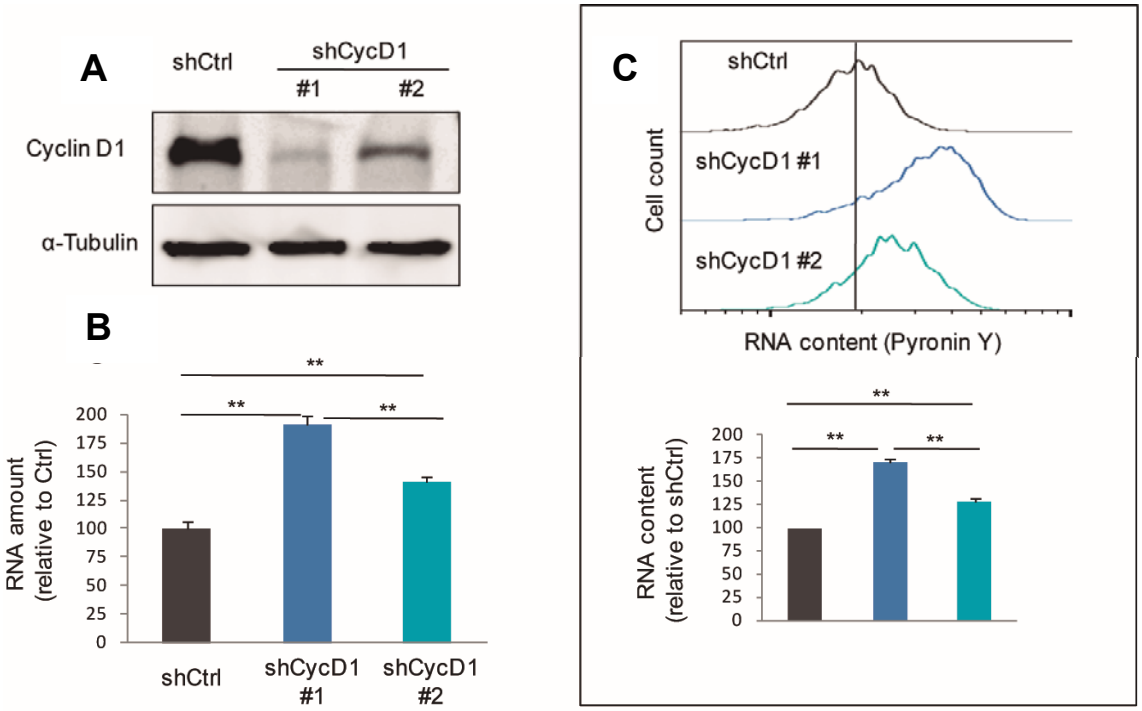


Figure 48. **Cyclin D1 silencing results in an increment of the total RNA content in GRANTA-519 cells** A) Western blot analysis of cyclin D1 in control (shCtrl) and Cyclin D1-depleted (shCycD1 #1 and #2) GRANTA-519 cells. α-Tubulin was used as loading control. B) Amount of total RNA content extracted from one million cells in control and cyclin D1-depleted GRANTA-519 cells. Results are shown relative to shCtrl as means ± SEM of eight independent RNA-extraction experiments (**:p val<0.01, two-tailed Student's t-test). C) RNA quantification by pyronin Y staining in control and cyclin D1-depleted GRANTA-519 cells.

Only cells in G1 phase were considered for the analysis. Top panel: FACS profile of a representative experiment. Bottom panel: bar graph displaying pyronin Y mean signal of four independent experiments. The data is shown relative to shCtrl as means ± SEM. (**:p val<0.01, two-tailed Student's t-test)

The cytometric analysis of pyronin Y we chose the G1 population in order to quantify the total RNA of the correspondent cell lines. However, since cyclin D1 has an essential role in G1/S transition and, as we were aware, cells modulate their total levels of RNA across cell cycle, we wanted to know whether the effect on total RNA content were detected independent o the cell cycle phase. For that purpose, we also quantify the Pyronin Y signal in G2/M population selected by Hoestch staining (Figure 49 A, B). The reduction/increase in total RNA was almost the same both in overexpression or downregulation models, so we could conclude that cyclin D1 effect on total transcriptome are independent of the cell cycle phase.

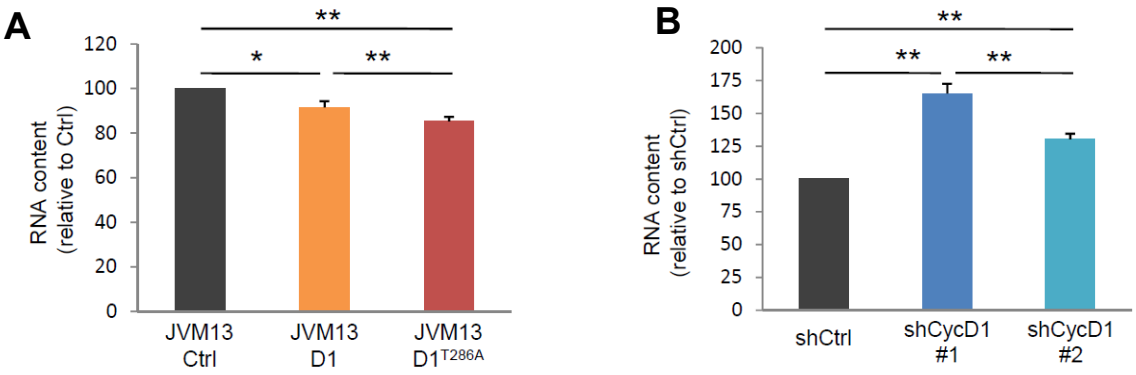


Figure 49. **Cyclin D1 effect on total RNA is independent of the S-phase.** A) RNA quantification by pyronin Y staining in JVM13 inducible cell lines; JVM13-Ctrl, JVM13-D1 and JVM13-D1^{T286A}. Cells were treated with doxycycline (0.1µg/mL) for 24 hours and only cells in G2/M phase (as indicated by Hoechst co-staining) were considered for the analysis. Pyronin Y signal is shown relative to Ctrl as mean ± SEM of three independent experiments. Statistical significance was determined by two-tailed Student's t-test. (*: p val<0.05, **:p val<0.01). B) RNA quantification by pyronin Y staining in control and Cyclin D1-depleted GRANTA-519 cells. Only cells in G2/M phase (as indicated by Hoechst co-staining) were considered for the analysis. The mean pyronin Y signal is shown relative to shCtrl as mean ± SEM of three independent experiments. Statistical significance was determined by two-tailed Student's t-test (*: p val<0.05, **:p val<0.01).

2.6 Cyclin D1 correlates with lower RNA amount in MCL and MM cell lines

. We wanted to test whether the association between cyclin D1 levels and the total RNA content was present in MCL cell lines. For that purpose, we quantified the amount of cyclin D1 by western blot and the RNA content by pyronin analysis of MCL cell lines (Figure 50 A). This allowed us to identify a highly significant negative correlation between the cyclin D1 protein levels and the total RNA content measured by cytometry. This association remained significant when the exogenous cyclin D1 overexpressing cells were included in the analysis (p val<0.01) (Figure 50 B).

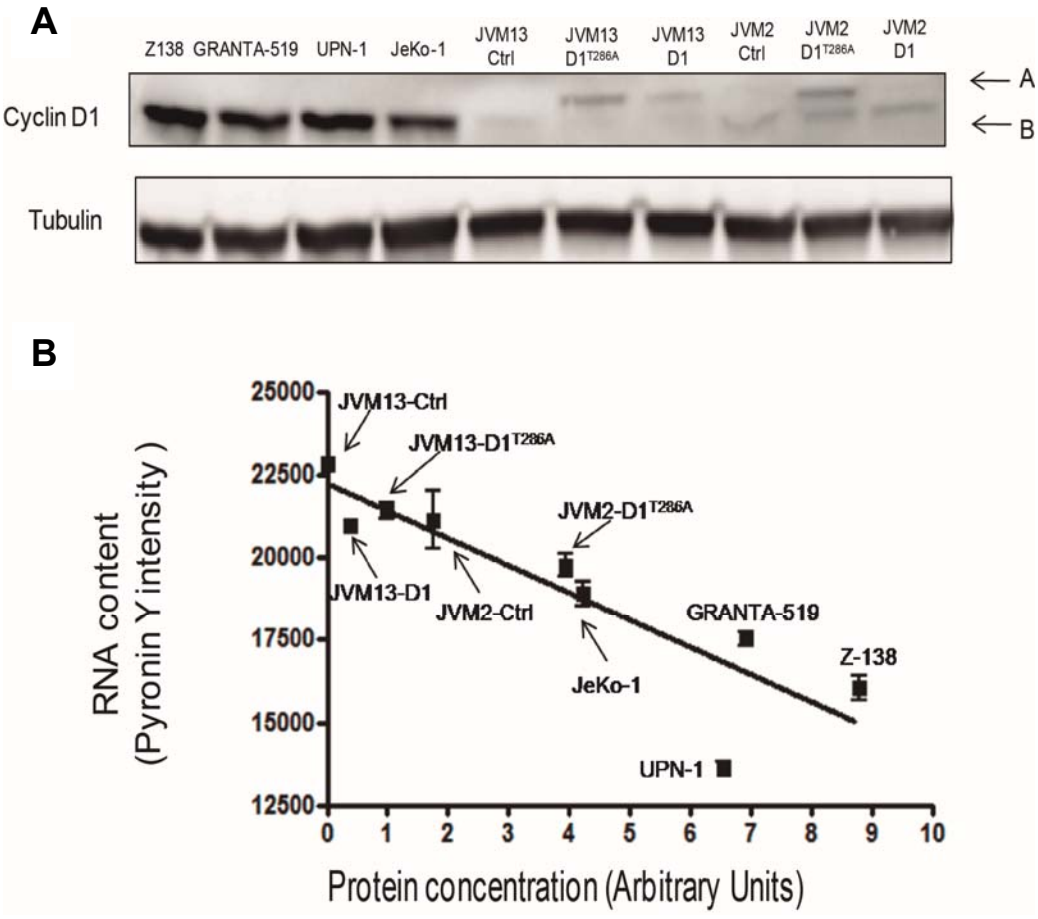


Figure 50. **Cyclin D1 levels correlate with total RNA content in MCL cell lines and lymphoblastic models.** A) Western blot showing the protein amount of cyclin D1 in different MCL cell lines and overexpressing models. Endogenous cyclin D1 (B) and overexpressed-tagged cyclin D1 (A) are shown. α-Tubulin was used as loading control. B) Correlation between cyclin D1 protein levels and the intensity of pyronin staining in MCL cell lines and retroviral inducible models. The data shows the Pyronin value Ctrl as means ± SEM of four independent measures. Black line represents the linear regression line.

This data encouraged us to test whether cyclin D1 could be provoking the same effects in other cancers with overexpression of cyclin D1 such as multiple myeloma. Cyclin D1 amplification by the t(11;14) translocation is detected in up to 30% of multiple myeloma primary cases, but it can be detected up to 40-50% of all cases by molecular techniques (Lesage et al., 2005). We selected seven MM cell lines with different levels of cyclin D1 expression to study whether cyclin D1 also correlates with total RNA in these cell lines. . Two of them (U266 and KMS12PE) harbor a translocation that determines high cyclin D1 levels (Figure 51A). However, the cyclin D1 levels observed in MM cell lines were lower than the ones observed in MCL cell lines (except to JVM2). In spite of the reduced number of MM cell lines analyzed, there was an association between the presence of cyclin D1 expression and the amount of total RNA, although it did not reach statistical significance (Figure 51B). So, the analysis of MM cell lines confirmed in a different cell model the negative correlation between cyclin D1 protein levels and the total RNA cell content. In summary, all these results provide strong evidences suggesting that cyclin D1 overexpression may induce, in a dose-dependent manner, a reduction in the amount of total RNA.

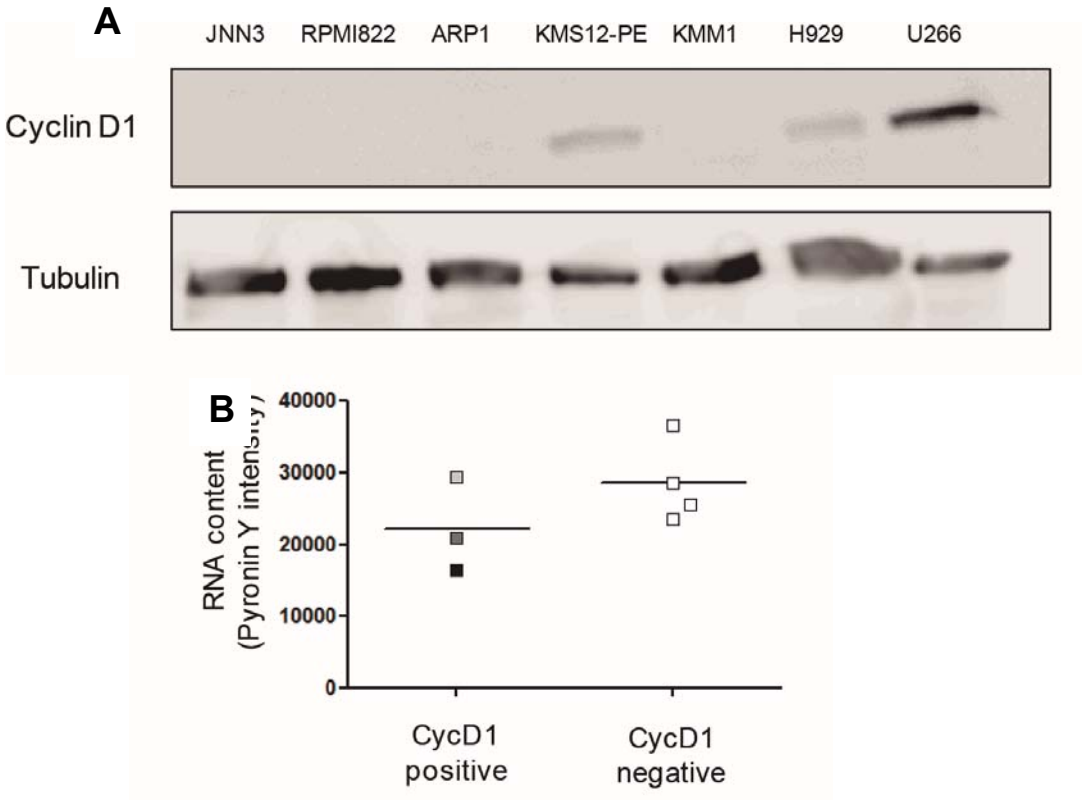


Figure 51. **High levels of cyclin D1 correlate with lower RNA content in MM cell lines.** A) Western blot showing cyclin D1 in different multiple myeloma cell lines. α-Tubulin was used as loading control. B) Pyronin intensity of seven multiple myeloma (MM) cell lines, including three cell lines expressing cyclin D1 protein. The cell lines are colored according the cyclin D1 levels (black: high, grey: medium, and light grey: very low, white: no expression).

2.7 Effects of cyclin D1 overexpression on messenger RNA in lymphoid cells

We next intended to investigate whether cyclin D1 overexpression also determined a global messenger RNAs downregulation. At this point, the absence of differentially expressed genes obtained by expression microarrays were totally in line with a global downmodulation of the quantity of total RNA per cell following cyclin D1 overexpression. The use of microarrays would be actually compromised under this scenario, since conventional microarray studies compare the same amount of RNA between conditions (Loven et al., 2012). To overcome this limitation, we took advantage of the NanoString's nCounter technology, able to perform a direct digital quantification of cell extracts. We analyzed a pre-defined panel of 48 genes in cell extracts from three different amounts of cells from the cyclin D1 overexpression model (JVM13 and JVM13-D1^{T286A}).

The high correlation observed between mRNA counts and the number of cells confirmed the suitability of this approach to test the global effect of cyclin D1 (JVM13-Ctrl, r=0.969±0.016; JVM13-cD1^{T286A}, r=0.987±0.007) (Figure 52A). Cells overexpressing cyclin D1 showed lower gene expression levels than controls cells at the three different amounts of cells. In this experiment, thirty-six genes out of the 48 genes were considered to have mRNA counts above the background in control cells. Interesting, the 36 genes expressed in control cells showed a downregulation in cyclin D1 overexpressing cells ($p<2.2E-16$) (Figure 52A). To further analyze the global impact

of cyclin D1 in mRNA levels we analyzed the expression levels of a pre-designed panel of 236 cancer related genes in JVM13-D1^{T286A} cell line following cyclin D1 induction. Control cells expressed 154 of these 236 genes. The average expression of these genes was lower in JVM13-D1^{T286A} (Figure 52B). Remarkable, 94% (n=145) of the genes expressed in control cells showed lower mRNA levels in JVM13-D1^{T286A} (Figure 52C). Altogether, these results strongly suggest that the binding of cyclin D1 to gene promoters leads to a global transcription down-modulation of expressed mRNAS.

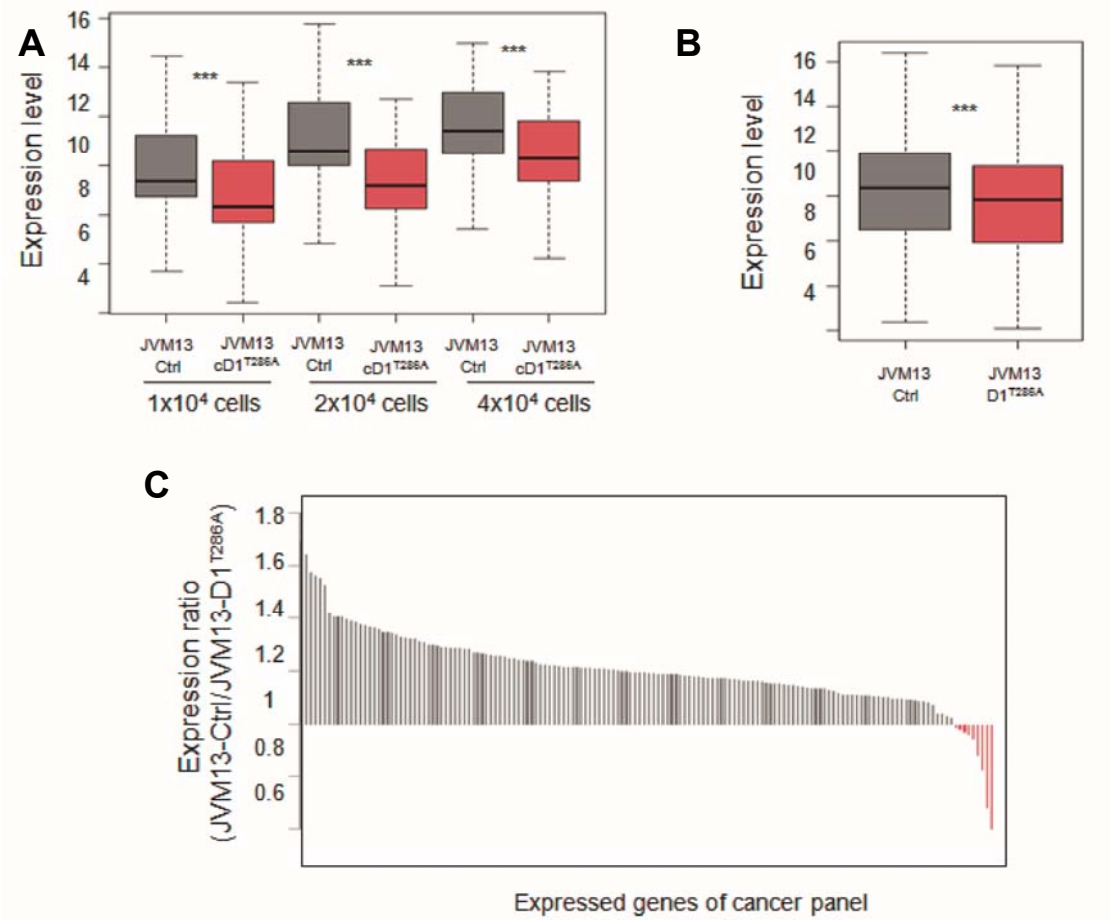


Figure 52. **Cyclin D1 overexpression produces a global downmodulation of mRNAs in lymphoid cells.** A) Boxplot displaying nCounter-based gene expression data of a 48 gene panel analyzed in JVM13-Ctrl and JVM13-cD1^{T286A} cells. Cell extracts from three different amounts of cells, counted by cell cytometry, are represented on the X axis. The nCounter counts of expressed transcripts (counts > 30) from two independent experiments are shown in log2 scale on the Y axis (***:p val<2E-16, two-tailed Student's paired). B) Boxplot displaying the mean ratio of nCounter counts of the Gene Cancer Panel analyzed in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cell extracts from 4x10^4 cells were analyzed. The nCounter counts of expressed transcripts (counts > 30) from two independent experiments are shown in log2 scale on the Y axis (***:p val<2E-16, two-tailed Student's paired). C) Barplot displaying nCounter-based gene expression data of the Gene Cancer panel analyzed in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cell extracts from 4x10^4 cells were analyzed. The nCounter counts of expressed transcripts (counts > 30) from two independent experiments are shown in log2 scale in the Y axis. Genes are sorted from the highest to the lowest expression ratio, both upregulated genes (grey) and downregulated genes (red) over JVM13-Ctrl.

2.8 Relationship between Cyclin D1 overexpression and RNA Pol II occupancy in promoters and gene body

To explore whether the cyclin D1 dependent transcriptome down-modulation was associated with changes in the RNA polymerase II (Pol II) chromatin profile we performed Pol II ChIP-seq experiments in control (JVM13) and cyclin D1 overexpressing cells (JVM13-D1^{T286A}). First of all, genomic visualization of these two ChIP-seq tracks showed Pol II peaks were well-defined and they appeared mainly in promoter (Figure 53A). We first decided to study whether Pol II occupancy changed upon cyclin D1 overexpression. For this reason, we correlate the normalized tag density of Pol II peaks in the control cell line vs the JVM13 expressing the mutant form of cyclin D1 (Figure 53B). According to the occupancy of cyclin D1 at promoters, there was a high correlation between both ChIP-seqs. However, Pol II peaks in overexpressing cyclin D1 JVM13 seemed to have a greater number of tags than in control cells (Figure 54A).

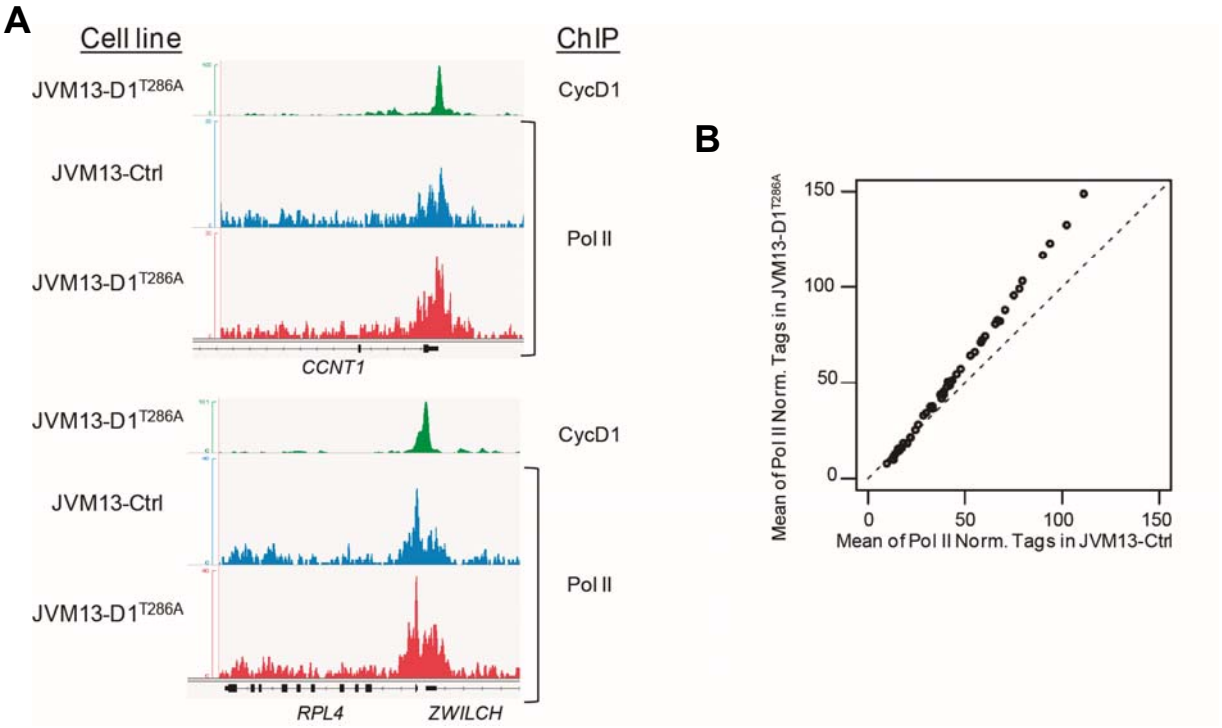


Figure 53. **Pol II ChIP-seq shows promoters co-occupancy by cyclin D1 and Pol II proteins.** A) Pol II occupancy on three representative cyclin D1-bound genes. Pol II (8GW16) profiles are shown in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cyclin D1 binding pattern in JVM13-D1^{T286A} is also represented in green. B) Correlation between normalized Pol II ChIP-seq tag density at promoters in JVM13-Ctrl and JVM13-D1^{T286A}. Promoters were sorted into 50 equal-size groups based on ChIP-seq tag densities in JVM13-Ctrl. Dashed line marks diagonal.

Concordantly with the association between cyclin D1 chromatin binding and the transcription levels, we observed a strong correlation between the tag density of cyclin D1 and Pol II, together with the colocalization of both profiles around the TSS (Figure 54B). Interestingly, JVM13-D1^{T286A} cells showed higher Pol II loading at promoters, and this difference was more evident in highly expressed genes too. Therefore, the observed transcriptome downmodulation was not due to a reduced Pol II recruitment to

promoters. We hypothesized that the observed transcription downmodulation could response to changes in Pol II pause-release.

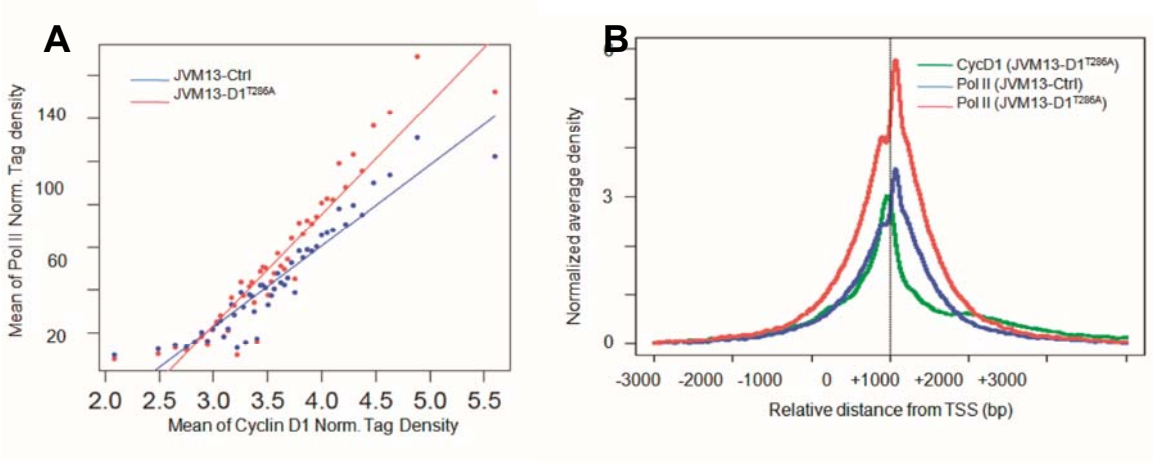


Figure 54. **Cyclin D1 overexpression causes an increase in the promoter-proximal pausing of the Pol II.** II A) Correlation between normalized cyclin D1 ChIP-seq tag density in JVM13-D1^{T286A} and the Pol II ChIP-seq tag density at promoters in JVM13-Ctrl and JVM13-D1^{T286A}. Promoters are sorted into 50 equal-size groups based on ChIP-seq tag densities of cyclin D1. X-axis represents mean cyclin D1 normalized tags of the promoters in JVM13-D1^{T286A}. Y-axis represents Pol II tag density in both cell lines. Solid lines show the linear regression between cyc D1 and Pol II in promoters. B) Average signal profiling of Pol II occupancy around the TSS (+/- 5kb) of cyclin D1 bound genes in JVM13-D1^{T286A} both in JVM13-Ctrl and JVM13-D1^{T286A} inducible cell lines. Cyclin D1 binding profile in JVM13-D1^{T286A} is also shown.

To further validate our hypothesis regarding cyclin D1 affecting pause-release from promoters, we decided to analyze the phosphorylation status of Pol II's C-terminal domain (CTD) upon cyclin D1 overexpression. Cyclin D1 overexpressing cells displayed similar levels of total Pol II protein than control cells (Figure 55A; Pol II N-20), supporting the idea that the higher promoter occupancy by Pol II was not due to the presence of higher Pol II levels. Interestingly, the phosphorylation status of the CTD was different in cyclin D1 expressing cells and control cells. Cyclin D1 overexpressing cells had higher levels of hypophosphorylated Pol II or Ser5 Pol II protein recognized by the 8WG16 antibody (Figure 55A) (Brookes and Pombo, 2009). These Pol II forms are associated with paused polymerase or early elongation complexes. However, cyclin D1 overexpressing cells showed lower levels of Ser2 phosphorylation, a modification associated with active elongation (Figure 55A). This Pol II phosphorylation pattern was compatible with a reduced Pol II pause release in cyclin D1 overexpressing cells. To investigate whether the Pol II pause release was affected by the overexpression of cyclin D1 we established the pausing index by performing the ratio between the Pol II occupancy at promoter and gene body regions (Zeitlinger et al., 2007).

The pausing index is a distribution of all the pausing ratios in genes. The pausing ratio for a certain gene is calculated as the division of the number of tags around the TSS and the number of tags in gene body (Figure 55B). The density of Pol II at proximal promoters or gene bodies may be modified by different treatments including protein overexpression or drug inhibitors determining a shift of the pausing ratio. . In our study, cyclin D1 overexpression increased the gene pausing ratios (Figure 55C). This result is consistent with the reduction in the mRNA content following cyclin D1 overexpression

and the Pol II phosphorylation pattern shift. Altogether, these results suggest that cyclin D1 overexpression determines a global transcriptome downmodulation by interfering with the RNA Pol II elongation process.

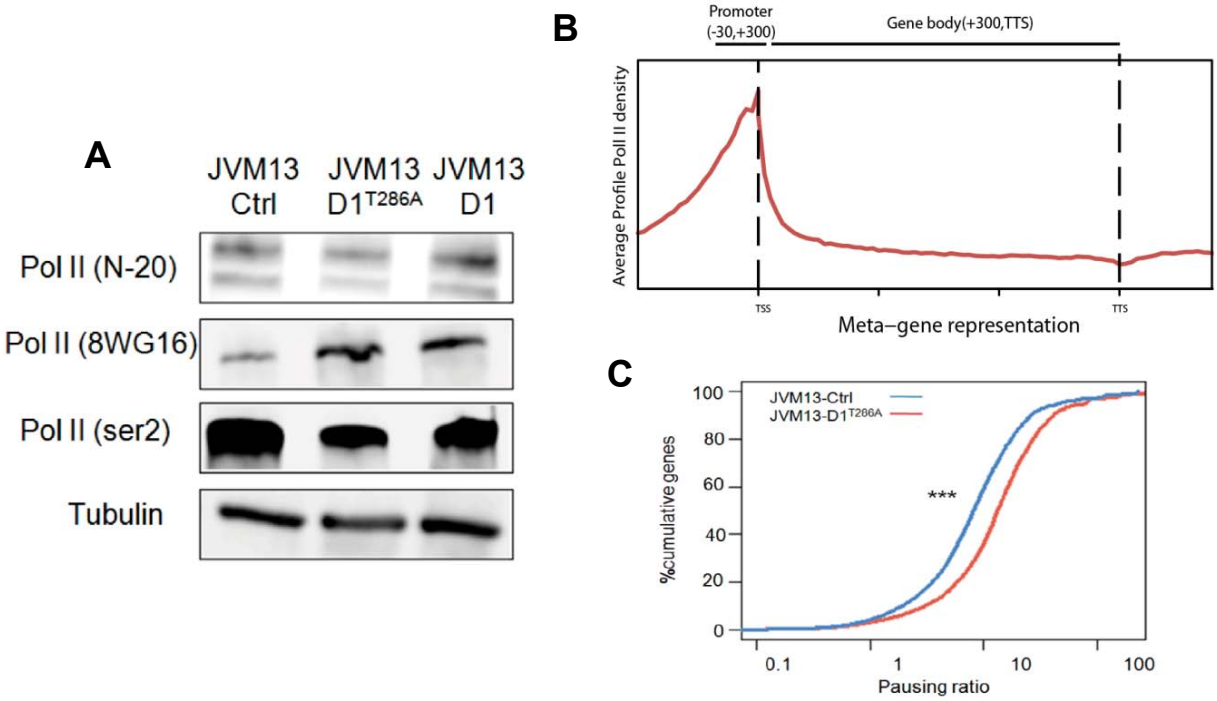


Figure 55. **Cyclin D1 overexpression increases Pol II promoter-proximal pause and decreases elongation.** A) Western blot showing different phosphorylated forms of Pol II in JVM13-Ctrl, JVM13-D1^{T286A} and JVM13-D1 inducible cell lines. A representative western blot (n=3) for each antibody is represented. α-tubulin is used as loading control. B) Schematic representation of the calculation of the pausing ratio for a certain gene in our study. C) Plot representing the pausing index in JVM13-Ctrl and JVM13-D1^{T286A} cell lines. It illustrates a right-handed shift of pausing ratio at all genes with cyclin D1 in their promoter (-5kb, TSS) after cyclin D1 induction in JVM13-Ctrl and JVM13-D1^{T286A} cells (***:p val<2E-16, Kolmogorov-Smirnov test).

2.9 Cyclin D1 functional interaction with CDK9 causes synthetic lethality in JVM13 and MCL cell lines

The strong association observed between cyclin D1 levels and the transcription dysregulation led us to investigate whether this effect could be mediated by an off-target effect of the overexpressed cyclin D1 onto the CDK component of the transcription machinery. CDK9-Cyclin T and CDK7-cyclin H are the main participants in transcription, but just CDK9-Cyc T is related both to pause-release and elongation processes (Pombo et al). For this reason, we wanted to study if cyclin D1 can aberrantly interact with this CDK. A co-immunoprecipitation using cyclin D1 antibodies in Z-138 cell line showed an interaction of the cyclin with CDK9 (Figure 56A). This result suggests that cyclin D1 overexpression may dysregulate transcription by its binding to CDK9. The global transcription downmodulation caused by cyclin D1 overexpression could render cyclin D1 overexpressing cells more sensitive to drugs targeting the transcription machinery. To test this possibility we analyzed the response of control (JVM13-Ctrl) and cyclin D1 overexpressing cells (JVM13-D1 and JVM13-D1^{T286A}) to DRB (5, 6-Dichloro-1-beta-Ribo-furanosyl Benzimidazole), a transcription

inhibitor that causes premature chain termination and its major target is CDK9. We used DRB at concentrations (20μM and 40μM) below the levels reported as required to fully inhibit transcription (100μM).

Firstly, we tested four different MCL cell lines, two with high levels of cyclin D1 and low transcription levels (UPN1 and Z138) and two cell lines with lower cyclin D1 levels (JEKO and JVM2). The MCL cell lines with higher levels of cyclin D1 showed a significant increase of apoptosis following DRB treatment, an increase that was milder in low-expressing cyclin D1 MCL cell lines (Figure 56B). To establish if cyclin D1 overexpression directly plays a role in this apoptotic response, we explored whether cyclin D1 overexpression sensitizes cells to transcription inhibitors. We treated control (JVM13-Ctrl) and cyclin D1 overexpressing cells (JVM13-D1 and JVM13-D1^{T286A}) with DRB. This experiment demonstrated a significant increased sensitivity to DRB in cyclin D1 overexpressing cells compared to control cells (Figure 56C). These results suggest the existence of a potential synthetic lethality interaction between the overexpression of cyclin D1 and transcription inhibitors.

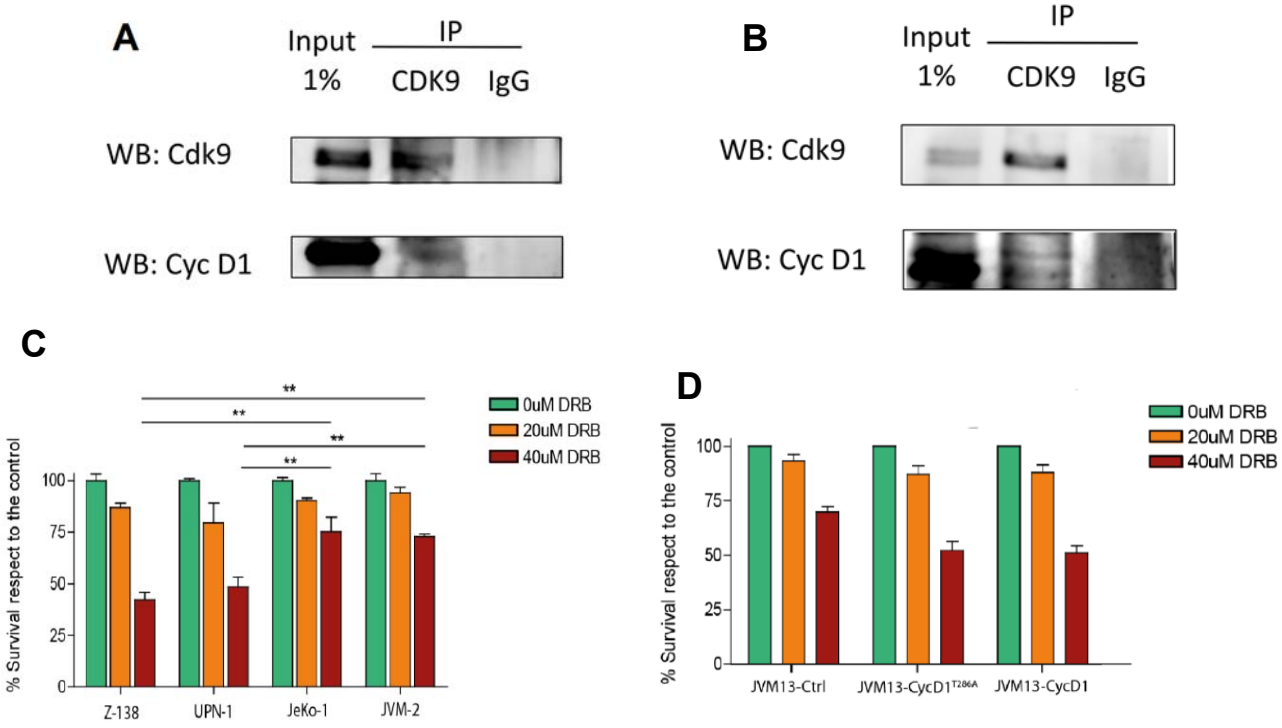


Figure 56. **Cyclin D1 interacts with CDK9 and its overexpression render cells sensitive to CDK9 inhibitors.** A) Co-immunoprecipitation experiment in Z-138 (A) and JeKo-1 (B) using antibodies against CDK9 or control IgG. Immunoprecipitated proteins were analyzed by Western blot analysis by blotting with cyclin D1 and CDK9 antibodies. Whole extract (We) was loaded as a control.c) Cell survival of MCL cell lines at 72 hours following transcription inhibition. Exponential growing MCL cell lines were treated with increasing concentrations of DRB. Results are shown as means ± SEM of four independent experiments. (**:p val<0.01) D) Cell survival of JVM13-Ctrl and JVM13-D1^{T286A} cells at 48 hours following transcription inhibition. After 24 hours of doxycycline induction, DRB was added to the indicated concentrations. Results are shown as means ± SEM of three independent experiments. (*: p val<0.05)

2.10 Drugability of Cyclin D1 interaction with transcription machinery in MCL and MM cell lines

DRB is a CDK9 inhibitor used for cell biology experiments at concentrations around the mM range. Although widely used for cell biology, it is not suitable for cancer treatment. Other transcription inhibitors are available and have been tested for cancer therapy, for instance triptolide. We treated four MCL and four MM cell lines with different levels of cyclin D1 with 40nM of triptolide. Forty-eight hours after drug treatment, MCL cell lines with high levels cyclin D1 and low transcription levels measured by pyronin displayed decreased survival. In fact, JVM-2, the MCL cell line with lower levels of cyclin D1, was the less sensitive to the drug. (Fig 57A). We observed a similar effect in MM cell lines. Cyclin D1 positive cell lines (U266 and KMS12-PE) displayed a significant increase of apoptosis after 48 hours of triptolide treatment (Fig 57B). On the other hand, cyclin D1 negative cells were not affected by the drug. Our results suggest the existence of a synthetic lethality between cyclin D1 overexpression and the consequent transcription downmodulation with the inhibition of the transcription machinery. Altogether, these results open a new specific therapeutic target for the treatment of cyclin D1 positive hematological cancers.

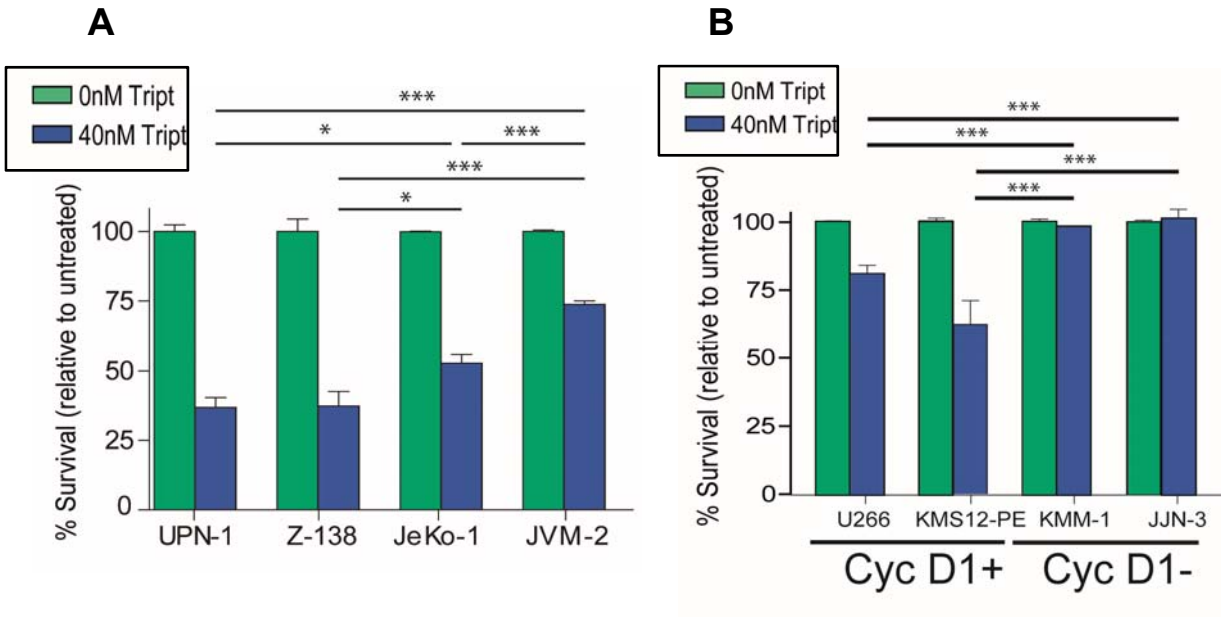


Figure 57. **Cyclin D1 sensitive MCL and MM cells to transcription inhibitors.** A) Cell survival of MCL cell lines at 48 hours following transcription inhibition. Exponential growing MCL cell lines were treated with 40nM of Triptolide (Tript). Results are shown as means ± SEM of four independent experiments. (* p val<0.05; ***p val<0 001). B) Cell survival of multiple myeloma cell lines at 48 hours following transcription inhibition. Exponential growing MM cell lines were treated with 40nM of Triptolide (Tript). . Results are shown as means ± SEM of four independent experiment s(*** p val < 0.001).

STUDY 2

Cyclin D1 oncogenic overexpression induces DNA replication stress and activates DNA damage response, correlating with aggressive clinicopathological features in MCL.

*Manuscript in preparation

1. Introduction

Genomic instability is a major driving force of tumorogenesis. It refers to an increased tendency of accumulating alterations in the genome during the life span of cells. It is counteracted by different mechanisms, including DNA-repair, cell cycle checkpoints and chromosome segregation control(Bartek and Lukas, 2001; Bartek et al., 2012; Halazonetis et al., 2008). Up to date, many events can give rise to this pathological condition such as alterations in control mechanisms, OIRS or replication stress.

MCL is a lymphoid neoplasm with striking high levels of chromosomal instability and proliferation. Early steps in MCL lymphomagenesis are thought to be orchestrated by t(11;14) translocation, causing cyclin D1 upregulation (Jares et al., 2007). The classical tumorogenesis model considers that cyclin D1 performs its oncogenic effect through the phosphorylation of RB and E2F release, promoting G1/S phase transition. However, emerging roles of cyclin D1 in other models question this simplistic concept. In addition to other roles of cyclin D1 now under investigation, aberrant overexpression during S-phase causes problems in rereplication and DDR activation (Gladden et al., 2006). These results point out a more complex role of cyclin D1 dysregulation in cell cycle in different models, although there is still little evidence about cyclin D1 role in replication stress in B lymphocytes (Shimura et al., 2013).

Taking together these findings, it may represent a strong rationale to investigate the functional role of cyclin D1 in replication stress and DNA damage activation in MCL. To date, there is little evidence showing cyclin D1 levels can directly contribute to DNA instability. In addition, the DDR activation in MCL has not been evaluated. We demonstrate cyclin D1 overexpression promotes proliferation and DNA replication stress in lymphoblastic B-cell models, activating the DDR. Our *in vitro* results led us to characterize the DDR activation of a small cohort of MCL primary cases in order to study the association of the DDR with clinicopathological data.

2. Results

2.1 Cyclin D1 promotes entry into S-phase but it increases the S-phase duration in a lymphoblastic cell line

Cyclin D1 is a cell cycle regulator critical for G1/S progression. However, its overexpression has given rise to contradictory results in different cell models. To clarify the oncogenic role of cyclin D1 overexpression in MCL lymphomagenesis we developed inducible overexpressing cyclin D1 cell models (Study 1). As we depicted before, we overexpressed in JVM13 cells either the highly stable nuclear form of cyclin D1 (JVM13-D1^{T286A}) or the wild-type form of the protein (JVM13-D1). We also overexpressed both cyclin D1 in JVM2 cells, a MCL cell line with low cyclin D1 expression.

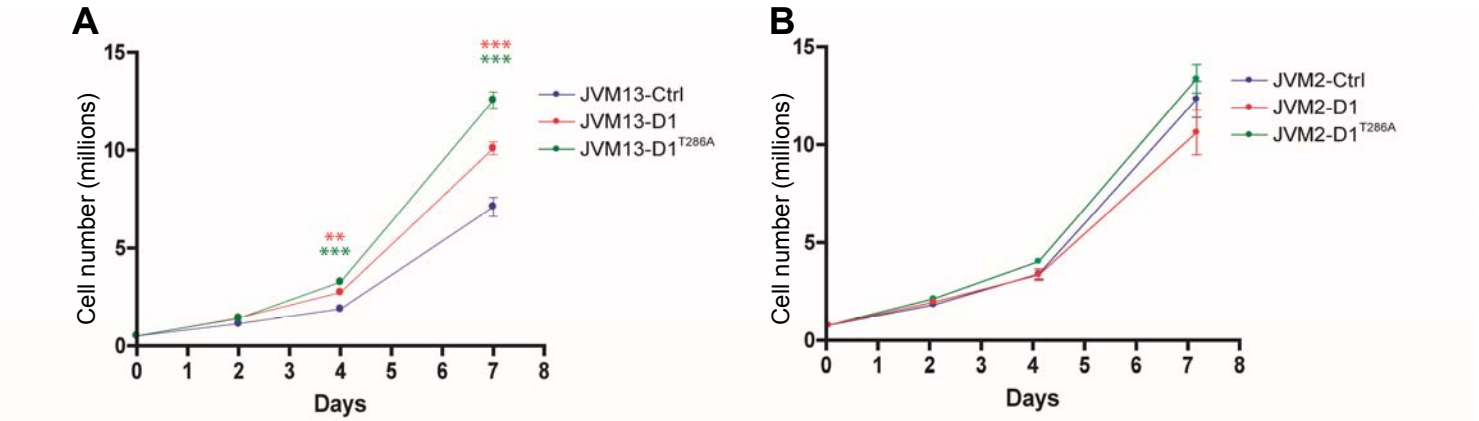


Figure 58. **Cyclin D1 overexpression increases proliferation in JVM13 cells.** A) Growth curve after 2, 4 and 7 days of cyclin D1 overexpression in JVM13. Results are shown as means ± SEM of three independent experiments. Statistical significance is calculated by t. test against the control (**:p val<0.01; ***:p val<0.001). B) Growth curve after 2, 4 and 7 days of cyclin D1 overexpression in JVM2. Results are shown as means ± SEM of three independent experiments.

Cyclin D1 overexpression enhanced cell growth when compared with cyclin D1 negative JVM13 cell line (p val<0.001). Moreover, the growth rate was significantly higher in JVM13-D1^{T286A} than in JVM13-D1 cells (Figure 58A). This difference might be related to the higher levels of cyclin D1 obtained in JVM13-D1^{T286A} cells (Study 1). Although we could detect a tendency to display increased cell growth rate in JVM2-D1^{T286A} cells compared to controls, it did not reach statistical significance (Figure 58B).

We wanted to characterize the cell cycle pattern associated to the increased cell growth induced by cyclin D1 overexpression. For that we decided to work with JVM13 model since the total absence of cyclin D1 protein in JVM3 cells would allow to study more clearly the effects of cyclin D1 overexpression. In addition, in this model we observed an association between the cell growth ratio and the amount of cyclin D1. We analyzed the proportion of cells in S phase following cyclin D1 induction in JVM13 cells for 24 hours. Cells were incubated with EdU to identify cells that were actively replicating genomic DNA. Cyclin D1 overexpression caused an increment of the percentage of cells undergoing DNA replication (EdU+ cells) in JVM13-D1 (46%) and JVM13-D1^{T286A} (47%) compared to control cells (29%) (Figure 59B).

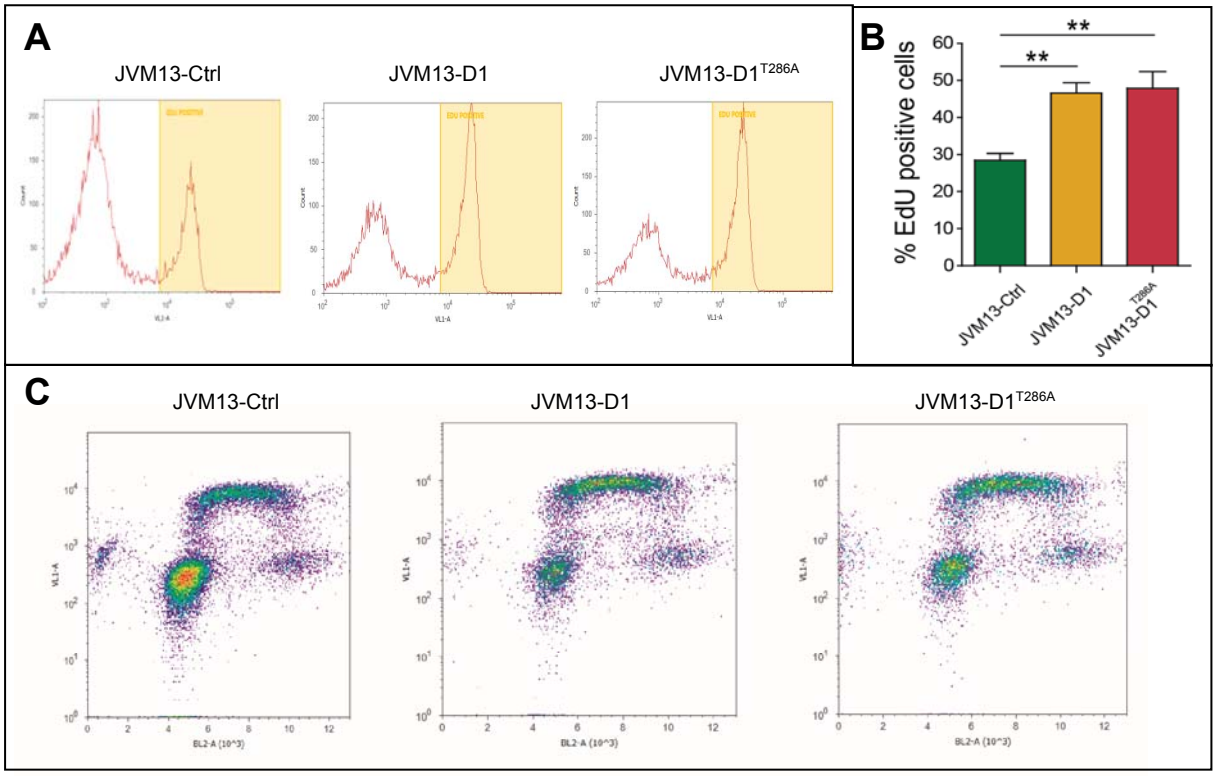


Figure 59. **Cyclin D1 overexpression increases the number.** A) FACS profile of EdU cytometry of a representative experiment of cyclin D1 overexpression in JVM13 cells after 24 hours of cyclin D1 overexpression. B) EdU positive fraction of cells after 24 hours of cyclin D1 overexpression. Results are shown as means ± SEM of three independent experiments**.** (non-paired t. test, *:p val<0.05; **:p val<0.01). C) Cell cycle profile analyzed by EdU/Pi staining of a representative experiment of cyclin D1 overexpression in JVM13 cells after 24 hours of cyclin D1 overexpression.

Although the increased S-phase fraction promoted by cyclin D1 overexpression was consistent with the observed cell growth induction (Figure 59A, B, C), there was certain disparity between these two measurements. The cell growth curve evidenced a 10-20% reduction of the estimated cell doubling time in the presence of cyclin D1 (JVM13-Ctrl: 1.84 days; JVM13-D1: 1.62 days; JVM13-D1^{T286A}: 1.51 days), whereas progression into S-phase was increased above 50%. This difference between the proportion of cells progressing from G1 to S and the estimated cell doubling time could indicate that the length of each phase is significantly affected by the overexpression of cyclin D1. We estimated the length of each phase taking into account the fraction of cells in each phase (Figure 59B) and the estimated cell doubling time. JVM13-Ctrl cells were 1.17 days in G1, while cyclin D1 positive cells stayed shorter, around 0.6 days. As expected, G2/M was similar in controls (0.23 days) and cyclin D1 cells (0.24 days). Consequently, JVM13-Ctrl cells lasted 0.44 days in finishing S-phase, while this process took 0.66 days in overexpressing cyclin D1 cells. These results suggest that cyclin D1 overexpressing cells enter faster to S phase, but undergo slower replication.

To validate this hypothesis, we performed a time course marking S-phase replicating cells with EdU and analyzing their progression through cell cycle. Cyclin D1 expression was induced by adding doxycycline to JVM13-Ctrl and JVM13-D1^{T286A} cells. Three hours after doxycycline induction, but before cyclin D1 was detected by western blot,

cells were labeled with a one hour pulse of EdU in order to identify S-phase cells. Samples were taken at different time-points and EdU positive cells were analyzed by PI cell cycle staining (Figure 60). Although at the initial time-point JVM13 cyclin D1 negative and positive cells had similar DNA content patterns, EdU positive control cells reached G1 phase shortly (T=6h).

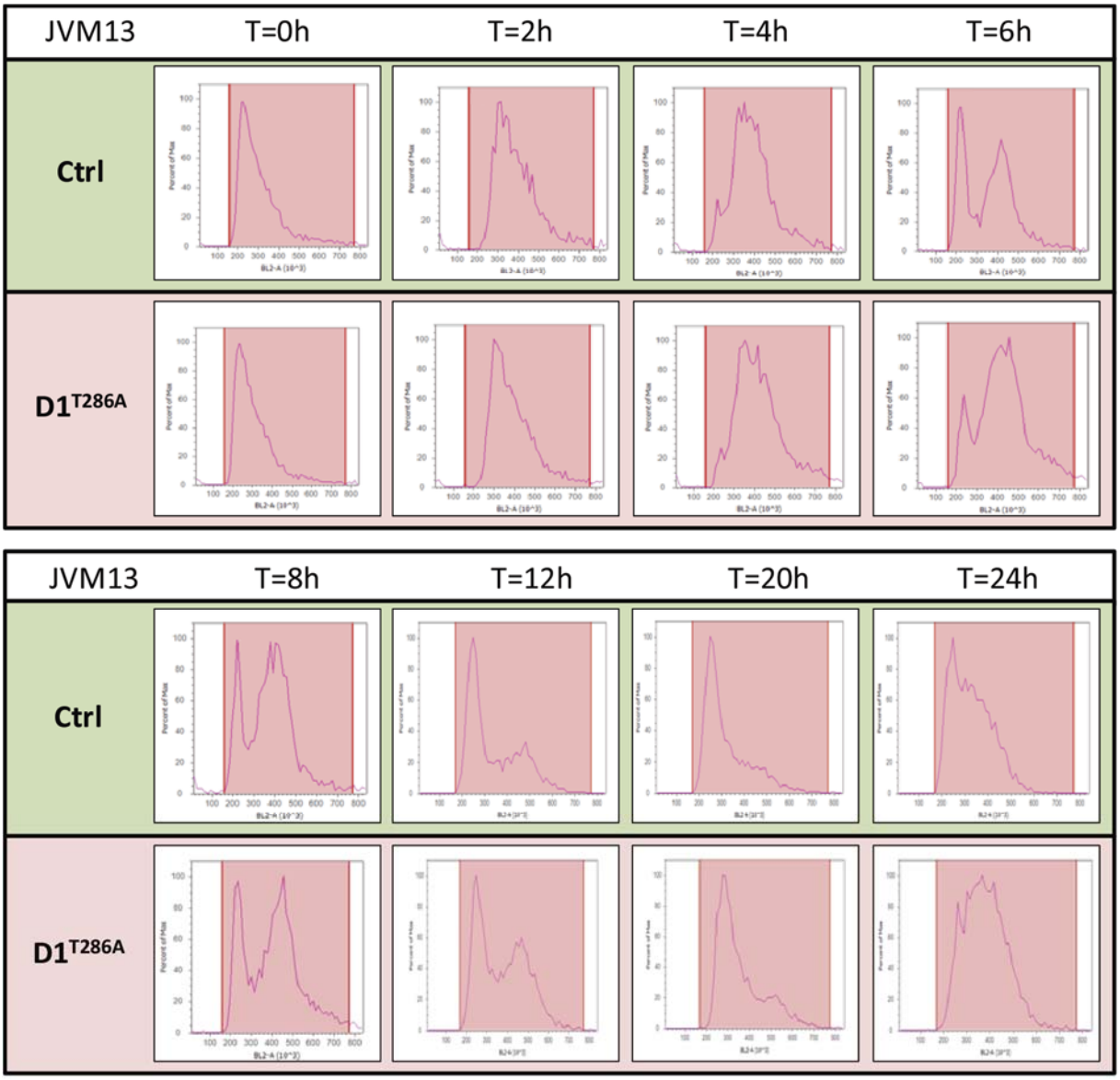


Figure 60**. Nuclear cyclin D1 overexpression slows S-phase progression and speeds up G1/s transition.** Time course showing the cell cycle profile of EdU positive cells analyzed by PI staining of a representative experiment. After three hours of doxycycline induction, JVM13-Ctrl and JVM13-D1^{T286A} cells where incubated for one hour for EdU. After EdU removal, EdU positive cells were analyzed at indicated time points. Details can be found in Methods section.

However, this experiment does not allow us to conclude if these cells are being blocked in late S-phase or in G2/M. In addition, we just interrogated mutated cyclin D1, so we do not know whether the wild type cyclin D1 also behaves in a similar way. To further explore whether cyclin D1 impairs progression through S-phase we performed a similar experiment, choosing only one time point, but labeling cells with BrdU to distinguish EdU positive cells that are still replicating. Cyclin D1 expression was induced by adding doxycycline to JVM13-Ctrl and JVM13-D1^{T286A} cells. Three hours after doxycycline

induction, cells were labeled with a one hour pulse of EdU in order to identify replicating cells. Ten hours after EdU labeling cells were labeled again with a BrdU pulse and the cell cycle distribution of EdU positive cells was analyzed. Remarkably, cyclin D1 overexpressing cells were mainly in late S and G2/M phases (EdU & BrdU positive cells), whereas control cells were largely in G0/G1 phase (Figure 61). Altogether, these results indicate that although cyclin D1 promotes cell cycle progression it determines a slowdown of the S-phase and an accumulation of cells in G2/M or late S-phase.

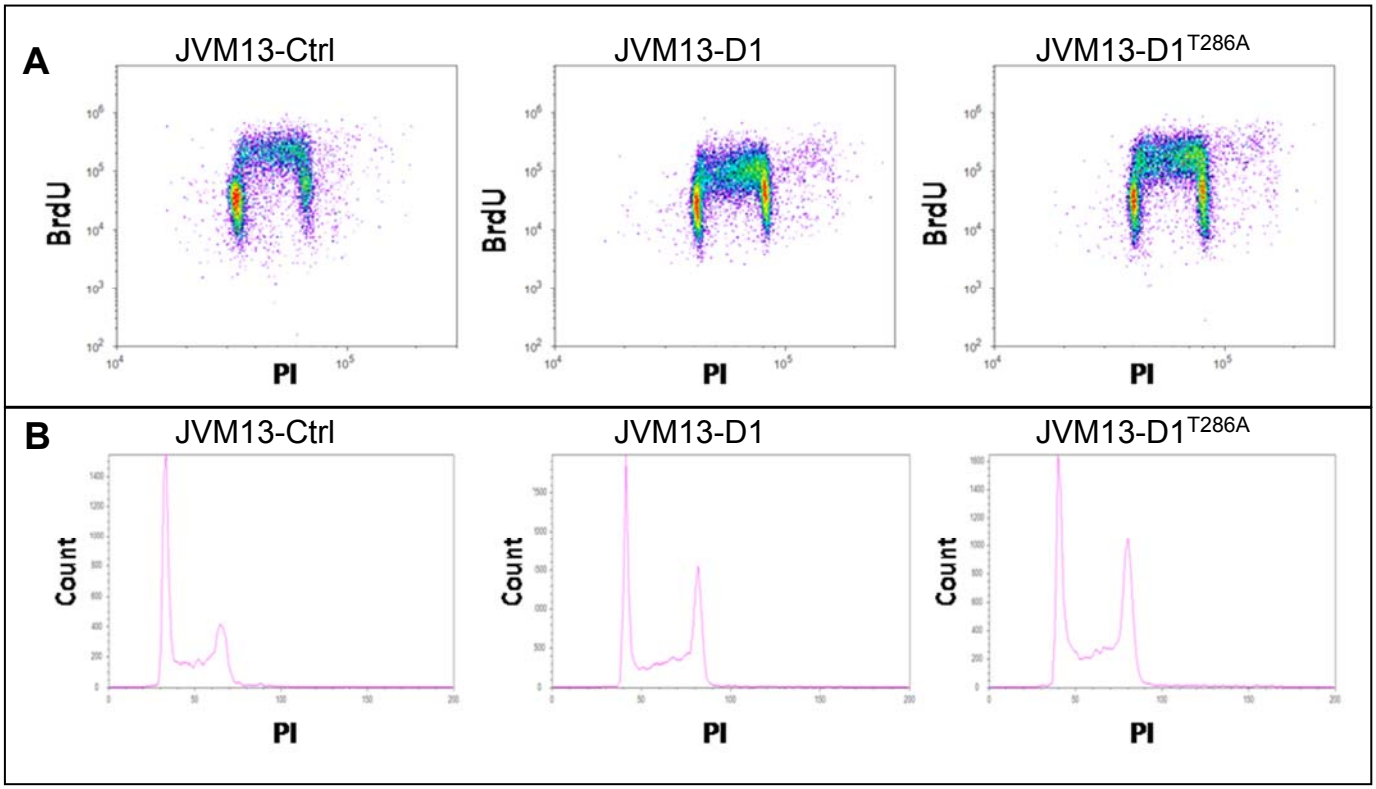


Figure 61. **Cyclin D1 overexpression slows S-phase progression** A) Cell cycle profile of EdU positive cells analyzed by BrdU/PI staining of a representative experiment of cyclin D1 overexpression in JVM13 cells after 13 hours of doxycycline induction. Details can be found in Methods section. B) Cell cycle profile of EdU positive cells analyzed by PI staining of a representative experiment of cyclin D1 overexpression in JVM13 cells after 13 hours of doxycycline induction. Details can be found in Methods section.

2.2 Cyclin D1 overexpression induces DNA replication stress

To further analyze the mechanism behind the S-phase slowdown mediated by cyclin D1 overexpression we investigated the speed of the DNA replication fork. DNA fiber assays were performed in JVM13-D1^{T286A} and control cells. Following 24 hours cyclin D1 induction cells were labeled with consecutive pulses of CldU and IdU. After 30 minutes incubation with IdU the length of the IdU tracks was measured. In three independent experiments JVM13-D1^{T286A} cells showed a lower elongation rate (shorter IdU track) compared to control cells that was translated into a significant reduction of the speed of DNA replication forks (p val< 0.001) (Figure 61).

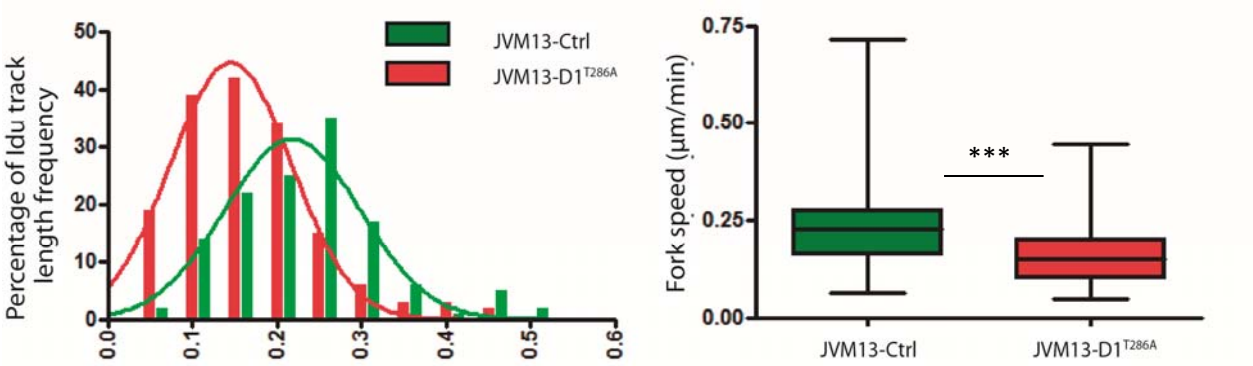


Figure 61. **Cyclin D1 overexpression slow down fork progression.** JVM13-Ctrl and JVM-13 D1^{T286A} were incubated with doxycycline for 24 hours and fiber assay was performed. IdU track length of 150 fibers from one representative experiment was measured. IdU track length distribution (left panel) and box and whiskers showing Min, Max, Median and first quartiles of fork speed value for one representative experiment. Statistical significance was calculated using non-paired t. test (***:*p val*< 0.001*)*. Three independent experiments were conducted and all three showed statistical reduction in fork speed (non-paired t. test, p val<0.001).

The reduction of the fork speed is compatible with the presence of DNA replication stress (Zeman and Cimprich, 2014). To confirm this point we analyzed in detail the fibers in order to identify and quantify other fiber features associated to DNA replication stress (Figure 62). We observed a significant higher number of stalled forks and a lower fraction of elongating fibers following cyclin D1 overexpression. Concordantly with the firing of dormant origins as response of cells to DNA replication stress we observed an increment of new activated origins. All these data suggest that cyclin D1 overexpression induce DNA replication stress.

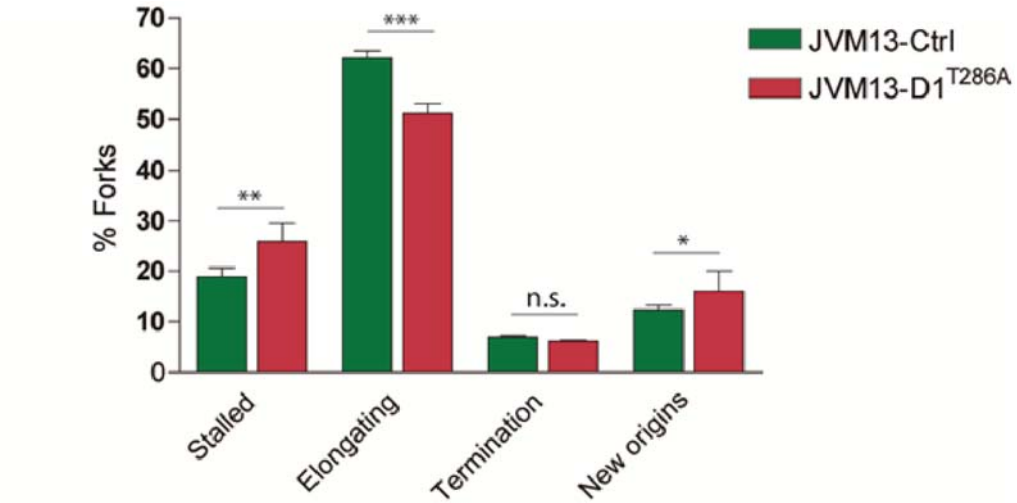


Figure 62. **Cyclin D1 overexpression impairs replication in JVM-13 cells.** JVM13-Ctrl and JVM-13 D1^{T286A} were incubated with doxycycline for 24 hours and fiber assay was performed. The percentage of replication stalled replication forks, new origin firing, elongating forks and terminating forks were counted. More than 600 fibers from three independent experiments were counted for each cell line. Results are shown as means ± SEM. Statistical significance was calculated using non-paired t. test (*:p val<0.05; **:p val<0.01; ***:p val<0.001).

Fork stalling has been associated to the presence of DNA damage. To confirm the increased presence of stalled forks that could compromise genome integrity we look for the presence of asymmetry forks that could arise from incomplete fork progression at one side of the replication bubble. To study this characteristic, we counted the new origins with two elongating forks observed in the three experiments. We established an asymmetry fork ratio by measuring both elongating forks of the fiber and dividing the length of the longer fork by the length of the shorter one. We displayed the log2 of this ratio to identify the distribution of symmetric fibers (log2 Ratio close to zero) or asymmetric fibers (log2 Ratio distinct to zero) in JVM13-D1^{T286A} and JVM13-Ctrl cells. (Figure 63). As shown in the Figure 63, JVM13 cells overexpressing D1^{T286A} showed a significant increase of the asymmetry ratio compared to JVM13-Ctrl (P val<0.008, Wilcoxon test). Moreover, cyclin D1 overexpression led to the accumulation of a population of highly assymmetric forks that represented close to 40% of the total anayzed forks in JVM13-D1^{T286A}. All these results together support the idea that cyclin D1 overexpression is slowing replication forks, forcing cells to stay longer in the S-phase to replicate its genome. Moreover, this impairment in the progression of the replication causes fork blockage and fork asymmetry. To sum up, cyclin D1 overexpression leads to an increment of the replication stress.

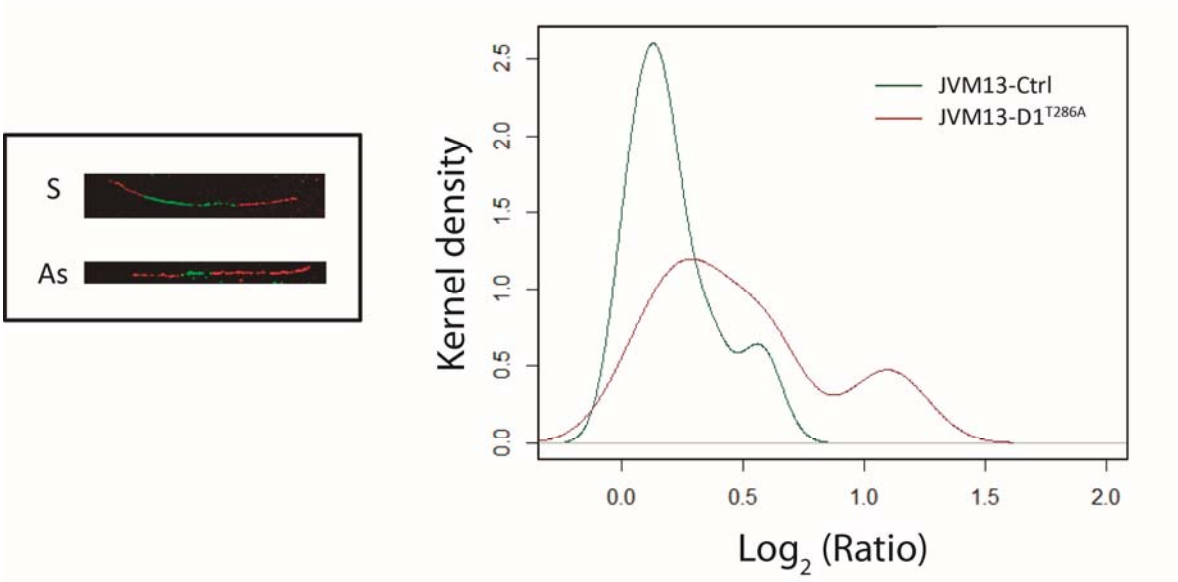


Figure 63. **Cyclin D1 overexpression increases the proportion of asymmetric forks in JVM-13 cells.** Examples of the new origin forks were symmetry (S) and asymmetry (As) detected in fiber experiments from figure 62 and 63 (Right panel). For this analysis, we considered the new origins from more than 450 analyzed fibers from 3 independent experiments showing a red-gree-red pattern. Kernel density of log2 ratio between the long and the short arms of the new origin, showing a population of asymmetric forks in JVM13-D1^{T286A} (Left panel).

To explore whether cylin D1 participates in RSR, we examined the ability of cyclin D1 positive cells to recover from a transient replication fork arrest. After 24 hours of 0.3 mM hydroxyurea (HU) with concomitant induction of cyclin D1, cells were released from the incubation. HU caused an increment of the 20% of cell death in JVM13-Ctrl cells (Figure 64). However, we could detect an increment in cell death up to 30% of cells in JVM13-D1^{T286A} cells. (Figure 64). With the HU conditions used for the experiment, replication is stopped in JVM13 cells (Data non shown). This experiment

confirms that cyclin D1 overexpression hampers JVM13 cells to recover from a cell stress.

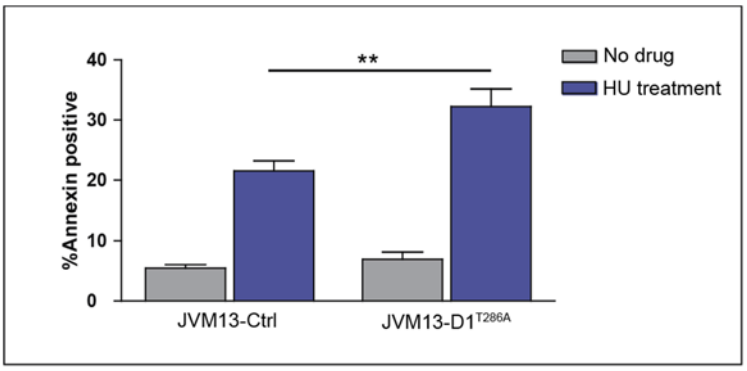


Figure 64. **Cyclin D1 overexpression impairs replication stress recovery.** A) Annexin V positive cells after 24 hours recovery upon HU-induced fork stalling for 24 hours. Results are shown as means ± SEM from four independent experiments. Significance was calculated using non-paired t. test (**:p val<0.01).

2.3 Continuous cyclin D1 overexpression causes DNA damage activation and genomic instability

The presence of DNA replication stress induced by cyclin D1 overexpression could compromise the genome integrity of the cells. In order to test whether continuous cyclin D1 overexpression would lead to DNA damage and the activation of the DNA damage response we induced cyclin D1 in JVM13 models (Figure 65). After four and seven days of cyclin D1 induction we analyzed the expression of γH2AX as a surrogate of the presence of DNA damage. After four days of cyclin D1 expression, γH2AX showed an strong induction that was related to the amount of cyclin D1. The increase of γH2AX was also observed after seven days of induction. In addition, the overexpression of cyclin D1 also led to the expression of pCHK2, a DDR gene involved in the response to double strand breaks. Altogether, our results suggest that the DNA replication stress induced by cyclin D1 overexpression could lead to the activation of the DDR.

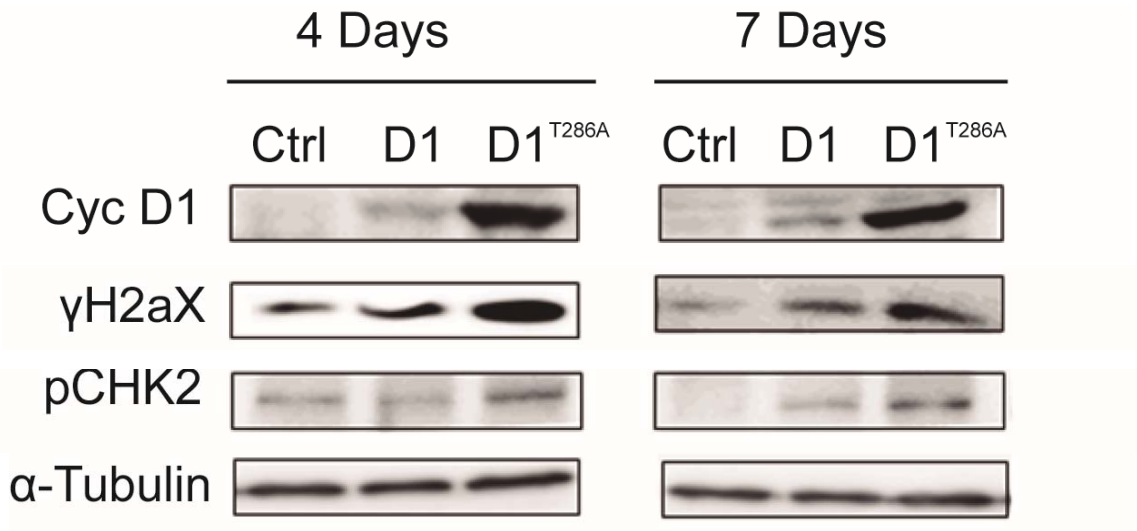


Figure 65. **Cyclin D1 overexpression activates DDR in lymphoid cells.** Cyclin D1 continues induction for 4 or 7 days in JVM13 lymphoblastic models increases γH2AX and pCHK2.

These results lead to think that cyclin D1 overexpression might be causing an increase in the number of polyploid cells. Since we did not analyzed this population in previous experiments we used PI cell cycle cytometry and evaluated the number of >4n cells after 48 hours of cyclin D1 induction. Cyclin D1 positive cells showed a higher number of tetraploid cells than JVM13-Ctrl. JVM13-D1^{T286A} cells showed 56% more tetraploid cells than the control cell line, indicating that in lymphoblastoid cell lines cyclin D1 is able to induce genomic instability and activate DDR (Figure 66).

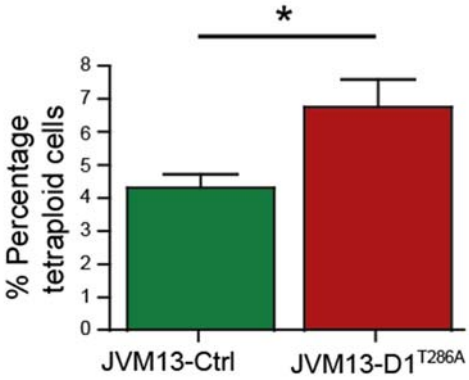


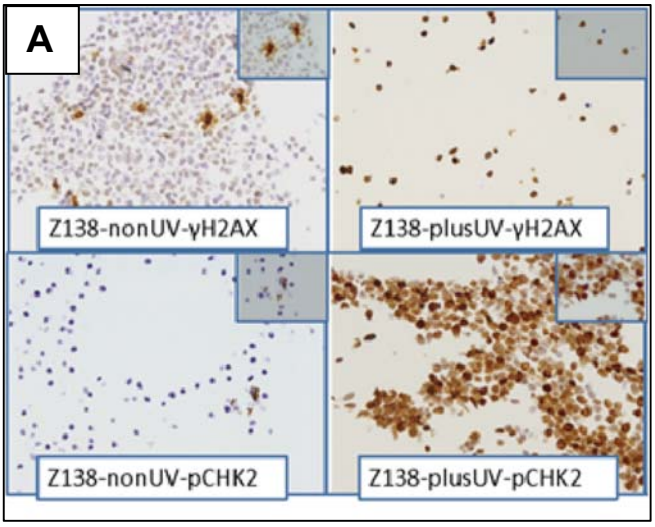
Figure 66**. Cyclin D1 overexpression increases tetraploid popultion.** >4n cells after 48 hours of cyclin D1 induction in JVM13-Ctrl and JVM13-D^{T286A} cells. Results are shown as means ± SEM from four independent experiments. Statistical significance was calculated using non-paired t. test (*:p val<0.05).

2.4 Activation of DDR components in mantle cell lymphoma correlates with clinicopathological features

We wanted to analyze whether the constitutive overexpression of cyclin D1 was associated with the detection of DNA damage and activation of the DDR in primary MCL. For that, we analyzed as a surrogate of DNA damage and DDR activation the expression of γH2AX and pCHK2 respectively. To standardize the detection of DDR markers by immunohistochemistry we used as positive control a MCL cell line (Z-138) irradiated by UV. After four hours recovery post-UV cellular pellets from untreated or treated cells were formol fixed and paraffin embedded. Strong, nuclear signal for both proteins was detected in 10% of the untreated cells. As expected, UV irradiation incremented the fraction of positive cells to 90% (γH2ax) and 70% (pCHK2) respectively (Figure 67A, B). To further evaluate the IHC detection, we analyzed the expression levels of the two DDR markers in normal tissues, including two reactive lymph nodes and two tonsils. In addition we studied small series of DLBCLs (n=10) since the expression of these DDR markers was previously reported for this lymphoma. Reactive lymph nodes and tonsils showed expression of γH2AX and pCHK2, only in few cells of the germinal centers. In our series of DLBCLs, the expression of γH2AX varied from 5% to 90%, with 6 of 10 cases showing positivity in more than 30% of tumor cells, while the expression of pCHK2 was around 10% in one case, as previously described (Derenzini et al., 2015).

Then, we analyzed the expression of γH2AX and pCHK2 in a series of primary MCL cases (n=37). For both markers, the staining was scored from 0 (negative) to 3 (very high). The cases with more than 10% of the cells with a staining value greater than 1 were classified as "high" signal group, whereas the cases with absence of expression

(0) or with a low percentage of positive cells (<10%) were defined as "low" signal group.
There was a strong positive association between the expression of γH2AX and pCHK2, since 14 out of 15 cases with high pCHK2 (93%) showed high γH2AX expression whereas only 10 out of 22 cases with low pCHK2 (45%) showed high levels of γH2AX (Person X^2, p val<0.005). (Figure 68B)



B

Antibody	Basal levels	UV
γH2AX	10%	90%
pCHK2	10%	70%

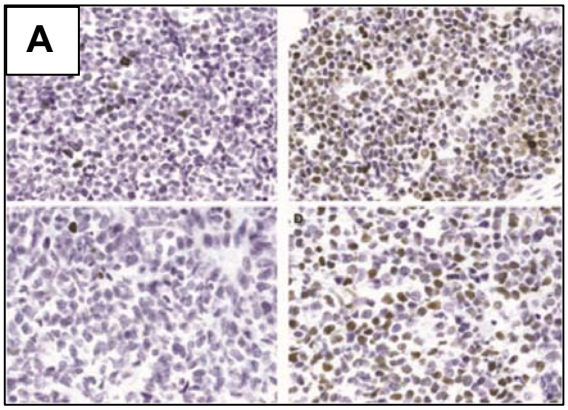
Figure 67. **UV-light is a good method to induce DDR activation in MCL cell lines.** A) Representative IHC pictures from paraffin-embedded Z138 cell line treated with 100J/m^2 UV and 4 hours recovery (plusUV) or without (nonUV) stained for pCHK2 and γH2AX. Higher expression levels of activated DDR pathway components were observed in UV-treated cells. B) Table summarizing the percentage of cells showing strong IHC staining in Z138 and conditions.

We wanted to correlate the expression of DDR markers with the clinicopathological characteristics of the tumors. Due to the low number of samples and the distribution of the staining, we classified the samples in two groups, high DDR activation (HDA) that include cases positive for both phospho-proteins (n=14; 37.8%) and low DDR activation (LDA) group that contains cases with low pCHK2 (n=22; 59.45%). Only one case was unclassified since it showed high pCHK2 with low γH2AX. Blastic and pleomorphic cases tend to show high expression of DDR markers, since five out of seven blastoid cases (71%) were included in the HDA group whereas only six out of 21 (28%) classical MCL were classified as HDA (Pearson X^2 , p val<0.05). Concordantly, a strong association between DDR activation and proliferation measured by Ki67 staining was detected. In that sense eight out of 10 HDA cases (80%) displayed high Ki67 (> 30% positive cells) expression whereas only six out 21 (28%) LDA cases showed high Ki67. These results suggest that high proliferative and more aggressive cases display higher levels of DNA damage and activation of DDR. (Pearson X^2 , p val<0.05).

MCL shows frequent alterations of genes involved in the DNA damage response and these alterations have been associated with increased chromosome instability. In our series we had information regarding *TP53* and *CDKN2* genetic alterations. . Eventually, we had mutational and/or copy number information for 26/36 cases (72%). Strikingly, eight of the ten HDA cases (80%) showed genomic alterations in *CDKN2A* and/or *TP53* , but only five out of 16 low LDR (31%) displayed alterations of these two loci

(Pearson X^2 , p val<0.01). Concordantly, HDA cases showed a significant higher number of copy number alterations than LDA cases (HDA, mean=15.9; LDA, mean=6.8, Wilcox test p val = 0.015). In addition, the HDA cases showed shorter overall
survival than LDA cases (p val<0.005). The high expression of any of the DDR markers also correlated to worse overall survival (Figure 69) and higher CNA (γH2AX , p val=0.0037; CHK2, p val=0.015) (Figure 70). Concordantly, HDA group had low overall survival compared to LDA.

A



B

	High pCHK2	Low pCHK2	Total
High γH2AX	14	10	24/37 (64.9%)
Low γH2AX	1	12	13/37 (35.1%)
Total	15/37 (40.5%)	22/37 (59.5%)	37/37 (100%)

Figure 68. **DDR markers correlate in MCL primary cases.** A) Representative IHC pictures from paraffin-embedded tissues stained for DDR markes. Negative (upper left) and positive (upper right) stain for γH2AX is shown, as well as negative (bottom left) and possitive (bottom right) for pCHK2. B) Table summarizing the percentage of primary MCL cases showing γH2AX or pCHK2 staining in our cohort.

Recently it has been described a variant of MCL with specific clinical and pathological characteristics like absence of lymph node affection, low number of chromosome abnormalities and a more indolent clinical behaviour. These cases may be recognized by the negativity for SOX11, a specific marker of conventional MCL. In our series, we knew the SOX11 expression in 34 cases. Twenty-six out of 34 (76%) MCL were SOX11 positive and eight were SOX11 negative (24%). Consistent with the association between the activation of DDR and the presence of more aggressive features, only one HDR sample was observed in the SOX11 negative group. Interestingly, this case has a high number of CNA (CNA=33) , including loss of 9p and *TP53* mutation.

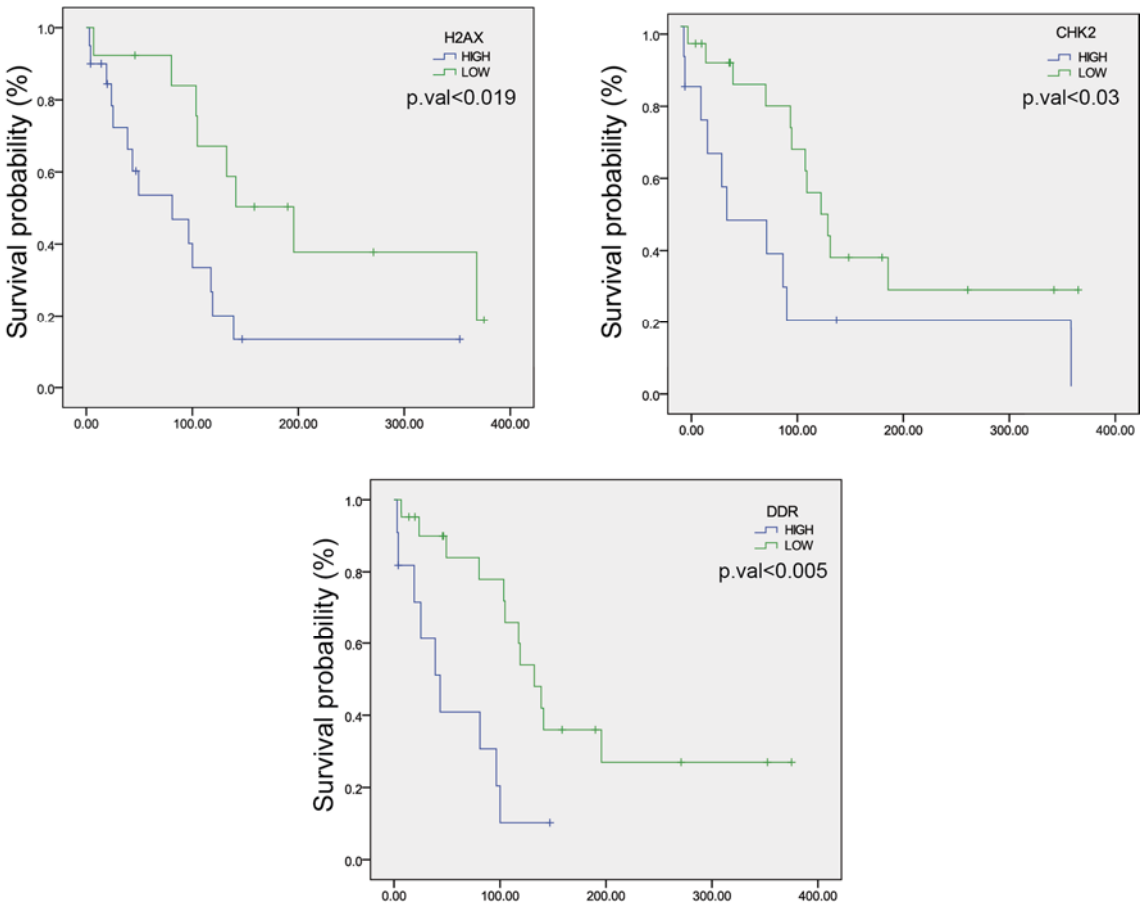


Figure 69. **DDR activation correlates with lower survival in MCL primary cases.** A) Kaplan Meyer curves of primary MCL cases stratified by H2aX (left) or pCHK2 (right) staining. P values were calculated by the Log-rank algorithm. B) Kaplan Meyer curves of primary MCL cases stratified by high or low DDR levels in MCL primary cases. P value was calculated by the Log-rank algorithm

We analyzed whether the association of DDR activation with aggressive features was observed when only the SOX11 group was considered (n=26). Fifty percent of the SOX11 positive cases were categorized as HDR. Information about copy number variations was available for 19 SOX11 positive cases. Similar to the whole series, the SOX11 positive cases classified as HDA group had higher number of CNA than the LDA group (*HDA, mean=14; LDA, mean=9, Wilcox test p val=0.022*).

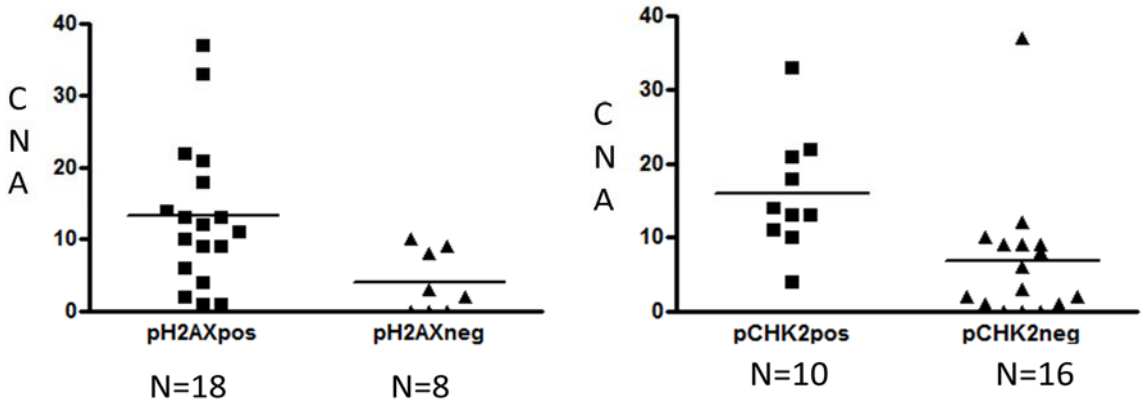


Figure 70. **Higher DDR activations correlates with** γH2AX **and pChk2 staining in MCL primary cases.** Copy number alterations (CNA) in γH2AX (left) and pChk2 (right) divided by their levels of staining.

Moreover, HDA cases showed poorer outcome in comparison to LDA group (Breslow test, p val=0.022, Figure 71) in SOX11 positive cases. All these data suggest that presence of DNA damage and activation of DDR is a common feature of primary MCL cases with a more aggressive behaviour.

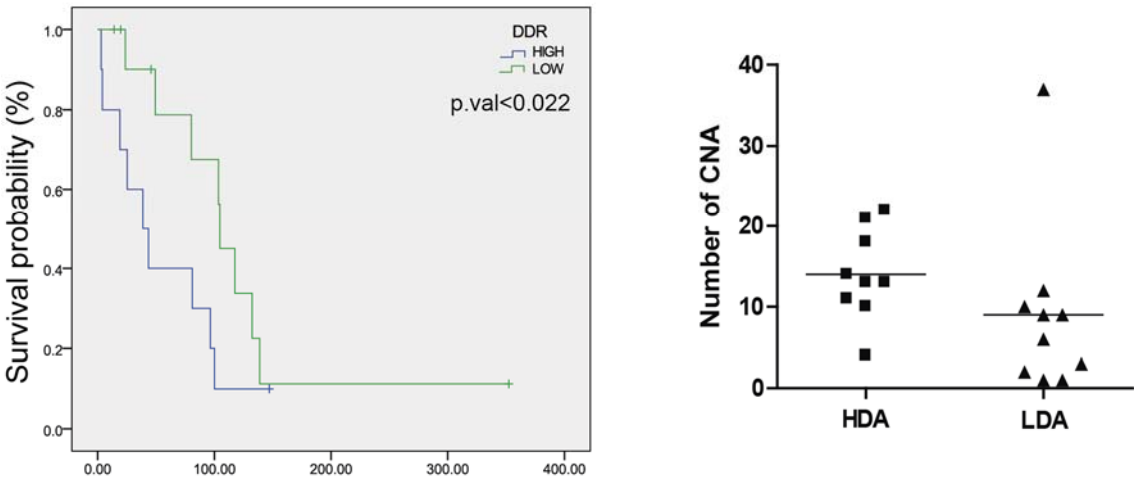


Figure 71. **DDR activation is a prognostic marker in SOX11 positive cases.** A) Kaplan Meyer curves of primary MCL cases stratified by high or low DDR levels (HDA or LDA group) in SOX11 positive cases. P value was calculated by the Breslow algorithm. B) Copy number alterations (CNA) in HDA and LDA SOX11 positive MCL primary cases.

STUDY 3

MCL cases with high proliferation accumulate an increased number of methylated CpGs

*The results of this study were published in December 2013: Enjuanes A, Albero R, Clot G, Navarro A, Beà S, Pinyol M, Martín-Subero JI, Klapper W, Staudt LM, Jaffe ES, Rimsza L, Braziel RM, Delabie J, Cook JR, Tubbs RR, Gascoyne R, Connors JM, Weisenburger DD, Greiner TC, Chan WC, López-Guillermo A, Rosenwald A, Ott G, Campo E, Jares P. **Genome-wide methylation analyses identify a subset of mantle cell lymphoma with a high number of methylated CpGs and aggressive clinicopathological features**, Int J Cancer. 2013 Dec 15;133 (12) :2852-63. doi: 10.1002/ijc.28321.

1. Introduction

Mantle cell lymphoma (MCL) is a B-cell neoplasia with a relatively short median survival (Swerdlow et al., 2016). This lymphoma is genetically characterized by the t(11;14)(q13;q32) translocation and cyclin D1 overexpression promoting cell cycle dysregulation. Besides this primary oncogenic event MCL cells may carry a high number of secondary molecular alterations that seem to contribute to the aggressive clinical course of the disease. Mainly, MCL displays mutations in proliferation and genomic instability pathways (Jares et al., 2007).

Increasing evidence confirms that acquired epigenetic abnormalities, like the genome-wide decrease in global DNA methylation (hypomethylation) and the concomitant increase of methylation (hypermethylation) in tumor suppressor genes (TSG), may be considered an important hallmark of human cancer (Egger et al., 2004; Jones and Baylin, 2007), including hematological malignancies(Esteller, 2008). Few studies have addressed the association of DNA methylation in MCL lymphomagenesis (Novak et al., 2009). In these regards, early single gene analysis in MCL identified low frequency or absence of methylation events in critical TSG commonly inactivated by mutation or deletion in MCL (Pinyol et al., 1998).

Despite these studies, the role of DNA methylation in MCL pathogenesis and its clinical relevance still remains an open question. In the current study, we sought to identify the potential role of *de novo* DNA methylation changes in the pathogenesis of MCL and to clarify their clinical relevance by performing a comprehensive genome-wide CpG island methylation profiling study, and correlating the DNA methylation status with gene expression, genomic alterations and clinical data in a large cohort of primary MCL tumors.

2. Results

2.1 DNA methylation profile of primary MCL

First, we perform a hierarchical non-supervised clustering analysis of all cases: 112 classic MCL (cMCL), 20 blastoid/pleomorphic MCL (bMCL), 6 cell lines and 30 normal samples. We identified two main groups, one containing the cell lines and the majority of primary MCL (80%), and the second comprising all the normal samples and a group of primary MCL (20%) (Figure 72A). The cell lines showed a CpG methylation pattern opposite to normal cells, whereas primary MCL evidenced a pattern similar to cell lines, but with a lower number of hypermethylated and hypomethylated CpG sites. Interestingly, the four sequential tumor samples obtained with a median interval of 5 years (range, 5-10 years) clustered together with their respective initial diagnostic specimens. Normal lymphoid samples had a more homogeneous methylation pattern than primary MCL (average correlation coefficient among normal samples r= 0.97; range= 0.05 vs primary MCL r=0.88, range=0.37). The tumor methylation heterogeneity was confirmed by the dispersion of the samples in a PCA analysis that in addition revealed a clear separation between normal samples and the majority of primary MCL (Figure 72B).

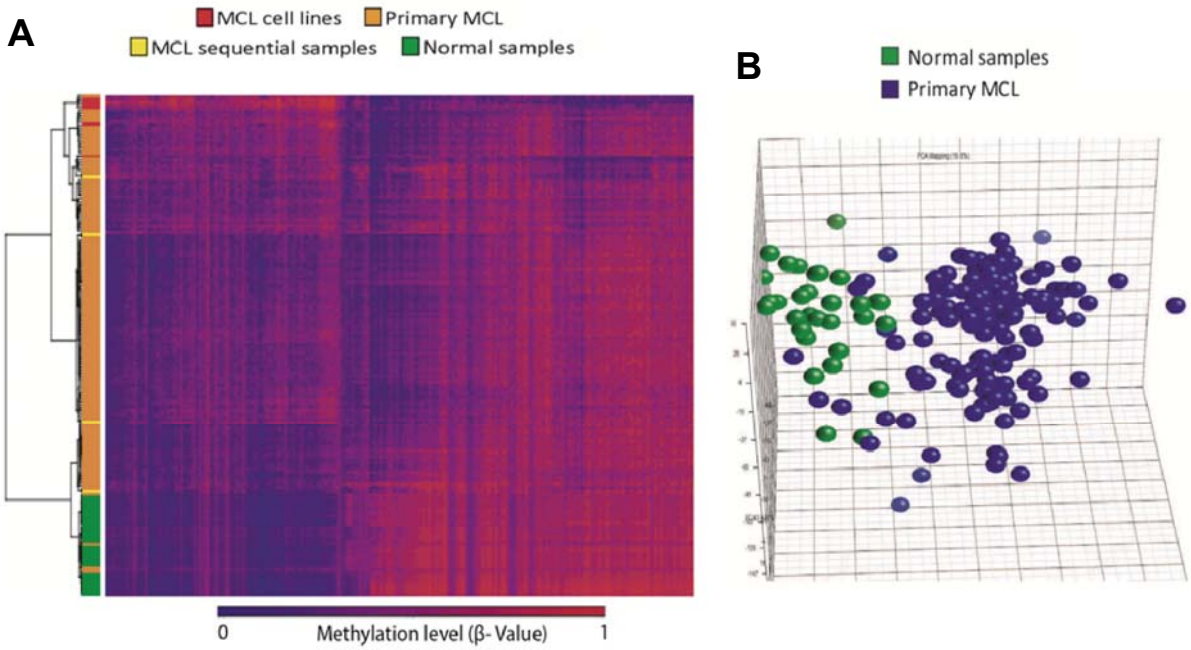


Figure 72. **MCL samples display a methylation profile different to normal lymphoid tissues.** A) Heat map of an unsupervised hierarchical clustering of 132 primary MCL, 4 sequential MCL, and 6 MCL cell lines together with 31 normal samples. The β-values of the 25th percentile of CpG probes with the highest standard deviation were used. B) A Principal component analysis (PCA) of primary MCL and normal samples clearly separates primary MCL (blue) from normal samples (green), and shows a more heterogeneous distribution of the tumors.

2.2 *De novo* DNA methylation changes in primary MCL

Our next step was to identify differentially methylated CpGs between primary MCL and normal samples. To identify highly significant differential methylation events between groups, we established that the average geometric difference of *β*-value should be at least 0.3 ($\Delta\beta \geq 0.3$) and showed an adjusted *p*-value of <0.001. We found hypermethylation and hypomethylation in 154 and 538 CpG residues, respectively (Figure 73A). Predictably, these CpGs classified MCL cell lines together and separated from the normal samples. cMCL and bMCL had a similar number of hypomethylated CpG (cMCL= 516, bMCL= 699) when compared to normal samples. However, bMCL showed a significantly higher proportion of hypermethylated CpG than cMCL (cMCL= 138, bMCL= 380; p val<0.0001) (Figure 73B). We observed that the majority of the hypo/hypermethylated CpGs found between cMCL and normal tissues were also detected differentially in bMCL vs normal samples.

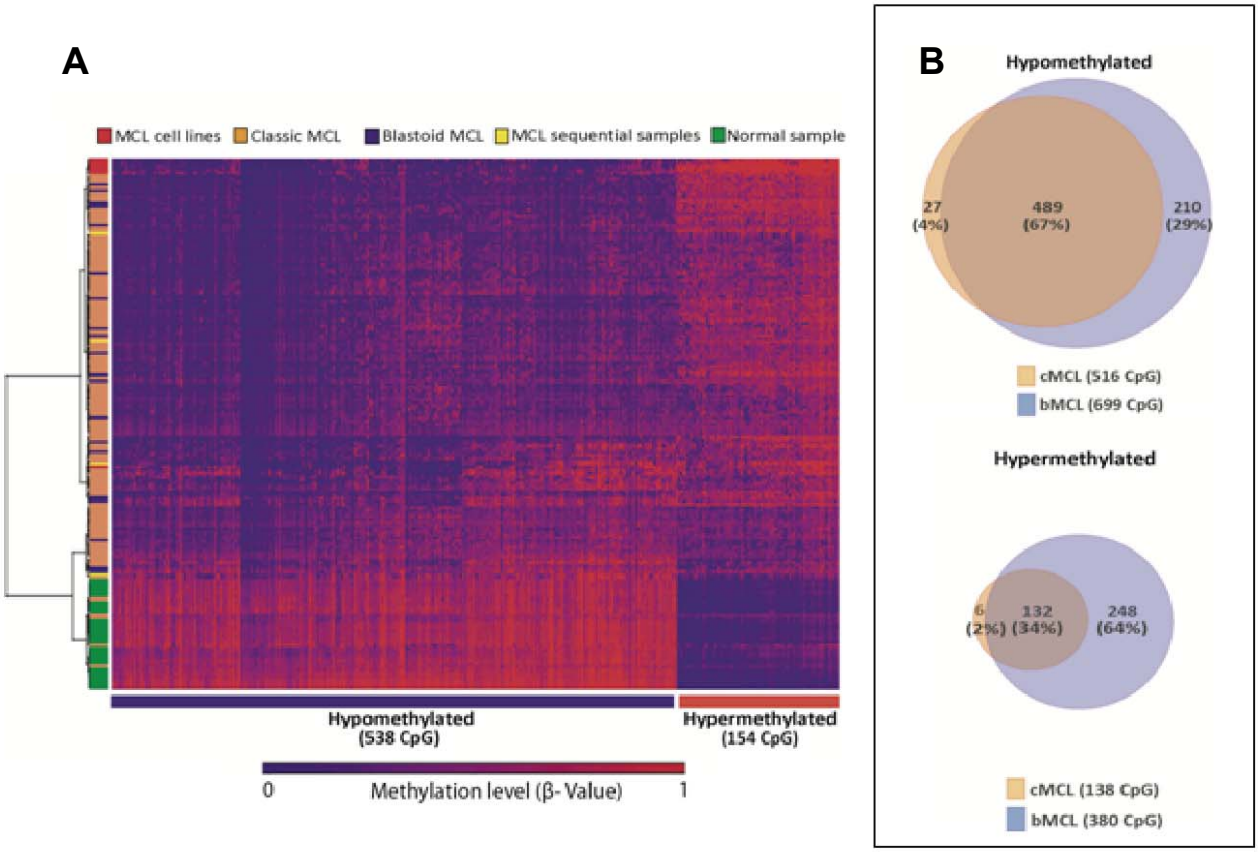


Figure 73. **Differentially methylated CpGs in primary MCL versus normal samples.** A) Heat map of a hierarchical clustering performed using the CpGs differentially hypermethylated (n=154) and hypomethylated (n=538) between primary MCL and normal samples; B) Venn diagrams representing the number of hypermethylated and hypomethylated CpG dinucleotides in blastoid and classical MCL when compared to normal samples.

One of the prominent facts derived from these results was the high heterogeneity observed among the primary MCL samples. Next, we aimed to define which of these epigenetic changes occurred *de novo* in MCL compared to normal samples. To do so, we took another approach considering that a CpG was *de novo* hypermethylated in a primary MCL when it displayed a β-value higher than 0.7 in at least 10% of primary

MCL and an average β-value below 0.4 in at least four out of the five categories of normal controls (Figure 74A). Following these criteria, a total of 551 CpG probes corresponding to 454 genes were considered *de novo* hypermethylated in MCL. CpG dinucleotides were considered *de novo* hypomethylated when displaying a β-value lower than 0.3 in at least 10% of primary MCL and an average β-value higher than 0.6 in at least four out of the five normal sample groups. A total of 947 CpG probes (including 63% of the CpG probes found in our previous algorithm) corresponding to 875 genes were selected as hypomethylated (Figure 74A). The clustering analysis of the cases using *de novo* hypermethylated and hypomethylated CpG revealed samples characterized by the accumulation of a higher number of hypermethylated and hypomethylated CpGs (Figure 74A). Accordingly, to this observation a positive correlation between the number of hypermethylation and hypomethylation events (r=0.737 and p val<0.001) was identified in primary MCL (Figure 74B). These results would suggest that, if epigenomic dysregulation occurs, it is translated in changes both in abnormal hypermethylation and hypomethylation.

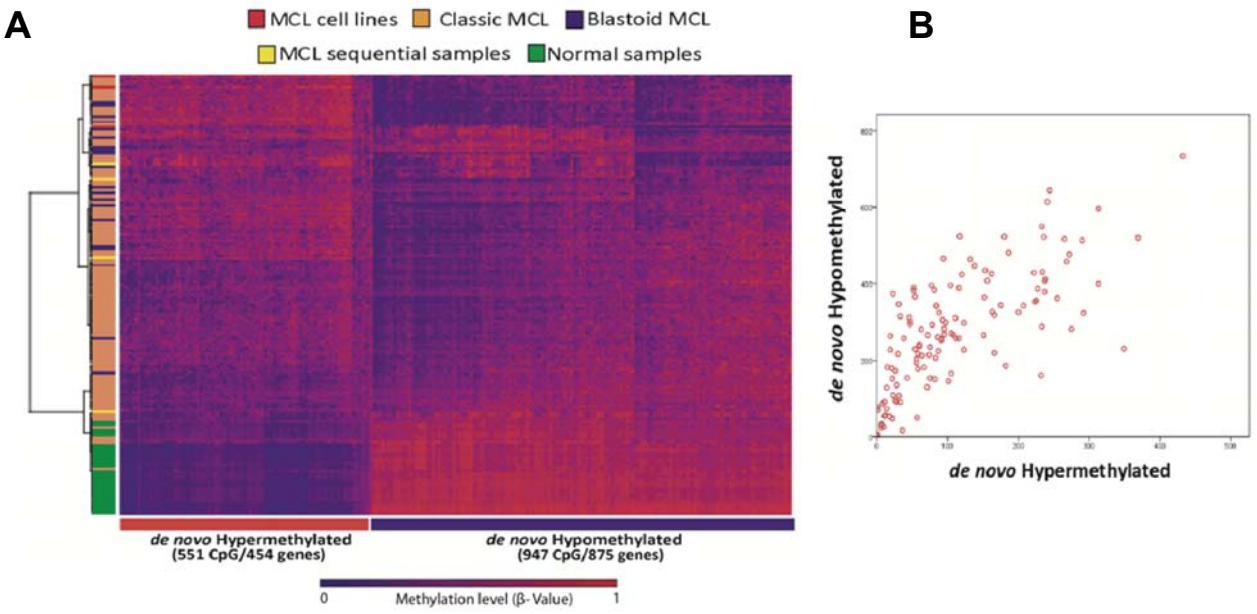


Figure 74. ***de novo* hypermethylated and hypomethylated CpG in at least 10% of primary MCL.** A) Heat map of a hierarchical clustering using the CpGs identified as *de novo* hypermethylated (n=551) and hypomethylated (n=947) ; B) Scatter-plot showing the number of *de novo* hypermethylated and *de novo* hypomethylated events per sample.

Although epigenetic alterations seemed to accumulate in the same subset of cases, we wanted to study if *de novo* hypermethylation and hypomethylation targeted the same kind of regions. Hypermethylation events mainly occurred in CpG sites within CpG islands (95%, p val<0.0001) and were located in promoters with high CG content (81%, p val<0.0001) (Figure 75A). On the contrary, the hypomethylation in primary MCL mainly occurred in CpG outside CpG islands (83.5%, p val<0.0001) and mapped to promoters with a low CG content (68%, p val<0.0001) (Figure 75A). In order to further elucidate the mechanisms beneath these different patterns between hypo and hypermethylation, we wanted to study how these CpGs could be targeted for *de novo* methylation in MCL cases. For that reason, we studied if they were targeted by EZH2

or presented H3K27metylation. This histone mark, typical of the enzymatic activity of EZH2, is well-described to have a role in development and silencing gene transcription. Concordantly, we observed a significant enrichment of hypermethylated genes targeted by EZH2 or showing histone repression marks in mature normal B cells (40%, p val<0.0001; and 47%, p val<0.0001 respectively). On the other hand, the set of hypomethylated genes did not show any enrichment of genes targeted by EZH2 or showing histone repression marks in mature normal B cells (Figure 75B). Taking everything together, *de novo* hyper and hypo methylation target different genomic regions, suggesting that they may happen through different mechanisms.

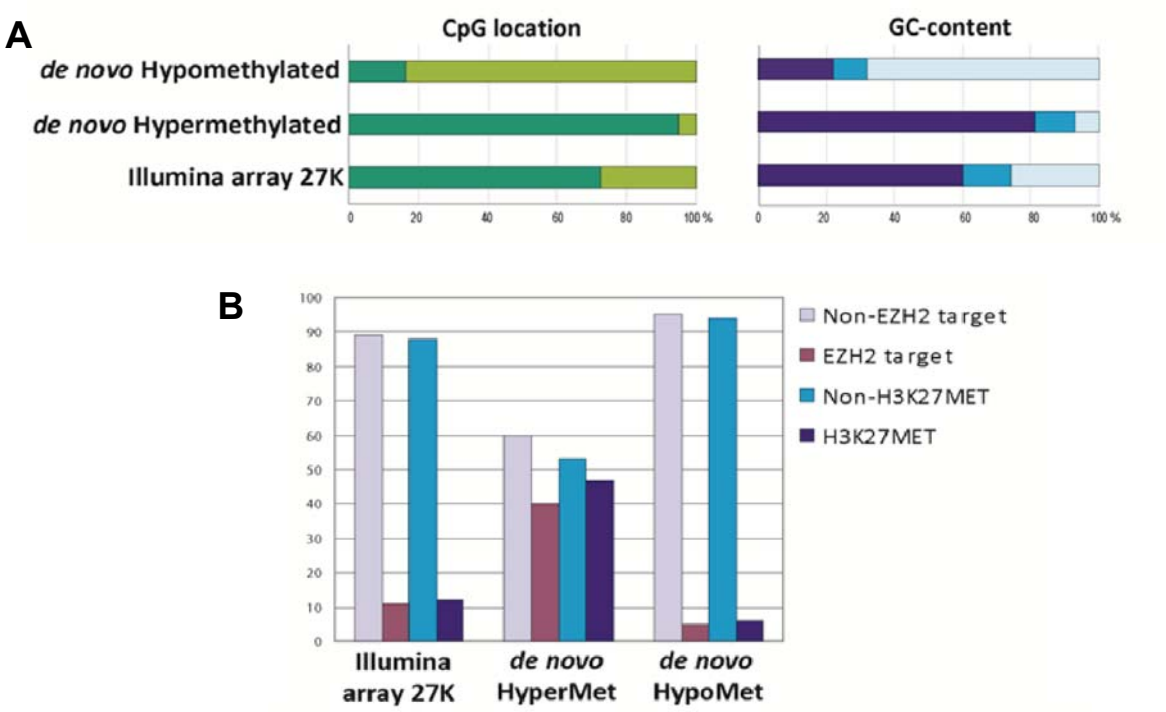


Figure 75. ***De novo* CpG hypermethylation occurs in different biological regions than *de novo* hypomethylation.** A) Bar graphs of the distribution of *de novo* hypermethylated and hypomethylated CpG according to CpG island association (CpG location, in green) and CG content (in blue), together with the distribution of the whole set of CpG present in the 27K Illumina array; B) Bar graphs of the distribution of the genes found as *de novo* hypermethylated or hypomethylated, and the genes interrogated by the 27k Illumina array, according to their description as targets of the EZH2 protein or as carriers of repressive histone marks in normal B-cells

2.3 Dysregulated pathways by *de novo* DNA methylation in primary MCL

Our next step was to study the pathways that are mainly targeted by methylation changes in MCL primary cases. Due to the enrichment in EZH2-target genes and the preferential hypermethylation in CpGs islands, we thought these methylation changes might dysregulate molecular pathways, rather than acting as a passenger event. According to our hypothesis, many of the hypomethylated promoters were related to general pathways like inflammatory and defense response pathways (p val<0.005). On the other hand, the hypermethylated list showed an enrichment in homeobox domain containing genes (p val<0.0001) and in genes related to transcription regulation and transcription factor activity (p val<0.0001). Interestingly, in the functional analysis using IPA, the WNT canonical pathway was significantly enriched (p val<0.025) with several

WNT antagonist genes hypermethylated. This pathway controls cell development, cell proliferation and migration and has been related to carcinogenesis. To further investigate this association, we assembled a homemade gene set containing 20 genes described as WNT inhibitors (Table 3).

Table 3. **Wnt inhibitors targeted by *de novo* hypermethylation in MCL cases.**

Wnt antagonist genes	nº of Methylated cases	% of Methylated cases
SOX9	87	65.91
SFRP1	45	34.09
SOX17	43	32.58
KREMEN1	23	17.42
DKK1	21	15.91
AXIN1	18	13.64
DKK3	12	9.09
SFRP2	12	9.09
ROR2	11	8.33
PITX2	10	7.58
RARB	10	7.58
CDH1	9	6.82
FRZB	6	4.55
FZD10	6	4.55
WIF1	5	3.79
DKK2	3	2.27
SFRP4	3	2.27
CDH2	2	1.52
APC	2	1.52
DKKL1	1	0.76

Nº of methylated gens	nº of Methylated cases	% of Methylated cases
>=1	104	78.79
>=2	79	59.85
>=3	56	42.42
>=4	43	32.58
>=5	32	24.24
>=6	21	15.91

This gene set showed a highly significant enrichment in the IPA analysis since 25% of the WNT antagonist were hypermethylated in primary MCL (p val< 0.0001). In fact, more than 78% of primary MCL showed hypermethylation of at least one WNT inhibitor gene and 30% of primary MCL showed hypermethylation of four or more genes. We verified by pyrosequencing the methylation status of *SOX9* and *SFRP1* promoter regions on GRANTA-519 and MCL cases (n=16), and normal samples (n=4).

2.4 Correlation between DNA methylation and gene expression

First methylation studies showed an inverse correlation between promoter methylation and transcription of the gene. To study the association between DNA methylation and gene expression we analyzed a subset of 79 cases with microarray expression data. A significantly inverse correlation between DNA methylation and gene expression was found more frequently than a positive correlation, consistent with the classical association between CpG methylation and gene expression (Fig 76).

In this regard, we focused on the genes that follow this tendency, aiming to identify whether hypermethylation can have a role in MCL pathogenesis regulating gene expression. A total of 103 genes (22%) showed significant mRNA downregulation in hypermethylated cases but only 3% (n=13) were upregulated in these cases. On the

contrary, the study of 839 hypomethylated genes interrogated by the expression array did not evidence an inverse relationship between gene expression and the methylation degree, since a similar number of hypomethylated genes showed gene expression upregulation (n=68) or downregulation (n=72). These results were in line with the widespread concept that increased CpG methylation is associated with decreased expression. Moreover, this seems to indicate that abnormal hypermethylation could have a role in malignant gene expression silencing,especially taking into account that hypermethylation often targets polycomb-regulated genes.

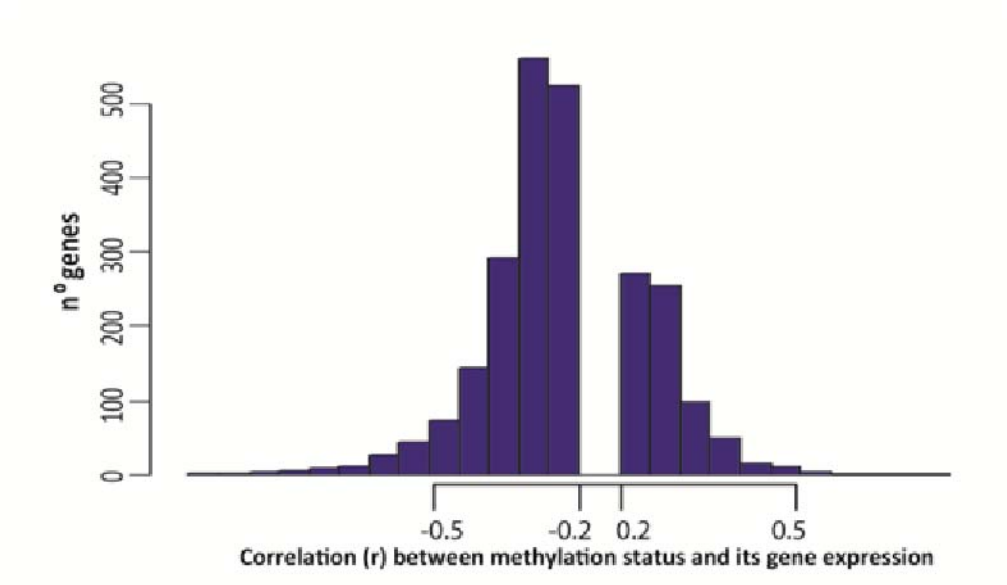


Figure 76. **Histogram of the frequency distribution of the correlations between the β-values and gene expression levels obtained in 79 primary MCLs**

In fact, our next step was to study this set of downregulated genes showing hypermethylated CpGs in promoters. Interestingly, these genes were able to distinguish a cluster of samples characterized by lower expression levels. The implications this may have in MCL disease were further confirmed looking into the clinical behavior of these two groups of MCL primary cases, showing that cases with these downregulated genes had a significantly poor prognosis (p val=0.0037) (Fig 77). Altogether, hypermethylation seem to generally correlate with gene silencing, which could lead to cancer progression and worse clinical behavior. Promoter hypomethylation, on the other hand, seems not to be associated with this global trend.

2.4 *De novo* CpG island hypermethylation distinguish two different groups of MCL with particular clinico-pathological characteristics

All the previous results seemed to suggest that hypermethylation occurs more frequently in blastoid variants, correlating with gene expression and with prognostic impact. For this reason, we aimed to study in depth our series. The histogram of the number of hypermethylated genes per sample revealed a statistically significant bimodal distribution (p val<0.0001) (Figure 78A).

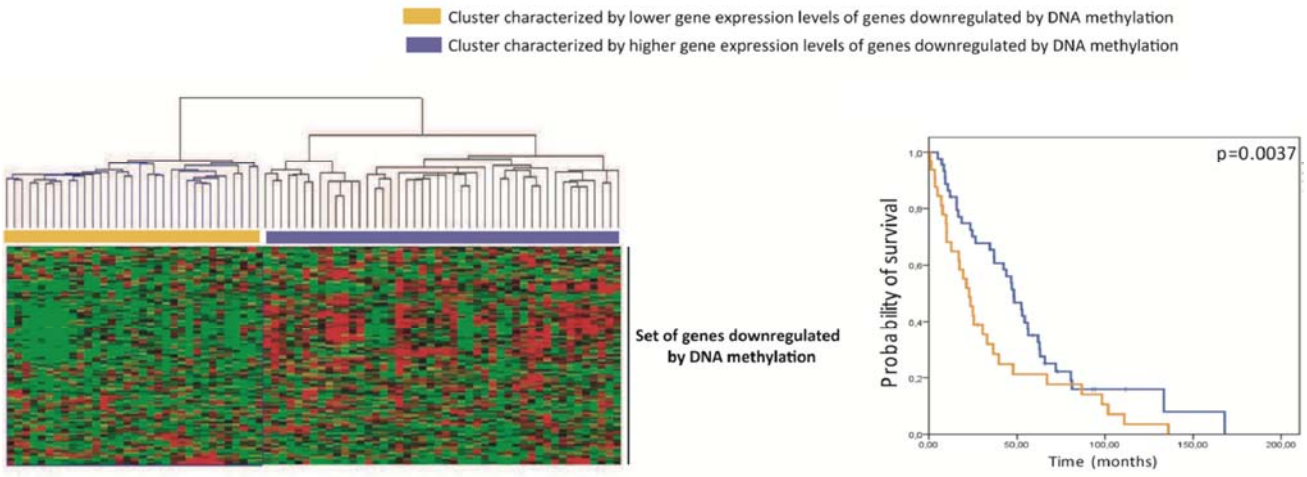


Figure 77**. Low expression of genes silenced by *de novo* hypermethylation in MCL cases correlate with worse survival.** Hierarchical clustering using the genes which methylation was associated with a significant reduction of their gene expression levels. The samples were grouped in two major clusters (left panel) and Kaplan-Meier survival curves according to the two clusters (right panel)

Based on this bimodal distribution, 87 samples with a number of hypermethylated CpGs lower than 120 were classified as the low methylation profile group (LMP), whereas 40 cases with a number of hypermethylated CpG higher than 150 were classified as the high methylation profile group (HMP). When both groups were compared against normal samples, the HMP group showed, as expected, a clear global dysregulation of DNA methylation with a high number of both hypermethylated and hypomethylated CpG dinucleotides ($\Delta\beta \geq 0.3$, adjusted P-value<0.001). Interestingly, the HMP group had also a five-fold increase in the proportion of hypermethylated CpG sites when compared to the LMP tumors (p val<0.0001), indicating that the HMP MCL were prone to accumulate preferentially hypermethylated CpG dinucleotides. This not only corroborates the existence of two groups of cases based on the hypermethylation events, but also it suggests that the differences between these two subsets of MCL are very significant, what may indicate different mechanisms contributing to pathogenesis. We hypothesized that these two groups, defined by its methylation profile, have differences in its transcriptomic profile. In fact, using supervised clustering analysis, we identified an important number of differentially expressed genes (n=300, FDR=5%) between the LMP (n=52) and HMP (n=24) tumors (fig 7B). Interestingly, among the genes upregulated in the HMP group, there was an outstanding enrichment in cell cycle related genes since 39% of all the overexpressed genes belonged to this ontology category (p val<0.0001), whereas the genes upregulated in LMP MCL showed an enrichment in T-cell differentiation and activation genes, although they did not reach statistical significance (p val=0.268). This not only supports that promoter hypermethylation as a main oncogenic force in MCL, but also seems to indicate a link between dysregulation of epigenomic profiles and proliferation.

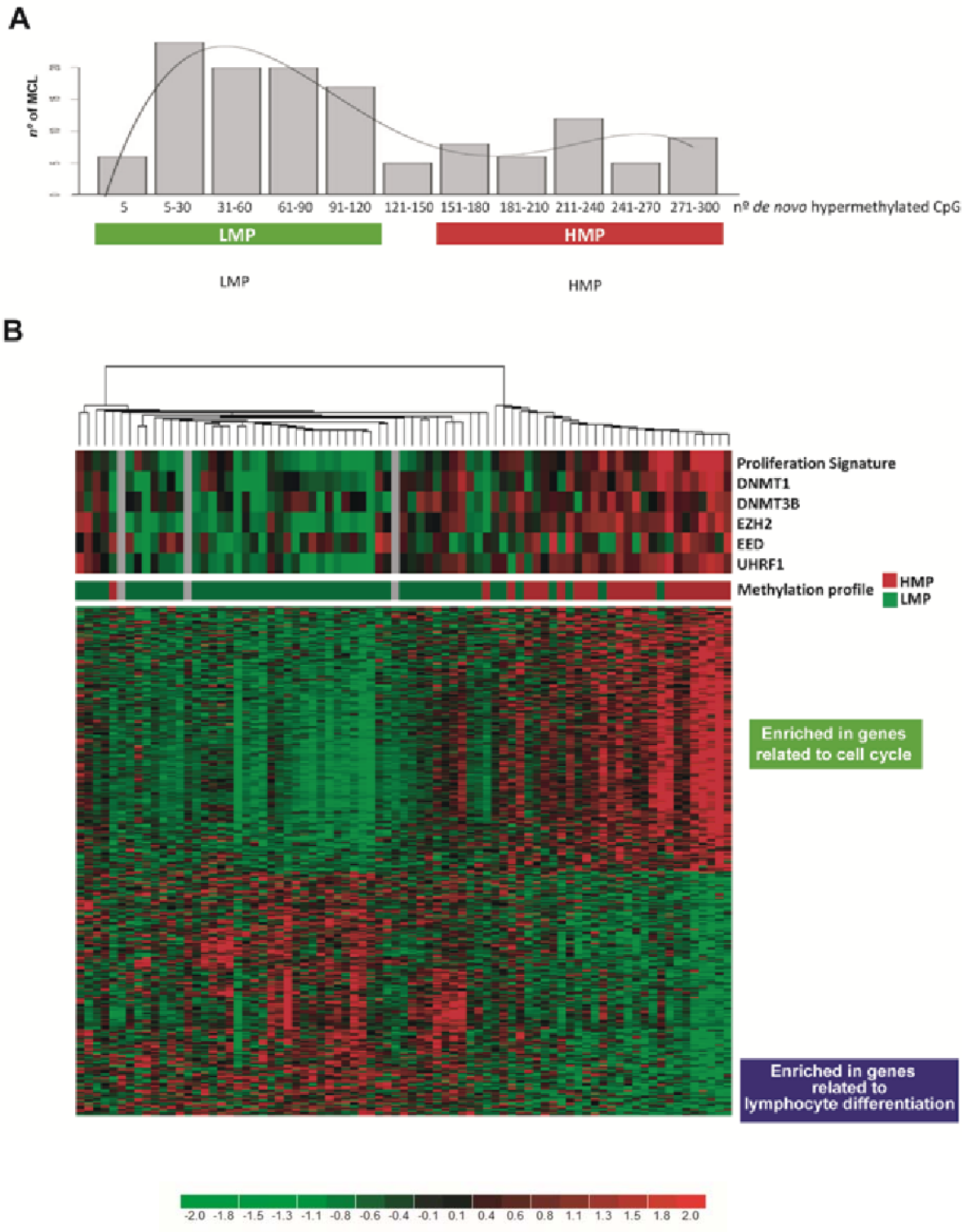


Figure 78. **Distribution of CpG island hypermethylation events in primary MCL. A)** The histogram of the number of hypermethylated CpG per sample shows a bimodal distribution of MCL cases. The samples with more than 150 hypermethylated CpG were classified as High Methylation Profile (HMP) and cases with lower than 120 CpG were defined as Low Methylation Profile (LMP). Five cases with a number of hypermethylated CpGs between 121 and 150 remained unclassified. **B)** Heat map of a hierarchical clustering using the genes that were differentially expressed between LMP and HMP samples. Grey indicates unclassified samples. The proliferation signature, together with gene expression levels of *DNMT1*, *DNMT3B*, *EZH2* and *EED* are displayed in the upper heat map. Genes highly expressed in HMP were significantly enriched in cell cycle genes.

To finish, we supposed this highly deregulated group of cases may have transcription changes in genes involving DNA methylation. For that purpose, we analyzed the differential expression of cytosine-5-methyltransferases and polycomb repressor genes between HMP and LMP cases. Interestingly, *DNMT1* (p val=0.005) and *DNMT3B* (p val=0.034) and two members of the polycomb repressive complex 2, *EZH2* (p val<0.0001) and *EED* (p val=0.03), were significantly overexpressed in HMP cases (Figure 78B). These results would indicate that modifications in gene expression can be in close relation with changes in epigenomic profile.

2.5 CpG methylation profile and clinicopathological parameters

We next decided to study the clinical association between epigenomic changes and the impact on the clinicopathological features of the patients. First of all, we analyzed globally the accumulation of hypermethylated and hypomethylated CpGs and its association with genomic instability. A significant association was observed between the number of hypermethylated loci and high number of genomic alterations (p val=0.003). Hypomethylation, on the other hand, was not associated with proliferation or genomic complexity (Fig 79A)

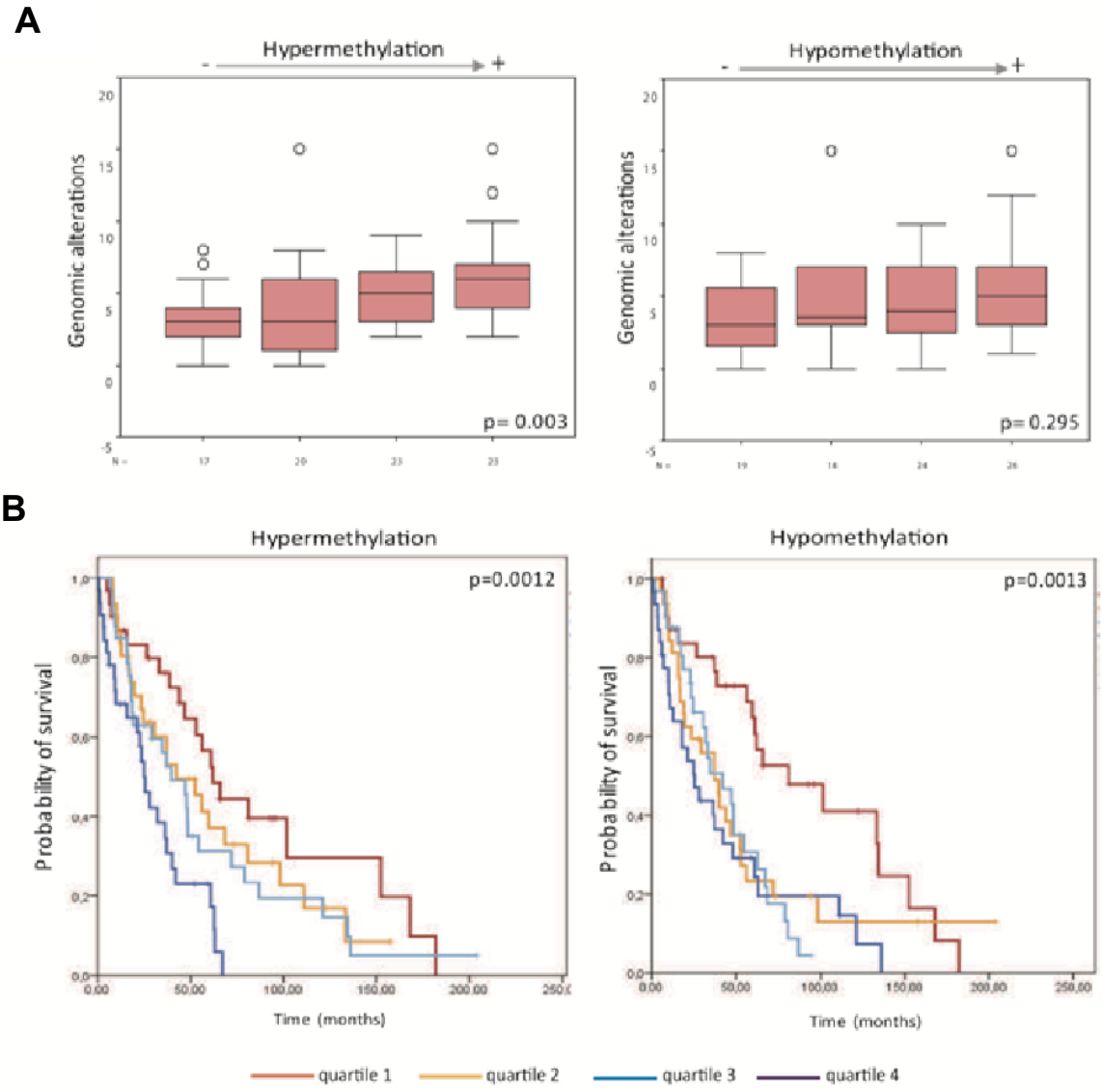


Figure 79. Association of *de novo* epigenetic events with genomic alterations and worse survival. A) Box plots representing the median and range of the number of chromosome alterations for patients stratified in quartiles according to the number of hypermethylated CpG (left) and hypomethylated CpG (right). Circles represent outliers. **B)** Kaplan-Meier survival curves for patients stratified in quartiles according to the number of hypermethylated CpG (left) and hypomethylated CpG (right).

This result is quite controversial, since we observed that these two phenomena tend to accumulate in the same cases. In this regard, both hypermethylation and hypomethylation were correlating with worse clinical course (Figure 79B). When we stratified patients in quartiles using the number of hypermethylated CpG, a significant association was observed with the overall survival of the patients (p = 0.0012 in hypermethylation, p val=0.0013 in hypomethylation). From the Kaplan-Meyer curves, we observe that the quartile with the highest number of abnormalities is also the one showing the worse prognosis. This led us to think about the possibility this group correspond to the HMP group.

In agreement with all the previous results, the HMP tumors displayed a significant higher number of chromosome alterations (p val=0.005) (Figure 80A). Moreover, genomic alterations were found more often in the HMP than in the LMP group (58% vs 28%, p val=0.03). Concordantly, we observed that HMP patients showed a worse prognosis (p = 0.0001) than LMP patients (Figure 80B).

2.6 *De novo* hypermethylation and proliferation in MCL primary cases

Many of the previous evidences showed a functional relationship between proliferation and *de novo* hypermethylation. For instance, *de novo* hypermethylated, silenced genes are enriched in cell cycle functions. For this reason, we decided to study the proliferation signature in our MCL series. As expected, proliferation signature was increased in cases harboring hypermethylated CpGs (p val=0.001) (Fig 81A). Hypomethylation was not statistically associated with proliferation, although a tendency was observed (p val=0.077). When proliferation signature was studied in the HMG, it appeared highly increased (p val=0.001) (Figure 81B). Taking together these results, high proliferative cases present more dysregulated epigenomic profiles, affecting especially aberrant *de novo* hypermethylation.

Interestingly, the *CDKN2A* locus was targeted by hypermethylation (38 *vs*.6%; p < 0.001) or 9p21 deletion (33 *vs*.9%; p < 0.001) more frequently in HMP than in LMP cases. When both phenomena were considered, 63% of the HMP cases showed hypermethylation of *CDKN2A* locus or 9p21 deletion compared to 15% of LMP cases (p < 0.0001). *CDKN2A* is a gene codifying for p16, a CDK4 inhibitor. This opens a scenario where nearly two thirds of cases of a tumor characterized by a strong cyclin D1 expression, concomitantly inactivate the G1/S pathway through *CDKN2A* alteration. Therefore, we next aimed to see if these effects of hypermethylation and proliferation could be independent markers of prognosis or they have similar clinical implications. When the methylation status (HMP *vs*. LMP) was compared to the proliferation signature in a bivariate COX regression analysis, only the proliferation signature remained as a significant predictor for poor overall survival (relative risk: 1. 086; 95% confidence interval: 1.054–1.119; p *val*< 0.001). Consequently, although proliferation

remains as a key point in MCL lymphomagenesis, this reinforces the idea that aberrant hypermethylation and proliferation may be functionally related.

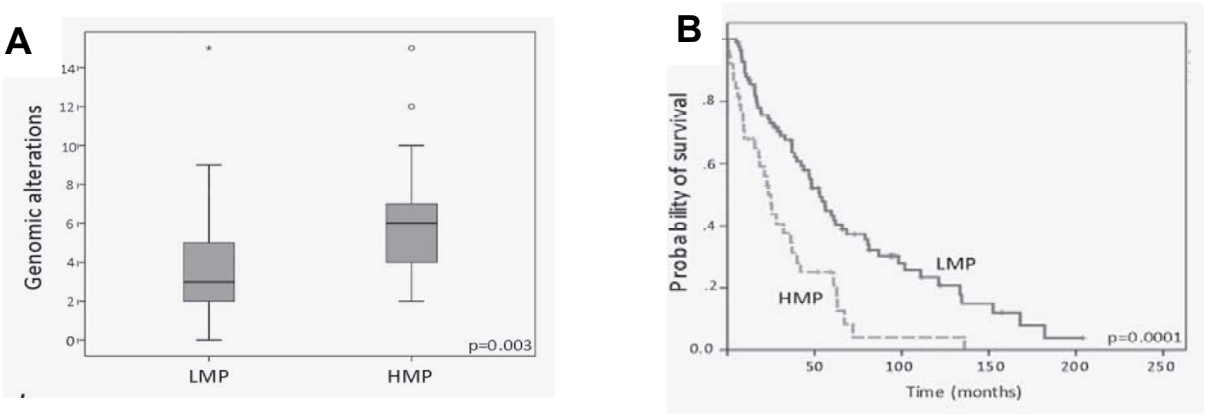


Figure 80. LMP associates with higher number of chromosomic abnormalities and worse survival. A) Box plots representing the median and range of the number of chromosome alterations for patients classified as HMP or LMP. Circles and starts represent outliers; B) Kaplan-Meier survival curves for patients classified as HMP or LMP.

Taken together all these findings, it seems that aberrant hypermethylation of CpG at promoter regions had a stronger influence on the biological behavior of MCL than hypomethylation. This effect may be mediated by the pathological silencing of tumor suppressor genes related to proliferation and cell cycle. However, dysregulated proliferation could be not only the consequence, but also the cause of *de novo* epigenomic changes that lead to cancer progression.

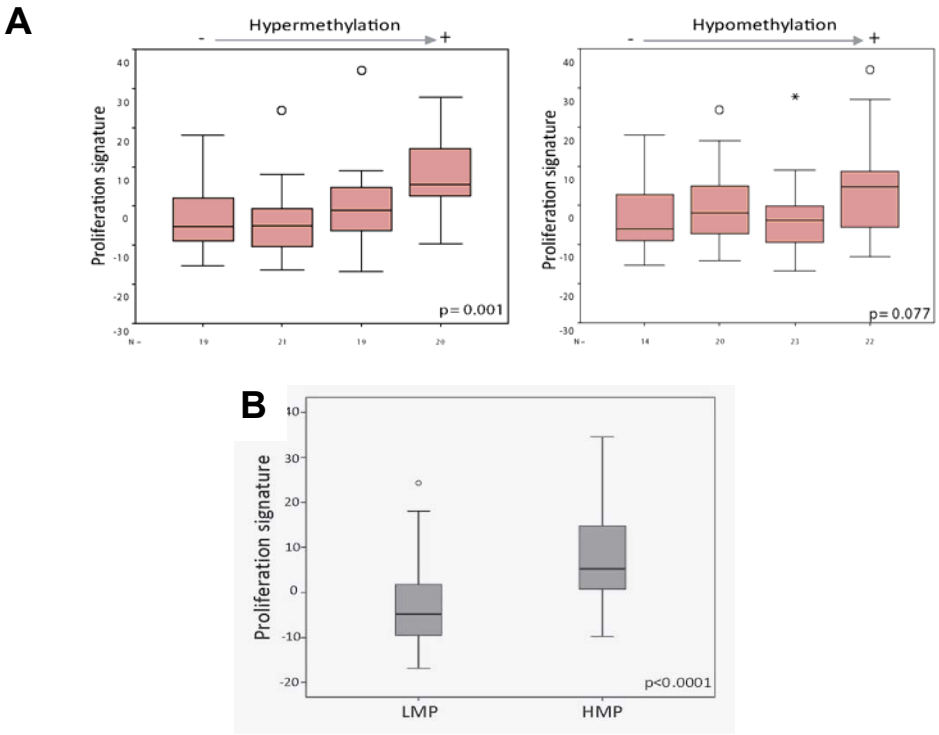


Figure 81. **Higher proliferation correlates with more CpG hypermethylation and HMP phenotype. A)** Box plots representing the median and range of the proliferation signature for patients stratified in quartiles according to the number of hypermethylated CpG (left) and hypomethylated CpG (right). Circles and stars represent outliers B) Box plots representing the median and range of the proliferation signature for patients classified as HMP or LMP. Circles represent outliers.

DISCUSSION

Cyclin D1 plays a central role in cell cycle and it is frequently upregulated by different genomic alterations including amplifications in breast and airway tumors (Callender et al., 1994; Jares et al., 1994; Zukerberg et al., 1995) and chromosomal translocations in mantle cell lymphoma (MCL) and multiple myeloma (MM) (Pruneri et al., 2000). Defects in cell signaling pathways also induce cyclin D1 oncogenic overexpression in a larger number of tumors. All these evidences turn cyclin D1 into one of the most recurrently overexpressed oncogenes in human tumors (Ciriello et al., 2013; Musgrove et al., 2011). In MCL, cyclin D1 overexpression is due to the t(11;14) translocation. This genetic event is considered the first oncogenic hit in MCL pathogenesis.

This PhD project has aimed to study the new cyclin D1 functions besides its canonical cell cycle role and its implication in MCL lymphomagenesis. This aggressive lymphoma is an exceptional model to study the contribution of the different cyclin D1 roles during lymphomagenesis. This model not only allow us to study cyclin D1 roles in B-cell biology, but it also leads us to study the relationship between this cyclin and the pathways frequently dysregulated in MCL. To achieve these objectives, we have combined *in vitro* experiments and primary samples in order to investigate the potential role of cyclin D1 as a transcriptional regulator and as a promoter of persistent DNA damage response (DDR) activation in MCL cells and lymphoblastic B cells overexpressing exogenous cyclin D1. Besides, we have investigated the epigenetic profile of MCL, focused on DNA methylation. The epigenetic changes have been associated with the clinicopathological characteristics of the tumors, including their proliferation activity, suggesting that cyclin D1 may lead to higher proliferation and epigenetic dysregulation.

In **Study 1**, we investigated whether cyclin D1 plays a transcription regulation role in lymphoid tumor cells. To this purpose, we used an integrative analysis combining data from ChIP-Seq and RNA-Seq in four MCL cell lines, characterized by the extremely high and constitutive overexpression of cyclin D1 due to the t(11;14) translocation. Our genomic analysis of cyclin D1 chromatin interaction revealed an outstanding and somewhat unexpected number of cyclin D1 binding sites, which preferentially occurred at promoters close to the transcription start sites. These promoters presented active histone marks (H3K27ac) and open chromatin DNAse I patterns.

Previous studies had demonstrated that exogenous cyclin D1 can bind to promoters, especially those enriched in CpG islands (Bienvenu et al., 2010). In these ChIP-Chip experiments, cyclin D1 occupied more than 900 promoters. This study only detected 382 downregulated/upregulated transcripts (FDR<0.05) upon cyclin D1 overexpression in CCND1-/- retinas, suggesting that cyclin D1 overexpression induced little changes in gene expression. The interaction of cyclin D1 with chromatin was also observed by ChIP-Seq in cyclin D1 rescued mice embryonic cells *CCND1*-/-. In this study, Casimiro and colleagues (2012) described 2840 cyclin D1-bound regions, a low number of peaks compared with the 40.000-45.000 intervals we found in MCL (Casimiro et al., 2012). These two works analyzed the binding of exogenous cyclin D1 overexpressed using constitutive overexpression constructs. In our hands, the level of expression reached with these types of vectors is not comparable with the high cyclin D1 amount detected in MCL cell lines. Since our results pointed out that the number of cyclin D1 peaks positively correlated with cyclin D1 amount, low cyclin D1 levels might explain the

extraordinary difference in the high number of peaks found in MCL cell lines and the reported in previous studies. Despite the difference on the number of peaks, the functional annotation of the cyclin D1 bound promoters gave rise to similar conclusions (Bienvenu et al., 2010; Casimiro et al., 2012; Casimiro et al., 2015). Cyclin D1 binds to genes regulating ribosomes, cell proliferation or RNA processing. In fact, 6 out 7 top functional clusters were shared among our study and the one performed by Casimiro and colleagues (2015). This suggests that cyclin D1 might control similar pathways even in different tissues and species. Interestingly, all these functions are regulated by genes highly transcribed, strengthening the notion that, in the three studies, cyclin D1 also occupies promoters of abundantly expressed genes. In addition, 50% of the transcription factors (TFs) predicted to interact with cyclin D1 in MCL cell lines were in common with the ones obtained by Casimiro and colleagues (2015) in mice fibroblast cell lines overexpressing cyclin D1. Moreover, the 43% of genes bound by cyclin D1 in MCL cell lines were also bound in this cell model. The high degree of overlap between our studies points out that cyclin D1 may act as a global transcriptional regulator independently of the tissue.

The analysis of the relationship between cyclin D1 occupancy at promoters and chromatin states in the same MCL cell line (Z-138), together with the RNA-Seq data, demonstrated that cyclin D1 binds globally to active promoters, and these interactions are proportional to the mRNA abundance of the targeted genes. We also observed that cyclin D1 was recruited to promoters that are pre-configured in an active state in the absence of cyclin D1, and the recruitment seems to be proportional to the expression levels of the targeted genes. This binding behavior was strikingly similar to the one reported for MYC in human tumor cells and mouse primary lymphocytes (Lin et al., 2012; Nie et al., 2012; Sabo et al., 2014; Walz et al., 2014). Both proteins display an extensive genome-wide chromatin binding, with the peaks centered on the transcription start site and preferentially occupying promoters of highly expressed genes. Furthermore, both proteins show a unimodal distribution in a peak-density histogram, opposite to the bimodal profile expected for a conventional transcription factor. In addition, we detected a dose relationship between the number of peaks and the quantity of cyclin D1, as the one reported for MYC. Moreover, the observed cyclin D1 recruitment to promoters of genes actively expressed in the absence of cyclin D1 reminds the binding of MYC, during B-cell activation, to genes already expressed in naïve resting B-cells. All these characteristics attributed to MYC have been described in two recent reports (Lin et al., 2012; Nie et al., 2012), which even questioned the existence of a specific signature of genes regulated by MYC. These works postulate that, instead of controlling a specific gene signature, MYC directly upregulates all transcripts from genes poised for transcription in a given cell, seemingly without any specificity. This phenomenon was called “global transcription amplification”. As a result of this global effect, MYC overexpression leads to an increase of total RNA (Lin et al., 2012; Nie et al., 2012; Sabo et al., 2014).

Recent publications have questioned the MYC-dependent global transcriptional activation, suggesting that this upregulation is an indirect effect of a specific gene signature regulated by MYC that, in turn, causes the transcription amplification (Sabo et al., 2014; Walz et al., 2014). The main evidence against a global response mediated by MYC is the small, but significant, number of transcripts downregulated upon MYC

overexpression. However, some authors point out that specific transcript downregulation upon MYC expression is an actual indirect effect of MYC global amplification (Kress et al., 2015). Further studies must clarify the link between the global and specific functions of global transcription modulators. Recently, the quantification of the MYC cellular levels and the correlation between gene expression and promoter occupancy at different MYC concentration have led to propose a model based in the presence of different promoter affinities for MYC that could reconcile both views (Lorenzin et al., 2016). At low physiological levels MYC would bind to high-affinity promoters, and, eventually, it would invade all the active promoters when expressed at high levels. This promoter and/or enhancer invasion may have functional regulatory roles in normal cell development. In a recent study (Pauklin et al., 2016), it has been shown that cyclin D1 promoter occupancy has an important role in stem cell differentiation, a situation that resembles the global regulation executed by MYC in embryonic stem cells (Rahl et al., 2010).

Surprisingly, despite the significant similarities between cyclin D1 and MYC, the overexpression of cyclin D1 in lymphoid cells was responsible for a significant reduction of RNA content, which was proportional to cyclin D1 protein levels. Cyclin D1 overexpression caused a decrease in transcription levels, while cyclin D1 silencing increased the total RNA amount per cell. As far as we know, this is the first time that a global transcription downregulatory effect has been reported for a protein outside the basal transcription machinery. We used two different strategies to study RNA levels, including the pyronin Y staining that allows to measure the RNA content associated to different cell cycle phases. Our data showed that RNA downregulation was independent of the cell cycle phase. This point is important, since we have observed in **study 2** that cyclin D1 induces changes in the cell cycle profile, an effect corroborated in other publications (Kim and Sederstrom, 2015).

The analysis of MCL cell lines confirmed the negative correlation between cyclin D1 protein levels and the total RNA cell content. We also detected this trend in MM cell lines, although it was not statistically significant, likely due to the low number of cell lines analyzed. Since cyclin D1 levels in MM cell lines are much lower than in MCL cell lines, it could be possible that cyclin D1-dependent global downregulation is more limited and more difficult to be detected in MM. Moreover, many studies have demonstrated that cyclin D1 association with clinical features is different in MM compared to MCL, thus maybe cyclin D1 is not playing the same function in MM than in MCL pathogenesis (Lesage et al., 2005). In any case, the detection of a similar tendency despite the lower cyclin D1 levels in MM cell lines, lead us to think in the possibility that other tumors with cyclin D1 overexpression can share the same characteristics. Further studies in cancer models with cyclin D1 amplification, such as breast or esophageal cancer cell lines, may elucidate whether cyclin D1 overexpression might exert a similar role in other tumors.

One controversial result came from the expression microarray analysis upon cyclin D1 induction, which showed a limited effect of cyclin D1 on gene expression. However, the use of microarrays or RNA-Seq is not an adequate approach to study changes in gene expression when total RNA content is down/upregulated since both techniques require the use of a defined amount of RNA to compare conditions without normalizing by the

number of cells. In these cases, two different strategies can be used: the use of spike-in normalization combined with microarrays/RNA-Seq, or technologies that allow digital quantification of gene expression directly in cell extracts (nCounter technology) (Loven et al., 2012). Spike-in normalization requires the use of a mixture of synthetic RNAs (spikes) that must be added to the sample according to the number of initial cells used for total RNA extraction. The spiked RNAs would be measured at the same time than the other transcripts by microarrays or by RNA-Seq experiments, allowing the samples to be normalized according to the number of cells. In our case, we found that the Nanostring platform was adequate for our studies because we could directly work with cellular extracts derived from counted cell pellets, avoiding bias in RNA extraction and quantification, cDNA transcription or spike addition. We used two independent gene sets, and both of them showed that cyclin D1 overexpression also determined a downregulation of most mRNA transcripts. Therefore, we suggested that cyclin D1 interaction at promoters decreases the expression levels producing a significant reduction of the cellular mRNA content.

Our data confirms the importance of performing a correct normalization during gene expression profiling analysis, especially when proteins that may change global expression patterns are studied (Chen et al., 2016; Loven et al., 2012). Chen and colleagues (2016) showed that the main technologies used for gene expression fail to detect the widespread increase of transcription upon MYC overexpression when inappropriate normalization was used. In this case, the use of a normalization that does not take into account the cell number would lead to the erroneous interpretation that a similar number of genes are up or downregulated upon expression of a global modulator. Some authors recommend the use of spike-in normalization as default standard for all expression experiments, but others limit its use to experiments where gross changes in RNA levels are anticipated (Bar-Joseph et al., 2012). When cell counting is problematic, as for expression experiments from solid tumors or tissues, DNA content may be used as a surrogate. If this is the case, ploidy and DNA replication profiles must also be characterized to prevent the introduction of a DNA content-based artifact. Our results suggest that this new normalization should be applied by default, since it is difficult to guess which proteins may affect globally or specifically to transcription *a priori*. This problematic issue is of major importance in cancer transcriptomics, since significant effort is being devoted nowadays to study cancer gene expression profiling using standard normalization methods (Berger et al., 2010; Lapointe et al., 2004; Ramaswamy et al., 2001). Since proteins like MYC or cyclin D1 are dysregulated in many cancers, global transcription modulation can be likely see in different cancers and, consequently, an adequate gene expression profile normalization should be taken into consideration for a correct interpretation of the results (Loven et al., 2012). Moreover, we detected that doxycycline concentrations frequently used in inducible models (1µg/mL) cause a global downregulation in lymphoblastic cell lines (data not shown). This is consistent with previous reports showing that doxycycline may alter metabolism and proliferation (Ahler et al., 2013), In our experiments, we used 0.1µg/mL of doxycycline that was able to induce high cyclin D1 levels without promoting detectable reduction in the total RNA content of control cells. Our unexpected finding justify the use of spike-in normalization or digital expression counting in transcriptomic studies using high concentrations of doxycycline that may mask some effects that cannot be detected by microarray profiling.

Our next goal was to study how cyclin D1 regulates globally the transcriptional process. To answer this question we reviewed, once again, how MYC produces a global transcription amplification. MYC dependent transcription amplification was related to the capacity of MYC to increase the Pol II processivity at all transcribed genes (Lin et al., 2012; Nie et al., 2012; Rahl et al., 2010; Wolf et al., 2015). In fact, transcription amplification both in cancer and stem cells is mediated by the interaction of MYC with P-TEFb subunits Cyclin T1 and CDK9 *in vitro* and *in vivo* (Eberhardy and Farnham, 2002; Kanazawa et al., 2003; Rahl et al., 2010). Consequently, we analyzed the impact of cyclin D1 overexpression on the transcription machinery. The analysis of Pol II localization/occupancy following cyclin D1 overexpression confirmed that cyclin D1 co-localize at promoters with Pol II polymerase, and we observed an increment in the RNA Pol II pausing index. The analysis of critical phosphorylation events on the CTD of RNA Pol II confirmed that the phosphorylation pattern of RNA Pol II changed following cyclin D1 overexpression. Interestingly, the global downmodulation was associated with a reduction of the Ser2 phosphorylation that takes place during active elongation mediated by CDK9. Taking this into account, we postulated that the phosphorylation pattern shift and the global transcription downmodulation detected in our study could be mediated by the physical interaction of cyclin D1 with CDK9. Co-immunoprecipitation experiments confirmed the interaction between cyclin D1 and CDK9 protein. Despite the well-defined CDK4/6-cyclin D interaction, cyclin D1 has been detected forming stable complexes with other CDKs. Mass-spectrometry studies of cyclin D1 interactors (Bienvenu et al., 2010; Jirawatnotai et al., 2011) showed that exogenous overexpressed cyclin D1 binds to CDK1, CDK2 and CDK5 in different cancer cell lines and normal cells. However, some of these CDK interactions have been reported in a single study. Firstly, Bienvenu and colleagues showed that cyclin D1 interacts with CDK11 in normal cells, a binding that Jirawatnotai et al could not detect. However, this study finds that cyclin D1 can bind to CDK3 in cancer cells, a CDK related to G1/S transition. Interestingly, the cyclin D1 binding to non-canonical CDK partners has been reported to inhibit the targeted CDKs (Higashi et al., 1996). In a similar way, cyclin D1 could sequester CDK9 in inactive complexes compromising the function of CDK9 during transcription activation.

CDK9 disruption would not only explain the global mRNA downmodulation that we detected using Nanostring technology, but also the decrease in the total RNA content. About 90% of the total RNA is constituted by ribosomic RNA (Conesa et al., 2016), produced by Pol I enzyme. Although it is not completely characterized, new studies have shown that CDK9 inhibition also affects ribosomic RNA production. Since total RNA is composed mainly by ribosomic RNA (Conesa et al., 2016) whose transcription is mediated by RNA Pol I, the dysregulation of the RNA Pol II mediated by cyclin D1 could not fully explain why the transcriptional downmodulation was detected when the total RNA content was measured. However, some authors have shown that CDK9 dysregulation not only decreases globally mRNA levels (in our study, detected using Nanostring technology) but also it impacts on the total RNA content affecting ribosomic RNA production. For example, CDK9 pharmacological inhibition or silencing decreases the production of 32S, 18S and 28S rRNAs dramatically (Burger et al., 2013). The role of CDK9 in rRNA transcription seems to be conserved in other organisms, where the disruption of the CDK9 homologues impairs rRNA processing (Coordes et al., 2015). Interestingly, MYC not only displays its global transcription regulation through its

interaction with CDK9, but also participates in rRNA processing (Gargano et al., 2007). In fact, a new study showed the colocalization between CDK9 and MYC at the periphery of the nucleolus, what allowed to the authors to suggest that CDK9 might be involved in rRNA processing via recruitment by MYC (data not shown) (Oqani et al., 2016). Further analyses should clarify in more detail how cyclin D1 interferes with the activation of the transcription machinery, whether other CDKs are compromised, and whether off-target effects may be a common phenomena following the pathogenic overexpression of other cyclins.

The aberrant downmodulation of transcription opens the possibility to explore the therapeutic potential of transcription inhibition in cancer cells with a compromised transcription machinery (Derheimer et al., 2005; Redell and Tweardy, 2005; Villicana et al., 2014). The inhibition of transcription has been described for the treatment of cancer based in the rationale that many tumors require a high transcription potential (Lin et al., 2012; Luo et al., 2009). However, we hypothesized that cells could have a transcriptional activity threshold below which they could not survive. Consequently, cells with a global transcription downregulation could be potentially targeted by transcription inhibitors. The higher sensitivity to a CDK9 inhibitor observed in MCL cell lines with lower RNA content per cell, together with the increased sensitivity displayed by lymphoid cells following cyclin D1 induction, confirmed the existence of a synthetic lethality between cyclin D1 levels and transcription inhibition. This lethal interaction opens opportunities for new treatment strategies in cyclin D1 overexpressing tumors, such as MCL or MM. In our study, triptolide was evaluated as a candidate for translating our results to pre-clinical studies. Triptolide is a diterpene triepoxide extracted from the plant *Tripterygium wilfordii* that inhibits transcription at micromolar concentrations (Leuenroth and Crews, 2008). It impairs the binding of XPB subunit to TFIIH (Titov et al., 2011) and induces a fast proteasome-dependent degradation of RNAPII (Titov et al., 2011; Vispe et al., 2009). Previous studies have shown that triptolide induces apoptosis in multiple myeloma cell lines (Huang et al., 2012; Wen et al., 2012). Notably, MM1S, a MM cell line overexpressing cyclin D1, was very sensitive to low concentrations of triptolide (Huang et al., 2012)

Preclinical studies have shown that combined depletion of cell cycle and transcription CDKs most effectively induces apoptosis in cancer cells (Cai et al., 2006). In the light of our results, CDK inhibition could be an effective target in MCL, not only because of the cell cycle inhibition, but also due to the inhibition of transcriptional CDKs. Interestingly, the pan-CDK inhibitor flavopiridol has minor clinical activity as a single agent, but it is currently under investigation in MCL in combination with other drugs (Lin et al., 2010). In this sense, a recent study has reported that SNS-032, a CDK7/9 inhibitor, induces cytotoxicity in MCL cells (Chen et al., 2010). Interestingly, the SP-53 MCL cell line, which displays very low levels of cyclin D1, was the less sensitive cell line to SNS-032 (Wang et al., 2009). Other studies must validate the underlying mechanism beneath this synthetic lethality between cyclin D1 overexpression and transcription inhibition. Some studies described the potential activation of *TP53* upon transcription donwregulation as the main cause of increased mortality (Derheimer et al., 2005). However, in our study MCL cell lines were sensitive to transcription blockage independently of the *TP53* mutation status (UPN-1 is *TP53* mutated, while Z-138 is wt

TP53) (Beà et al., 2013), thus other pathways may mediate cell apoptosis upon transcription inhibition.

Early studies showed that the RNA content could be a potential prognostic factor (Preisler et al., 1994; Urashima et al., 1992). Interestingly, Urashima and collaborators (1992) detected that leukemic cases with lower levels of RNA/DNA were more aggressive than cases with higher RNA levels. These studies not only show that low levels of RNA are not always related to quiescence, but also that cells with lower RNA/DNA signals correlated to poor survival. Our study has proved that pyronin Y can be an easy and fast method to study total RNA/cell. Previous studies used acridine orange as a staining method to determine RNA content a more difficult and less reliable technique than the pyronin Y staining we have standarized. The cyclin D1-total RNA correlation shown in our study, together with the studies claiming that cyclin D1 levels in MCL have a prognostic impact (Rosenwald et al., 2003), make us think that pyronin Y staining could be an interesting approach for studying the total RNA content per cell and evaluate its prognostic value in primary MCL cases.

One important issue we do not address in the present work is whether cyclin D1 transcription role is dependent or not on CDK4/6 binding. However, our mechanistic hypothesis, where cyclin D1 would be inactivating CDK9 in the means of a off-target binding, would support the idea that the cyclin D1-mediated transcriptional changes are CDK4/6 independent. This idea is reinforced by a recent report showing that cyclin D1 K112E mutant was binding DNA and regulating transcription exactly as the wild type form (Casimiro et al., 2015). This mutant form of cyclin D1 binds CDK4/6 but it does not activate the CDKs. Other studies have also evidenced that cyclin D1 transcription role is CDK4/6 independent (Bienvenu et al., 2010; Coqueret, 2002; Pauklin et al., 2016). Moreover, CDK4 levels in MCL are not correlating with the high level of cyclin D1 upregulation. CDK4 amplification is found in less than 10% of MCL cases (Hernandez et al., 2005), especially in blastoid variants. Consequently, all these evidences lead us to hypothesize that cyclin D1 may play a transcriptomic function independent of CDK4 in the molecular pathogenesis of MCL.

To our knowledge, it is the first time that a global role in transcription has been revealed for cyclin D1. In fact, the global inhibitory model through CDK9 interaction is not against the more established idea of a specific role of cyclin D1 regulating specific genes (Fu et al., 2005b; Lamb et al., 2003; Lin et al., 2002a; Liu et al., 2006). The transcriptional output, consequently, would be a balance between the impaired global transcription and the specific action of cyclin D1 with specific TFs, as it occurs with the global transcriptional amplifier MYC (Nie et al., 2012). In this regard, some studies showed that cyclin D1 can bind specifically to some TFs and perform specific functions on gene expression regulation. In our study we have shown different motifs that are overrepresented by motif enrichment and colocalization analysis. Interestingly, six of the ten significant TF binding site motifs (*CREB1, E2F1, EGR1, GABPA* and motifs related to *FOXP2* and *SP2*) were also detected in previous studies (Casimiro et al., 2012). Consequently, cyclin D1 may bind to similar regions through the same TFs in different models, controlling the expression of a specific signature of genes. Most of these reported interactions have been associated to a negative regulation of gene transcription at specific locus (Coqueret, 2002). Herein, we have shown that cyclin D1

also interacts physically with transcription factors in MCL cells. However, these interactions hardly could explain the cyclin D1 dependent global transcription downregulation we have observed.

It is generally believed that transformed cells require active transcription for proliferation and survival, and that certain oncogenes, ribosomal genes and components of the transcription machinery are overexpressed in tumor cells to maintain proliferation (Adhikary and Eilers, 2005; Cabarcas and Schramm, 2011; Drygin et al., 2010). Moreover, many different tumors present MYC amplification, what may lead to a burst in transcription (Lin et al., 2012; Nie et al., 2012). However, we show in this work that an oncogene dysregulation (cyclin D1 overexpression) determines a transcription downmodulation. The cyclin D1 dependent global transcription donwmodulation raises the question of its potential role in MCL oncogenesis. Although this aspect requires further studies, we may speculate that the increased pausing index promoted by cyclin D1 overexpression may facilitate the generation of genomic instability by increasing the probability of conflicts between DNA replication and transcription machineries. The collision between these machineries may cause an increase in DNA breaks as a consequence of replication fork stalling and collapse leading to recombination and chromosome rearrangements (Gaillard et al., 2013). In fact, this mechanism not only would explain a new oncogenic role for cyclin D1 overexpression, but also a plausible mechanism of increased cell death upon transcription inhibition. Transcriptional stress response (TSR) and DDR are discussed below in the light of the results of **Study 2**. In addition, cyclin D1-mediated reduction of the transcriptome program of a cell could have an oncogenic impact by decreasing the levels of active tumor suppressor genes (TSG), which are frequently inactivated or downregulated during oncogenesis. The effect on proto-oncogenes would be limited since the activation of these genes usually requires dominant mutations or genetic alterations that lead to increased activity (Figure 82).

In conclusion, in **Study 1** we have identified that oncogenic cyclin D1 overexpression produces a global transcriptome downmodulation by increasing RNA Pol II pausing. This dysregulation might, in part, be mediated by the interaction of the cyclin D1 with CDK9 that would interfere with the normal activation of transcription elongation. The global transcription downmodulation induced by cyclin D1 overexpression seems to generate a synthetic lethality interaction that may be exploited therapeutically.

In **Study 2**, we addressed whether cyclin D1 overexpression, the initial oncogenic hit in MCL pathogenesis, is able to induce DNA replication stress in B cells that would end up activating the DDR. We demonstrated *in vitro* that cyclin D1 overexpression increased cell growth promoting a rapid S-phase entry. Despite the increased S-phase entry of cyclin D1 overexpressing cells, these cells showed a slower S-phase progression. This result was in agreement with our hypothesis suggesting a potential DNA replication dysregulation by cyclin D1 overexpression in B cells. In this thesis we

demonstrate that cyclin D1 slows down the replication forks and cause replication stress. Our results led us to characterize the DDR activation in primary MCL.

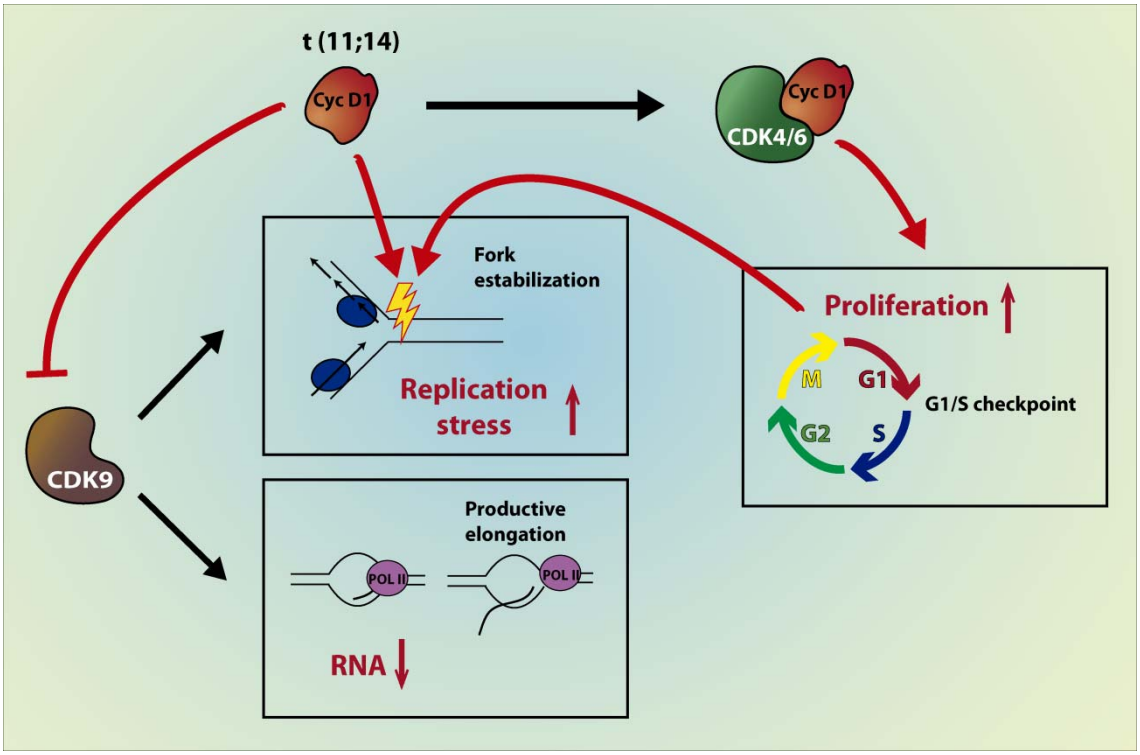


Figure 82. **Proposed model for cyclin D1-dependent transcription inhibition, a side effect of its oncogenic overexpression**. The classical cyclin D1 tumorogenesis model considers that it binds to CDK4/6 and promotes G1/S phase transition. However, the overexpressed cyclin D1 may interact with CDK9 and interfere with transcription elongation and fork stabilization. Replication stress may further increase through the direct interaction of cyclin D1 with the replication machinery or dysregulated proliferation induced by CDK4/6-cyclin D1 complexes. Text in black, normal physiological functions mediated by CDK9 and CDK4/6 respectively. Text and arrows in red: Functional consequences upon cyclin D1 overexpression.

Cyclin D1 aberrant overexpression has been reported to be able to inhibit DNA synthesis in cancer cell lines, through direct binding to CDK2 and PCNA, a DNA polymerase clamp loader (Fukami-Kobayashi and Mitsui, 1999). Our study represents the first evidence that cyclin D1 overexpression interferes with the DNA replication of B cells. A recent study supported the idea that cyclin D1 overexpression may dysregulate DNA replication by downregulating replication fork progression (Shimura et al., 2013). In this study, radiation-resistant cells increased cyclin D1 expression, which led to a decrease of the DNA replication fork speed. In fact, cyclin D1 overexpression in HeLa and liver cells also caused slowing down of fork progression. Our investigation of the DNA replication fork dynamics showed, for the first time, that in addition to decrease the fork speed progression, cyclin D1 causes fork stalling and new origin activation, phenotypes associated with the response of the cell to DNA replication stress. These results clearly show cyclin D1 overexpression induces the replication stress response (RSR). Moreover, cyclin D1 increases the number of asymmetric forks, which has been related to genomic instability (Mazouzi et al., 2014).

Recently, it has been reported that cyclin D1 silencing caused replication stress in MCL cell lines, increasing γH2AX phosphorylation (Mohanty et al., 2017). Consequently, cyclin D1 silencing causes apoptosis in MCL cell lines (Mohanty et al., 2017; Weinstein et al., 2012). However, the authors did not completely evaluate other fork parameters associated with replication stress. Moreover, some of the functional experiments were performed with shRNA inducible systems that showed low levels of cyclin D1 silencing and also certain degree of silencing was observed in the absence of the inductor. In addition, the protective role of cyclin D1 in genome integrity reported in this study was limited to the inducible knockdown system generated in UPN-1 cells, and the authors were not able to confirm it in other cell lines. The dependence of MCL cell lines on cyclin D1 for growth and survival, could compromise the cyclin D1 silencing model to sort out *de novo* functions induced by cyclin D1. In this regard, it seems more reliable to use lymphoblastic models based on the overexpression of cyclin D1 upon doxycycline treatment, in order to better clarify the cyclin D1 oncogenic functions.

How does cyclin D1 overexpression activate RSR and lead to chromosomal instability (CIN) in lymphoid cells? First of all, cyclin D1 may impair the fork progression through direct interaction with the replication machinery. Indeed, cyclin D1 association with DNA replication factors such as PCNA (Fukami-Kobayashi and Mitsui, 1999; Prosperi et al., 1994; Xiong et al., 1992), together with its capacity to interact with chromatin, lead us to think that cyclin D1 may be recruited to replication forks, for example through PCNA, and it would prevent replication fork movement. Secondly, cyclin D1 overexpression causes problems in origin licensing and rereplication (Aggarwal et al., 2007), which induces DNA damage in different models (Li and Gilmour, 2013; Vaziri et al., 2003). Interestingly, our group described that the presence of an unbalanced licensing (high levels of Cdt1 and Cdc6 without high expression of geminin) correlates with an accumulation of a higher number of chromosome alterations in MCL (Pinyol et al., 2006). In addition, the tumors with *TP53/CDKN2A* inactivation that showed unbalanced licensing had significantly higher levels of cyclin D1 than tumors with a normal licensing signature.

However, the results obtained from **Study 1** and **Study 2** lay out a novel hypothesis for the mechanism underneath cyclin D1-mediated DDR and replication stress. Cyclin D1 seems to be able to bind different CDKs, and we showed for the first time cyclin D1 may interact with CDK9 in MCL cell lines, impairing its transcription functions. Interestingly, recent studies have demonstrated a new role for CDK9 in replication stress response through its interaction with cyclin K (Yu et al., 2010). CDK9 forms a heterodimer with a regulatory subunit, cyclin T1, T2a, T2b or K, to form the main component of the P-TEFb complex. Cyclin K interacts with CDK9 *in vitro* and *in vivo* (Fu et al., 1999; Lin et al., 2002b), and its expression is activated through p53 in response to DNA damage by UV light and ionizing radiation (Mori et al., 2002). Recently, a study analyzed the effects of CDK9 silencing (Yu et al., 2010). Even in the absence of exogenous damage, replication forks stalled and collapsed when CDK9 was silenced, leading to the formation of DNA double-strand breaks (Liu et al., 2010; Yu et al., 2010). The authors determined that cell cycle recovery after a replication challenge was impaired in cells silenced for cyclin K, but not when cyclins T1 or T2 were silenced. Our results suggest that the overexpressed cyclin D1 may dysregulate CDK9 function, displacing its canonical cyclin partners. Indeed, cyclin D1

overexpression had similar biological effects that CDK9 silencing. For instance, CDK9 silencing causes an increase in γH2AX in cells in S and G_2 phase, suggesting the DDR induction during replication (Yu et al., 2010). In our study and others (Aggarwal et al., 2007; Pontano et al., 2008; Shimura et al., 2013), cyclin D1 caused DDR and RSR activation also during S-phase. Definitive evidence of the potential effect of cyclin D1 overexpression impairing the different CDK roles will require further experiments.

Cyclin D1 dysregulation of transcription may also provoke higher replication stress due to an increased number on collisions between transcription and replication machineries. In fact, oncogenes like cyclin E induce RSR by increasing the number of collisions between both machineries (Jones et al., 2013). In **Study 1** we detected that Pol II pausing index on promoters is increased upon cyclin D1 expression. Therefore, this effect on the transcription machinery may generate problems during DNA replication. In this regard, a very recent study showed that global transcription amplification leads to an increase in genomic instability and retarded fork progression, mediated by the formation of R-loops structures (Kotsantis et al., 2016). R-loops are nucleic acid structures composed of an RNA–DNA hybrid and a displaced single-stranded DNA (Santos-Pereira and Aguilera, 2015). These structures appear essentially at promoters and termination transcription sites (Ginno et al., 2012; Sollier and Cimprich, 2015) and they have a role in different physiological processes, such as regulating gene expression, Ig class-switch recombination, DNA repair and DNA replication. However, their dysregulation might be responsible for increasing genomic instability. Interestingly, Kotsantis and colleagues lay out the possibility that decreasing transcription output could lead also to an increased R-loop formation and genomic instability (Pauklin et al., 2016). Thus, an increase in Pol II pausing mediated by cyclin D1 overexpression could induce R-loop formation. Recently, it has been described a role of RAD51 in R-loop formation. As described before, cyclin D1 activates RAD51 functions participating in RAD51 recruitments in DNA repair process. This suggests that cyclin D1 may also activate RAD51 role in R-loop formation (Wahba et al., 2013). Interestingly, another study showed that estrogen stimulation of cyclin D1 positive breast cancer cell lines led to an increment of R-loops in estrogen-induced promoters, leading to higher DDR (Stork et al., 2016). In this regard, cyclin D1 is also known as an activator cofactor of ER, even in the absence of ligand (Neuman et al., 1997). Consequently, cyclin D1 overexpression in ER-positive cancer cells could also lead to an increment in R-loops and genomic instability. Moreover, highly expressed genes tend to accumulate R-loops (Wahba et al., 2016), what would be a indirect evidence suggesting that cyclin D1 binds to promoters with increased levels of R-loops. Further experiments aiming to evaluate the genomic distribution of R-loops following cyclin D1 overexpression are needed to clarify our hypothesis..Finally, some studies claim that the dysregulated accumulation of R-loops may also modify the epigenetic of the promoter. R-loops accumulate naturally at the G-rich 5′-UTR regions immediately downstream of the CpG-non-methylated promoters in humans (Ginno et al., 2012). It has been proposed that the displaced ssDNA in the R loop acts as a signal to recruit either the protective H3K4me3 or DNA demethylases complexes (Ginno et al., 2012). In addition, this work proves that R-loops protect chromatin from being methylated. Pathological increment of R-loops, consequently, may participate in aberrant chromatin demethylation. Interestingly, we have shown in **Study 3** that MCL show a high number of hypomethylated regions, corroborated in other studies (Queiros et al., 2016).

Interestingly, our results differ from previous studies where it was showed that cyclin E, but not cyclin D1, was capable of inducing replication stress and CIN (Spruck et al., 1999). Since cyclin E functions downstream of cyclin D1, the effects on DNA replication and DDR mediated by cyclin D1 overexpression could just reflect the increase of cyclin E. However, several observations do not favor this idea. Firstly, cyclin $D1^{T286A}$ promotes errors in origin licensing and rereplication, while cyclin E/CDK2 kinase is proposed to trigger replication stress due to a premature S-phase entry before complete origin licensing (Loeb et al., 2005; Tort et al., 2006). In this regard, in our study we detected the impairment of fork progression upon cyclin D1 overexpression in cells that already performed correctly origin licensing and G1/S entry. As we have described, Cyclin D1 induction in JVM-13 cells during S-phase led to longer S-phase duration, in spite of the fact they had undergone normal G1 phases without cyclin D1 overexpression. Although we have not characterized how cyclin D1 affects replication during S-phase, a study claimed that total CDK2 activity is not increased by cyclin $D1^{T286A}$ (Aggarwal et al., 2007), indicating cyclin D1-RSR activation may not be dependent on CDK2 activity.

This DNA replication stress might represent a chronic event in the cells carrying the t(11;14) translocation. Chronic stress may lay out a scenario that may enable the acquisition of secondary alterations required for MCL lymphomagenesis (Jares et al., 2012). However, the activation of DDR in response to this stress would limit the generation of chromosome instability. Our results agree with that idea, since cyclin D1 induction leads to an increment of γH2AX and pCHK2 signal by western blotting, confirming that cyclin D1 overexpression might induce DDR activation. This hypothesis also agrees with the experiments showing that cyclin D1 oncogenic effects in tumor development *in vivo* can only be detected after long latency and incomplete penetrance (Casimiro et al., 2012; Wang et al., 1994). Interestingly, recent studies have demonstrated that cyclin D1 overexpression synergizes with *ATM* deletion to promote B-cell lymphomas and increase chromosomal instability (Vaites et al., 2014; Yamamoto et al., 2015). In this regard, *ATM* mutation is the most frequent mutation in MCL tumors (around 50% of cases), suggesting a selection pressure for MCL tumor cells to inactivate genes involving in DDR (Beà et al., 2013).

We hypothesized that cyclin D1 may not only increase replication stress, but also interfere with the normal RSR, maybe through its impairment of CDK9 functions. Normal RSR entails limited apoptosis and both G1 and G2/M blocking. To validate RSR in cyclin D1 overexpressing cells, we examined the ability of cyclin D1 positive cells to recover from a transient replication fork arrest. Twenty-four hours after removing HU, cells overexpressing cyclin D1 showed higher apoptosis, indicating impaired RSR response. In addition, G2/M phase was increased in cyclin D1 positive cells. One explanation would be that these cells are not arrested, but the longer S-phase enabled us to see an increase in G2/M phase. On the other hand, high levels of G2/M blocking may be indicative of higher DDR activation during S-phase, since the G2 checkpoint allows the cell to repair DNA damage before entering mitosis. The phenotype observed upon cyclin D1 overexpression was similar to the one obtained when members of the RSR pathway, such as ATRIP, ATR or CDK9, are silenced in cells recovering from replication stress induced by drugs (Yu et al., 2010).

We explored the hypothesis that chronic DDR activation due to the constitutive overexpression of cyclin D1 could be present in MCL primary cases. The IHC results of the **Study 2** demonstrated that MCL is a neoplasm that has aberrant activation of the DDR in primary tissue samples. In our patient cohort, about two thirds of patients showed high levels of the DNA damage marker γH2AX and almost one third showed pCHK2 activation. We observed a significant correlation between γH2AX and pCHK2 levels, constitutive DDR activation and a higher number of chromosome abnormalities and *TP53* or *CDKN2A* alterations. Moreover, patients showing high pCHK2 levels had a significant poorer overall survival compared to patients with low pCHK2.

Prolonged activation of DDR may sometimes lead to survival of malignant cells when they are able to bypass the cell growth barrier imposed by the TSGs, acquiring mechanisms of DDR tolerance (Huang et al., 2005). In our study, CHK2 activation was mainly detected in cancers with *TP53* alterations or/and 9p deletions. *CDKN2A*, located at 9p21, encodes p16 and p14ARF, a protein required for cellular response to chronic DDR (Bieging-Rolett et al., 2016). *TP53* is a very well known TSG and one of the most altered genes in cancer (Sengupta and Harris, 2005). Our *in vitro* results, together with the study in primary tumors, allowed us to propose a cancer progression model in MCL based on DDR activation, similar to the one described for solid tumors (Bartek and Lukas, 2003; DiTullio et al., 2002; Gorgoulis et al., 2005; Kshirsagar et al., 2012) (Figure 83) . We suggest a model where cyclin D1 overexpression due to the primary oncogenic event, the t(11;14) translocation, causes aberrant stimulation of cell proliferation and interferes with replication, leading to replication stress. Replication stress, in turn, could lead to DNA DSBs, activation of the DNA damage checkpoint and genomic instability (Spruck et al., 1999; Tanaka and Diffley, 2002; Vaziri et al., 2003). In this study, the absence of γH2AX stained helped us to detect less aggressive cancers or tumors at early stages (Gorgoulis et al., 2005; Kuo and Yang, 2008). This replication stress, either directly or through the formation of DNA DSBs, can activate the DDR, exemplified by the cases with H2AX and CHK2 phosphorylation. It is likely that persistent activation of DDR could represent a selectively pressure to the cells in order to acquire mutations of TSG involved in the DDR response such as *TP53* or *CDKN2A*, thereby contributing to tumor development (Bartek and Lukas, 2003; DiTullio et al., 2002). Eventually, TSG suppression would cause a cell release from the suppressive effects of the DNA damage checkpoint pathway, facilitating tumor progression despite the chronic activation of DDR pathway.

Recent findings in pathology could be consistently explained by our cancer progression hypothesis in MCL. Isolated reports have described cases with cyclin D1-positive MCL-like cells restricted to the mantle zone of hyperplastic follicles in otherwise reactive lymph nodes (Espinet et al., 2005; Nodit et al., 2003; Richard et al., 2006). This distribution of the atypical cells suggests that these lesions may represent an early step in the development of MCL, and the process has been termed "*in situ* mantle cell neoplasia". Both SOX11-negative and positive "*in situ* " lesions had a very indolent behavior (Carvajal-Cuenca et al., 2012). Although we did not investigate the expression of DDR activation in this pre-malignant stage, we hypothesize that these cases would display low levels of DDR activation.

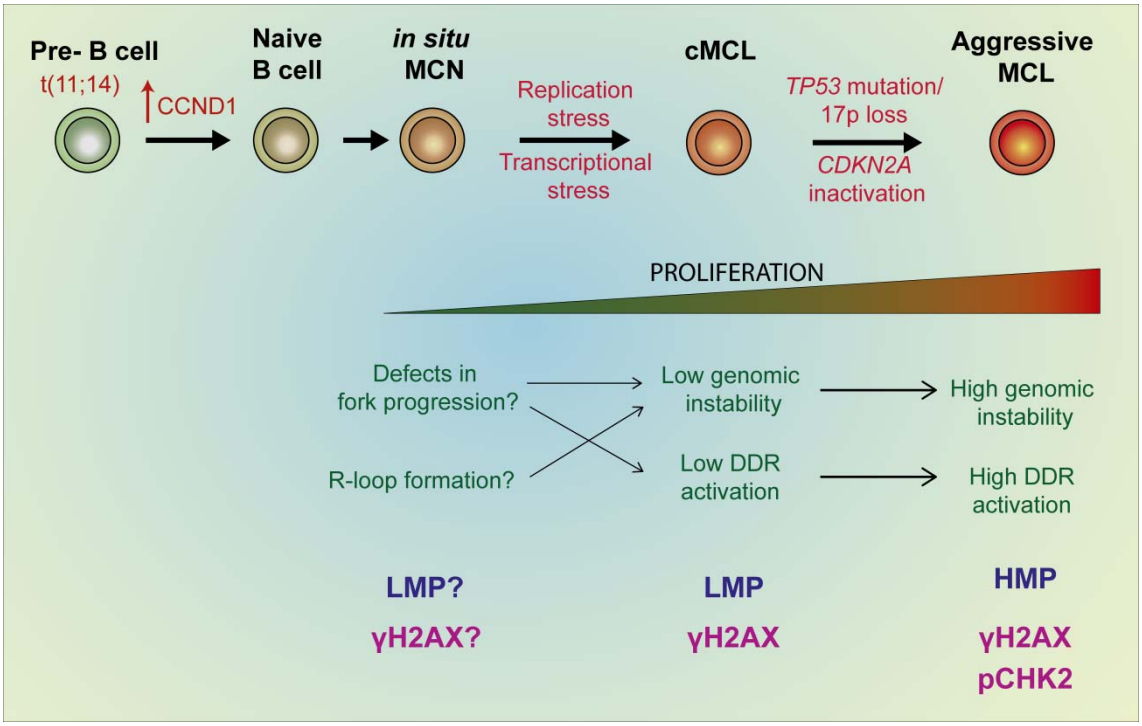


Figure 83. **Stepwise progression of MCL from initial oncogenic hit, the translocation t(11;14) to aggressive MCL progression.** Pre-B cells harboring the t(11;14) translocation increase cyclin D1 and arrive up to the naïve B cell maturation step. Increased proliferation, defects in fork progression and transcription downmodulation might occur in *in situ* mantle cell neoplasia. Replication and transcription stress may be causative agents in cMCL progression, thus low levels of DDR activation and genomic stability may be detected in those cases. TSG inactivation would lead to cancer progression, DDR tolerance, high genomic instability and aggressive morphologies. Primary cases may display very different epigenomic profiles. High aggressive cases display TSG-silencing by aberrant hypermethylation. Increasing proliferation, going from green (low) to red (high) is indicated with a triangle.

Our results also suggest a relationship between the DDR status and the expression of SOX11.77% (21/27) of SOX11-positive MCL cases presented γH2Ax activation, while only 33% of SOX11 negative cases presented γH2Ax activation (3/9 cases). Interestingly, we had molecular information of two of these three cases, showing that they had *TP53* deletion. These cases had very bad prognosis and complex karyotypes despite its SOX11-negative status. Consequently, DDR activation could be used as a biomarker to identify SOX11-negative cases with poor prognosis. Furthermore, SOX11 positive cases with higher DDR activation (γH2Ax and pCHK2 positive cases) also presented worse clinical outcome, suggesting that DDR tolerance may be an important characteristic in MCL progression. Altogether, the role of SOX11 in MCL progression and aggressiveness will have to be further reanalyzed in the context of DDR activation.

It has been recently reported that inherent DNA damage is a common trait shared by a variety of hematological cancer cell lines of both myeloid and lymphoid origin (Cottini et al., 2014). MCL is a lymphoma characterized by high proliferative index and high genomic instability. Recently, a study analyzed the DDR activation by DDR markers such as pCHK2 and γH2AX by IHC in different hematological diseases: Chronic lymphocytic lymphoma/Small Lymphocytic lymphoma, splenic marginal zone lymphoma, Burkitt lymphoma, Hodgkin lymphoma and diffuse large B cell lymphoma (Derenzini et al., 2015). They showed that diffuse large B cell lymphoma cases showed

almost 47% of γH2AX activation, with 5% of pCHK2 activation. The authors considered that, among all the hematological cancers studied, diffuse large B cell lymphoma was the one showing higher levels of DDR activation. However, this work did not include MCL, where we have detected higher levels of activated DDR. Reinforcing the idea that DDR activation is intrinsically playing a role in MCL pathogenesis, the genes more frequently mutated in MCL primary cases have well-established roles in DDR, for instance *ATM, CCND1, TP53, NSD2* or *UBR5* (Beà et al., 2013) and some are targeted by frequent losses (Table 1) . In addition, we found in **Study 3** that a great proportion of MCL cases targeted *CDKN2A* promoter by hypermethylation. In fact, 63% of aggressive cases with the hypermethylated phenotype has either deletion or promoter hypermethylation of *CDKN2A*, suggesting that there may be a selective pressure for TSG deletion in order to overcome DDR chronic activation in MCL.. .

The DDR activation depicted in **Study 2** opens the possibility to explore specific therapies targeting the compromised DDR signaling. Many studies showed that cancers with impaired DDR, TSG inactivation and high chromosomal instability may be dependent on compensatory repair strategies to prevent catastrophic DNA damage (Fece de la Cruz et al., 2015). Indeed, PARP-inhibitors used successfully in breast cancer follows this rational (Farmer et al., 2005). Current studies describe replication stress as an enabling characteristic intrinsic of tumor cells and, thus, synthetic lethality occurring between RSR inhibitors and the high basal levels of replication stress can be exploited therapeutically (Dobbelstein and Sorensen, 2015). In the lights o four results, we could think that drugs targeting the DDR, such as CHK1 inhibitors, could be a useful therapeutical approach for MCL treatment. In this regard, CHK1 inhibitors have been recently shown to be strongly effective as single agents in MCL, although its effect was increased in the presence of Wee-1 inhibitors (Chila et al., 2015). Reinforcing the role of cyclin D1 in the DDR and RSR activation in MCL, JeKo-1 cell line that was resistant to CHK1 inhibitors displayed less cyclin D1 concentration and restoration of cyclin D1 expression rendered cells more sensitive to the pharmacological inhibition (Restelli et al., 2015). Other strategies are also interesting to validate in MCL patients in the light of our results and others, such as ATR/PARP1 inhibition or topoisomerases inhibitors (Williamson et al., 2012).

In conclusion, our data demonstrate that cyclin D1 overexpression increases proliferation, what leads to the activation of RSR and DDR *in vitro*. MCL cases, displaying high cyclin D1 overexpression, have to deal with replication stress. This threatening condition may be the cause of the constitutive activation of the DDR pathway and is associated with poor prognosis. These findings suggest that genomic instability, inherent DNA damage and alterations in DNA repair pathways may represent an Achilles heel by which highly genetically unstable aggressive lymphoid neoplasms may be targeted therapeutically.

As a part of this thesis, in **Study 3** we identified a marked reprogramming of the methylation profile of MCL tumor cells compared to the normal lymphoid samples using DNA methylation genome-wide analysis of a large series of primary cases. This methylation pattern was characterized by extensive hypomethylation targeting CpG dinucleotides outside CpG islands and the hypermethylation of CpG islands, particularly in a subset of primary tumors with aggressive clinical and biological

features. Our results support a scenario characterized by a heterogeneous MCL methylation profile related to the biological behavior. Previous studies have found a more homogeneous pattern of methylation in primary MCL but probably these apparent different results may be due to the relatively low number of cases investigated in these studies that may not capture the complex biological behavior of MCL (Leshchenko et al., 2010). Very recently, Queiros and colleagues (2016) depicted that almost 300.000 CpGs can be modulated in MCL primary cases, representing around the 62% of the total CpGS interrogated by their array. This result not only shows the high level of heterogeneity between cases, but also indicates the high level of epigenetic dysregulation in MCL (Queiros et al., 2016). They also confirmed that DNA hypomethylation is a more common phenomenon than hypermethylation.

The distribution of *de novo* hypermethylated sites in CpG islands and loss of methylation in promoters with low number of CpG dinucleotides is concordant with the observations in solid tumors, hematological neoplasms and other studies in MCL (Loginov et al., 2017; Queiros et al., 2016). In a variety of cancers (Hinz et al., 2007; McCabe et al., 2012; Ren et al., 2012; Tonini et al., 2008), silencing by *de novo* hypermethylation or repressive histone marks frequently occur in promoters hypermethylated in stem cells by polycomb complex proteins (McCabe et al., 2012). In this regard, our results are in agreement with the established idea that polycomb complex proteins repress tumor suppressor genes (Dietrich et al., 2007; Gil and Peters, 2006) through the accumulation of DNA methylation in the promoter regions of their targets, altering their methylation profiles (Ohm et al., 2007; Schlesinger et al., 2007). In our study, *de novo* hypermethylation was significantly enriched in homeobox genes, genes targeted by *EZH2* or genes carrying inactivating histone marks in normal B-cells or in embryonic stem cells. Among different gene signatures, GSEA analysis showed a modest enrichment of the WNT pathway in cases with higher number of hypermethylated WNT inhibitor genes. Although we have not been able to show nuclear B-catenin expression by immunohistochemistry in a limited number of cases, other authors have shown the constitutive activation of the WNT pathway in primary MCL (Gelebart et al., 2008). Further studies should clarify the presence and role of WNT dysregulation in MCL pathogenesis, although some evidences have arisen in recent years (Vogt et al., 2017) . In addition, we have found that a number of known TSG, like *BCL11b, KLF11, KLF4, AHR, DAPK1, CDKN2A, DCC* and *SFRP1* among others, are methylated with a concomitant reduction in their mRNA levels. Concordantly, we observed that primary MCL showing lower expression levels of genes downregulated by DNA methylation showed a significantly poor prognosis. This observation supports the idea that these methylation events might be important in MCL lymphomagenesis.

The association between the hypermethylation and hypomethylation phenomena that we observed in MCL has already been reported in other neoplasias. However, our data reveal substantial differences between both phenomena in MCL, suggesting that DNA hypermethylation, although less frequent, might play a more important role in MCL lymphomagenesis. In that sense, we observed that DNA hypermethylation in MCL correlated to a significant downregulation of gene expression, and with several important clinicopathological parameters associated with aggressive behavior including blastoid morphology, chromosomal instability, proliferation signature and, concordantly,

shorter overall survival. Moreover, the DNA hypermethylation in our series, contrary to DNA hypomethylation, follows a bimodal distribution. These data would suggest that a subgroup of MCL (HMP) might display a CpG island methylator phenotype (CIMP), a phenomenon described in other neoplasms including B-cell lymphomas (Herman and Baylin, 2003; Martin-Subero et al., 2009). This group of MCL classified as HMP displayed a differential gene expression profile characterized by the upregulation of an extremely high number of cell cycle related genes compared to MCL samples considered LMP. Other studies confirmed our results regarding promoter hypermethylation in MCL (Queiros et al., 2016). However, Queiros and collaborators also found that much of the hypomethylation detected in primary MCL cases target intergenic enhancers and transcribed regions, not only heterochromatin regions. Enhancer demethylation could cause important transcription activation of distant promoters. These new findings are explained by the use of a different platform used for the methylation analysis. While in our study we used a 27k CpGs array, enriched in promoters and CpGs islands, Queiros and collaborators used 450K arrays, highly enriched in gene body and intergenic CpGs.

In the last years, new therapeutic options targeting the molecular mechanisms of MCL have been tested, including DNA methyltransferases and/or histone deacetylases inhibitors (Parekh et al., 2011). In that sense, treatment with vorinostat, a HDAC inhibitor, has shown important response rates in relapsed MCL (Kirschbaum et al., 2011). Interestingly, it has been shown that MCL cell lines are sensible to DNMT inhibitors and show synergy with HDAC inhibitors (Enjuanes et al., 2011; Leshchenko et al., 2010). Remarkably, cyclin D1 interacts with HDAC1 (Fu et al., 2005b) and causes the increase in repressive histone mark H3K27me3 in endoderm loci (Pauklin et al., 2016). Consistent with this fact, the HMP cases showed significantly higher mRNA levels of DNA methyltransferases (*DNMT1* and *DNMT3B*) and polycomb repressor genes (*EZH2* and *EED*). Our results would suggest that these epigenetic treatments could be tailored to MCL patients with HMP phenotype.

In **Study 3** we also reported a strong association observed between the accumulation of hypermethylated genes and the proliferation of the tumors. In that sense, the HMP MCL showed a significant higher proliferation signature than LMP samples. The correlation between proliferation and epigenetic dysregulation can be explained in two different ways. First of all, DNA hypermethylation might target the promoters of TSG and genes from cell cycle checkpoints and contribute to increase proliferation and MCL progression. Secondly, there are many evidences suggesting that increase proliferation can lead to epigenetic dysregulation (Uchida, 2016). For example, it has been reported that *CDKN2A* inactivation in breast tumors has been associated with higher proliferation, which ends up in increased TSG hypermethylation (Reynolds et al., 2006). In addition, disruption of the *CDKN2A*/*RB1/E2F* pathway, a key element of cell cycle control frequently targeted in MCL, has been described as a mechanism to dysregulate DNA hypermethylation, through the E2F-dependent upregulation of DNMT1 and polycomb repressors genes like *EZH2* (Jung et al., 2007; Pradhan and Kim, 2002; Reynolds et al., 2006). Highly proliferative MCL cases overcome cell cycle control and display higher E2F activity, what would increase epigenetic instability. Consistent with this, the HMP cases showed significantly higher mRNA levels of E2F1 and E2F2 (data non shown) (Figure 84).

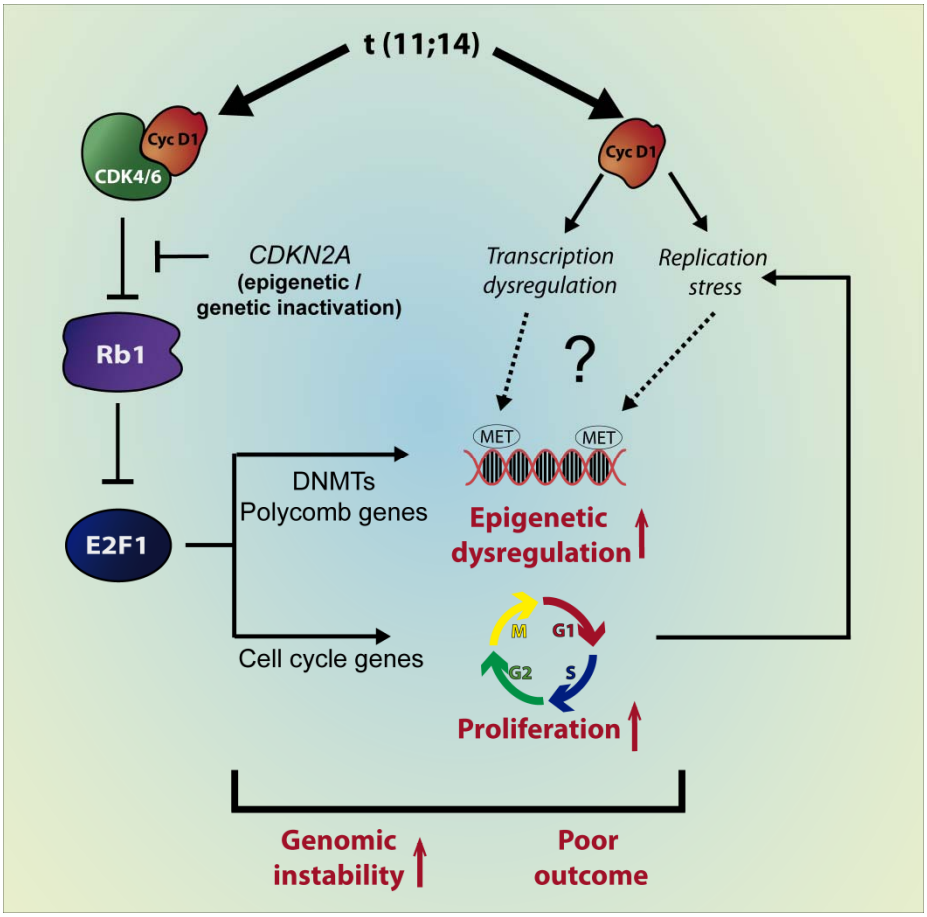


Figure 84. **Suggested model explaining cyclin D1 association with cell proliferation and DNA hypermethylation in MCL.** The dysregulation of cyclin D1 G1/S pathway (*CCND1/CDK4/CDKN2A/RB1)* by frequent genetic alterations is critical in MCL lymphomagenesis. These alterations would promote E2F activation that would be responsible for cell-cycle progression. In addition to cell-cycle genes, E2F might promote dysregulation of epigenetic regulator genes that might be involved in the inactivation of TSGs and important pathways. Dysregulated proliferation may contribute to replication stress and increased epigenomic instability. In addition, replication stress and transcription downregulation caused by cyclin D1 non-canonical pathways might have a role in epigenetic dysregulation.

We hypothesize that cyclin D1, directly or indirectly (for instance, increasing proliferation) may have an important role in epigenetic dysregulation. In addition to the direct interaction with chromatin remodelers, cyclin D1 also interacts with proteins such as PCNA (Fukami-Kobayashi and Mitsui, 1999), a replication factor that orchestrates DNA synthesis and plays an essential role on nucleosome assembly and establishment of epigenetic inheritance (Alabert and Groth, 2012). An attractive model is that PCNA binding enhances the local concentration of DNMT1 and facilitates rapid recognition of hemimethylated sites in the open structure of nascent chromatin. Consistent with this view, lack of PCNA-dependent DNMT1 recruitment does not reduce DNA methylation dramatically but slows methylation kinetics on newly replicated DNA (Schermelleh et al., 2007). Interestingly, higher levels of cyclin D1 interact with PCNA and impair its function in DNA replication (Fukami-Kobayashi and Mitsui, 1999). Our study has not focused on PCNA-cyclin D1 interactions, although future experiments may clarify its functional implications in replication stress and epigenetic dysregulation in MCL.

Recent evidences claim that not only proliferation, but also replication stress fuels epigenetic instability (Alabert and Groth, 2012; Khurana and Oberdoerffer, 2015). In light of the tight coupling of histone dynamics to fork progression, several types of 'epigenetic injuries' can be enhanced in response to replication stress. Firstly, fork stalling impairs recycling of parental histones (Jasencakova et al., 2010), which can lead to loss or gain of epigenetic information and aberrant gene silencing (Sarkies et al., 2010). Secondly, replication stress may alter histone modifications (Jasencakova et al., 2010; Nyce et al., 1986). In that sense, halting fork progression may promote gene silencing owing to unscheduled histone modification and recruitment of silencing factors in mammals (Mirkin, 2007). Interestingly, DNA hypermethylation levels increase in cells exposed to replication stress (Nyce et al., 1986). Lastly, fork collapse could lead to dramatic changes in chromatin reorganization (Zaratiegui et al., 2011). One example of these changes upon replication impairing is the global increase in H3K9me3 that cells experiment during senescence caused by replication stress (Di Micco et al., 2011). As H3K9me3 does not increase when senescence is induced by replication-independent damage, it is tempting to speculate that this increment occurs at sites of fork stalling. To finish, although little is known about the chromatin dynamics during DNA repair, homologous recombination activated upon DDR activation can have even more dramatic effects on the chromatin landscape (Alabert and Groth, 2012). Therefore, cyclin D1 may not only affect proliferation, but also speeding up changes in MCL methylome as a side effect of the replication stress generated.

In our study, we have brought together many evidences that point out that cyclin D1 overexpression play an important role in MCL biology besides its role in cell cycle. In fact, the results in our three studies enable us to present a more complete hypothesis about MCL pathogenesis (Figure 83). This thesis has elucidated that primary case with high proliferation show chronic DDR activation and higher hypermethylation of promoters, the HMP group. All these three characteristics (proliferation, DDR activation and *de novo* hypermethylated profile) correlate with poorer outcomes. Currently, MCL pathogenesis model suggest that cases with high levels of cyclin D1 are highly proliferative, aggressive and, usually, present blastoid morphology (Rosenwald et al., 2003). Even though we have not evaluated directly the quantity of cyclin D1 in primary cases, the more proliferative cases had high levels of epigenetic dysregulation. In addition, these tumors face a selective pressure to inactivate TSGs in order to overcome the DDR activation. Consequently, genomic instability accumulates and tumors acquire complex karyotypes and a high number of CNA. Cyclin D1 levels may be relevant for the pathogenesis, as this protein is involved in cell proliferation, genome instability and, as we proved in this work, in DNA damage activation and replication stress. Our work also characterized the global effects on transcriptional downregulation induced by cyclin D1, what may also affect epigenetics and/or replication stress response. In addition, the interaction of cyclin D1 with specific transcription factors in MCL may also represent an interesting, unexplored field. The transcriptional roles performed by cyclin D1 in MCL pathogenesis must be clarified in other studies,especially their contribution to oncogenesis and the underlying mechanisms.

CONCLUSIONS

1) Cyclin D1 displays a genome-wide binding pattern that preferentially targets active promoters. Cyclin D1 binding correlates with the transcription output of the bound genes.

2) Cyclin D1 behaves as a global transcription downregulator in MCL cell lines and lymphoblastic models.

3) Cyclin D1 overexpression increases Pol II pausing index likely impairing transcription elongation by the binding of the cyclin to CDK9.

4) Transcription inhibitors lead to extended apoptosis in MCL, MM and lymphoblastic models with high expression of cyclin D1, suggesting that this synthetic lethality may represent a novel therapeutic strategy for the treatment of aggressive cyclin D1-overexpressing lymphomas.

5) Cyclin D1 increases cell growth and S-phase entry when expressed in lymphoblastic cell lines.

6) Cyclin D1 overxpression induces a limited, but statistically significant, increment in the proportion of tetrapolyploid cells in lymphoblastic cell lines.

7) Cells overexpressing cyclin D1 have S-phase defects and signs of replication stress, including fork stalling, new origin activation, slower replication and longer times required for completing DNA replication.

8) Long incubation times of cyclin D1 induction lead to the phosphorylation of CHK2 and H2AX in lymphoid cells suggesting the activation of the DNA damage response.

9) MCL is characterized by a high level of DDR activation. Consequently, tumors display high levels of γH2AX and pCHK2.

10) MCL cases with high DDR activation correspond more frequently to blastic/pleomorphic variants and have poorer outcomes, higher copy number alterations, more frequent inactivation of TSGs i and increased proliferation.

11) The methylation analysis of MCL primary cases indicates that hypermethylation essentially targets and silences TSGs promoters of pathways related to cell proliferation, such as WNT pathway.

12) Extensive CpG hypermethylation in MCL cases correlates with increased proliferation signature, higher number of chromosomal alterations and poorer prognosis.

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APPENDIX

Appendix 1. Antibodies and reagents

Antibodies

Reference	Antigen detected	Company	W.B. Dilution	ChIP	Fiber / Cytometry
sc-8396	Cyclin D1	Santa Cruz	1:1000	X	
sc-753	Cyclin D1	Santa Cruz	1:1000		
13499	Phospho-Rpb1 CTD (Ser2) (E1Z3G)	Cell signalling	1:500		
sc-899	Pol II (N-20)	Santa Cruz	1:100		
920101	RNA Polymerase II(8WG16)	Biolegend	1:500	X	
sc-13130	CDK9 (C-20)	Santa Cruz	1:1000		
CP06-100UG	Alpha-Tubulin	Oncogene	1:10000		
A5341-100UL	Beta-actin	Sigma	1:5000		
2661S	pCHK2	Cell signalling	1:1000		
9716S	γH2AX	Cell signalling	1:1000		
sc-22810	GABPA	Santa Cruz	1:1000		
sc-350	ETS1	Santa Cruz	1:1000		
ab6336	BrdU	Abcam			X
347580	BrdU	Becton Dickinson			X

Secondary antibodies

Reference	Antigen detected	Company	W.B. Dilution	ChIP
P0217	Anti-rabbit (HRP conjugated)	DAKO	1:3000	
P0260	Anti-mouse (HRP conjugated)	DAKO	1:3000	
AP200P	Anti-Mouse light chain antibody- HRP	Millipore	1:1000	
7074	Anti-rabbit IgG, HRP-linked	Cell signalling	1:1000	
7076	Anti-mouse IgG, HRP-linked	Cell signalling	1:1000	

Control antibodies

Reference	Antigen detected	Company	W.B. Dilution	ChIP
sc-2025	Normal mouse IgG	Santa Cruz		X
sc-2027	Normal rabbit IgG	Santa Cruz		X

Reagents

Reference	Antigen detected	Company
H8627-1G	Hydroxyurea	Sigma-Aldrich
631311	Doxycycline	Clontech
P4864-10ML	Propidium Iodide (E1Z3G)	Sigma-Aldrich
P9172-1G	Pyronin Y	Sigma-Aldrich
94403	Hoechst 33258	Sigma-Aldrich

Appendix 2. Primers and sh Sequences

Primers		
Gene	***Position***	***Sequence***
XPC	Forward	TTTAAGGAGGTCGCTCGAAG
XPC	Reverse	GGCCATTTTTCCTGAGTCTG
RFC3	Forward	TAGCCTTTCCGTCCAAAATC
RFC3	Reverse	GGCCTACGCTTGAAAATCC
CCNT1	Forward	CCGAGTTAACAGCCAATATGC
CCNT1	Reverse	GTTCTCGCGGGAAGATACAC
CDC5L	Forward	CTTTGGCCAGAGTGGTTTG
CDC5L	Reverse	GATATTGGGTGGCTGAAAGG
POLE	Forward	CGCTCCTCAGAGACATGGA
POLE	Reverse	CAAATTTCTCCCCTGAAGCA
TIPIN	Forward	CTCACCTCACGCAGAAAACA
TIPIN	Reverse	CCCAGGAGTTCCCGAGTATC
MRE11A	Forward	GCAGGATCCGTGAAAAGAA
MRE11A	Reverse	AGAGCCGAACTGGACTTGAA
RPL4	Forward	CCAAACCACTCCTATTCCCT
RPL4	Reverse	TAGCCAACTCGTAATAAGACCA
Chr12negativeregion	Forward	CCATTGTAGGAGCCAAATCC
Chr12negativeregion	Reverse	ATTGAACACCAGCTCCCAAC

sh_Sequences		
Identifier	***Reference***	***Sequence***
shCycD1 #1	(TRCN0000295873)	CCGGCCACAGATGTGAAGTTCATTTCTCGAGAAATGAACTTCACATCTGTGGTTTTTG
shCycD1 #2	(TRCN0000295874)	CCGGACAACTTCCTGTCCTACTACCCTCGAGGGTAGTAGGACAGGAAGTTGTTTTTTG

Appendix 3. Transcription factors candidates to interact with cyclin D1 using motif analysis

TF	p value	Adjusted p value	TF matrix
STAT2	0.00E+00	0.00E+00	MA0517
KLF4	0.00E+00	0.00E+00	MA0039
FOXP1	0.00E+00	0.00E+00	MA0481
SP2	0.00E+00	0.00E+00	MA0516
ZNF263	0.00E+00	0.00E+00	MA0528
IRF1	0.00E+00	0.00E+00	MA0050
SPI1	0.00E+00	0.00E+00	MA0080
SP1	0.00E+00	0.00E+00	MA0079
KLF5	0.00E+00	0.00E+00	MA0599
STAT1	0.00E+00	0.00E+00	MA0517
ELF5	1.96E-303	1.16E-300	MA0136
EGR1	1.92E-297	1.13E-294	MA0162
ETS1	1.86E-294	1.10E-291	MA0098
ERG	1.78E-293	1.05E-290	MA0474
PRDM1	1.99E-282	1.18E-279	MA0508
FOXD3	8.23E-271	4.86E-268	MA0041
FOXP2	3.50E-260	2.07E-257	MA0593
MEF2C	1.60E-249	9.48E-247	MA0497
NFATC2	3.61E-247	2.13E-244	MA0152
MEF2A	4.30E-230	2.54E-227	MA0052
FLI1	2.91E-227	1.72E-224	MA0475
FEV	5.17E-227	3.06E-224	MA0156
EGR2	8.85E-226	5.23E-223	MA0472
PAX4	6.46E-221	3.82E-218	MA0068
KLF1	8.97E-214	5.30E-211	MA0493
EWSR1-FLI1	5.20E-207	3.07E-204	MA0149
ELF1	3.09E-203	1.83E-200	MA0473
FOXI1	2.07E-199	1.22E-196	MA0042
TBP	3.09E-199	1.82E-196	MA0108
HNF1A	1.08E-172	6.39E-170	MA0046
EHF	3.79E-172	2.24E-169	MA0598
FOXL1	7.76E-156	4.59E-153	MA0033
LHX3	7.87E-155	4.65E-152	MA0135
SPIB	5.69E-154	3.36E-151	MA0081
YY1	5.68E-153	3.36E-150	MA0095
HIF1A	1.36E-149	8.01E-147	MA0259
ARNT	1.36E-149	8.01E-147	MA0259
POU2F2	5.25E-147	3.10E-144	MA0507
E2F6	1.61E-144	9.51E-142	MA0471

MYC	3.35E-142	1.98E-139	MA0059
MAX	3.35E-142	1.98E-139	MA0059
SOX3	1.02E-141	6.03E-139	MA0514
FOXO3	3.25E-130	1.92E-127	MA0157
E2F3	8.23E-127	4.86E-124	MA0469
RREB1	2.79E-124	1.65E-121	MA0073
FOXF2	8.32E-112	4.92E-109	MA0030
POU5F1	1.36E-111	8.04E-109	MA0142
SOX2	1.36E-111	8.04E-109	MA0142
FOXQ1	5.33E-110	3.15E-107	MA0040
MYC	1.41E-107	8.33E-105	MA0147
BHLHE40	3.44E-103	2.03E-100	MA0464
FOXA2	1.89E-100	1.12E-97	MA0047
MAX	5.93E-100	3.50E-97	MA0058
MYOD1	7.90E-99	4.67E-96	MA0499
FOXO1	1.14E-98	6.73E-96	MA0480
TCF3	8.61E-95	5.09E-92	MA0522
ZFX	9.81E-95	5.80E-92	MA0146
ELK4	2.46E-94	1.45E-91	MA0076
ZNF354C	2.63E-93	1.56E-90	MA0130
SOX6	1.49E-88	8.79E-86	MA0515
USF2	4.37E-87	2.58E-84	MA0526
STAT4	1.49E-84	8.83E-82	MA0518
CDX2	3.06E-84	1.81E-81	MA0465
ARNT	6.93E-82	4.09E-79	MA0004
MYCN	1.23E-79	7.29E-77	MA0104
ZNF143	5.18E-79	3.06E-76	MA0088
STAT3	1.21E-77	7.14E-75	MA0144
RUNX2	5.11E-77	3.02E-74	MA0511
RUNX1	1.00E-75	5.93E-73	MA0002
GATA4	1.60E-75	9.48E-73	MA0482
GABPA	8.71E-71	5.15E-68	MA0062
GATA3	2.18E-63	1.29E-60	MA0037
STAT1	4.14E-62	2.45E-59	MA0137
MYOG	5.43E-61	3.21E-58	MA0500
FOXA1	1.62E-60	9.56E-58	MA0148
USF1	1.17E-59	6.91E-57	MA0093
E2F4	2.13E-58	1.26E-55	MA0470
SRY	2.05E-56	1.21E-53	MA0084
IRF2	1.66E-53	9.81E-51	MA0051
AR	7.91E-53	4.68E-50	MA0007
ZEB1	5.80E-51	3.43E-48	MA0103
FOXD1	2.74E-48	1.62E-45	MA0031
NFIL3	1.28E-47	7.55E-45	MA0025
TCF12	4.19E-46	2.47E-43	MA0521
STAT6	3.30E-45	1.95E-42	MA0520

NRF1	9.94E-45	5.87E-42	MA0506
E2F1	3.17E-44	1.87E-41	MA0024
SOX10	4.15E-43	2.45E-40	MA0442
HOXC9	5.25E-42	3.10E-39	MA0485
STAT5A	5.73E-40	3.39E-37	MA0519
STAT5B	5.73E-40	3.39E-37	MA0519
NHLH1	2.19E-36	1.29E-33	MA0048
TEAD1	1.78E-30	1.05E-27	MA0090
NKX3-1	4.34E-30	2.57E-27	MA0124
SPZ1	1.29E-28	7.62E-26	MA0111
GFI1B	4.14E-28	2.44E-25	MA0483
ZBTB33	1.44E-26	8.51E-24	MA0527
PRRX2	4.11E-24	2.43E-21	MA0075
MECOM	3.74E-22	2.21E-19	MA0029
TAL1	1.36E-19	8.06E-17	MA0091
TCF3	1.36E-19	8.06E-17	MA0091
NR2C2	4.96E-18	2.93E-15	MA0504
ARID3A	4.10E-17	2.42E-14	MA0151
PAX5	3.82E-15	2.26E-12	MA0014
GATA1	1.34E-14	7.92E-12	MA0035
HNF1B	3.21E-14	1.90E-11	MA0153
HOXA5	2.06E-13	1.22E-10	MA0158
CREB1	4.80E-12	2.84E-09	MA0018
SMAD2	1.06E-11	6.29E-09	MA0513
SMAD4	1.06E-11	6.29E-09	MA0513
SMAD3	1.06E-11	6.29E-09	MA0513
SMAD3	1.06E-11	6.29E-09	MA0513
ELK1	1.80E-11	1.06E-08	MA0028
FOXH1	5.32E-10	3.15E-07	MA0479
JUN	7.45E-10	4.40E-07	MA0489
HOXA9	7.79E-10	4.60E-07	MA0594
MZF1_1-4	1.07E-08	6.34E-06	MA0056
THAP1	2.67E-08	1.57E-05	MA0597
ARNT	4.61E-08	2.72E-05	MA0006
AHR	4.61E-08	2.72E-05	MA0006
FOXC1	2.09E-07	1.23E-04	MA0032
DUX4	2.58E-07	1.53E-04	MA0468
CEBPB	5.26E-07	3.11E-04	MA0466
BACH1	6.21E-07	3.67E-04	MA0591
MAFK	6.21E-07	3.67E-04	MA0591
JUN	1.76E-06	1.04E-03	MA0488
NFKB1	1.93E-06	1.14E-03	MA0105

Appendix 4. Functional analysis of TFs candidates to bind cyclin D1 using motif analysis

Term	p value
hsa05200:Pathways in cancer	2.28E-15
hsa05220:Chronic myeloid leukemia	3.83E-09
hsa05212:Pancreatic cancer	1.09E-06
hsa05221:Acute myeloid leukemia	4.81E-06
hsa05215:Prostate cancer	5.77E-05
hsa04630:Jak-STAT signaling pathway	1.71E-04
hsa04110:Cell cycle	3.79E-04
hsa04350:TGF-beta signaling pathway	5.36E-04
hsa04950:Maturity onset diabetes of the young	9.20E-04
hsa04010:MAPK signaling pathway	9.20E-04
hsa05222:Small cell lung cancer	0.0040279
hsa05210:Colorectal cancer	0.0040279
hsa04012:ErbB signaling pathway	0.00456865
hsa04310:Wnt signaling pathway	0.00614686
hsa04062:Chemokine signaling pathway	0.01475413
hsa05211:Renal cell carcinoma	0.01715753
hsa05219:Bladder cancer	0.04262549
hsa05213:Endometrial cancer	0.06251666
hsa05223:Non-small cell lung cancer	0.06681328

Appendix 5. Top 25 TFs co-localizing with cyclin D1 binding intervals in common in MCL cell lines

Transcription factor	Odds ratio
E2f1	109.15
Hmgn3	83.36
Sp2	77.63
Sp4	64.07
Taf7	61.07
Ccnt2	57.4
Zeb1	54.17
Ets1	49.73
Ctcfls	40.8
Foxp2	38.14
Mbd4	31.83
E2f6	31.14
Six5	30.77
Mybl2	30.31
Tcf3	29.92
Atf3	29.06
Bcla	27.94
Creb1	27.6
Nanog	27.02
Egr1	26.98
Hey1	26.89
Pml	25.78
Mta3	24.06
Gabp	24.04
Taf1	23.94

Appendix 6. Published papers