Mechanism and dynamics of transgenerational epigenetic memory in *Caenorhabditis elegans*

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TESI DOCTORAL UPF / 2015

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Dla mojej rodziny

Acknowledgments

It has been well over five years I arrived to Barcelona to do a PhD in Natural Sciences, which has been one of my dreams since I learned the basics of molecular biology and genetics back in high school. It has been a truly exciting ride; all thanks to the wonderful friends I have met here.

First of all I would like to thank my mentor Ben Lehner, whose commitment and passion for science has been truly inspirational to me. I consider myself extremely lucky to have ended up in your lab. You have been a great supervisor! Thank you for giving me so much of your time and support, for letting me pursue my own crazy ideas and for showing so much excitement about this project, but also all the other ones that I have undertaken along the way. I always felt I had your back and it meant a lot to me. It was really a treat working with you, designing experiments, discussing the results, arguing about interpretations and models and finally writing it up. Good times, and many lessons learned.

Thanks to all the past and the current members of the Lehner Lab, for being such a lovely bunch of smart, kind and awesome people; you were like a second family to me. You guys are the best! A special thanks goes to the worm breeders who picked, chunked and bleached worms side-by-side with me through all these years. First, to Alejandro who thought me many essential life-skills such as how to identify an L4 hermaphrodite by morphology of its vulva and where to get good burgers and piscolas in Barcelona. He also contributed to this work by creating the *daf-21* reporters and was in fact the first to point out that their expression is heritable. Many thanks to Marcos for being such a wonderful and jolly companion as much for chatting about science as for shenanigans. Big thanks to Kadri who deserves credit for showing the effect of *div-1*

knockdown on *daf-21* reporter and for bringing a joyful atmosphere to our worm corner. Jenny, I also need to thank you for all the help you gave me throughout these years! You always had the time form and gave me a lot of great advice. It's been so much fun working and partying with all of you guys, particularly on Fridays… (did someone say Friday!?) I will definitely miss it a lot once I leave (and this time for real, I mean it!).

I am also grateful to my fantastic collaborators Edu and Cristina, who contributed to the content of this thesis. Eduard Casas with help of Tanya Vavouri did a great job on analyzing the small RNA sequencing data. Cristina Hidalgo provided some of the experimental data and provided me with a limitless supply of worm-food. Check out her superb immunohistochemistry skills on page 115. Enormous thanks to Alsu, for being there for me and supporting me through the writing process and also to Alex and Alba who cheered along!

Wielkie pozdro dla mojej kochanej ekipy w Polsce, w szczególności dla Brzezina, Gołych, Agaty, Krasnej, Ruperta, braciszka Pawła, a w szczególności dla Agnieszki, za mega wsparcie podczas pisania tej pracy.

Na koniec chciałbym złożyć ogromne podziękowania dla moich rodziców, którzy zawsze we mnie wierzyli i dawali wparcie i miłość. To Wy nauczyliście mnie wiary we własne siły, wytrwałości w dążeniu do celu i tego by nie bać się spełniać własnych marzeń. Jestem wam na zawsze wdzięczny!

Abstract

Since Darwin and Lamarck, biologists have been intrigued by the possibility of the inheritance of environmentally-acquired traits. Examples of inter-generational transmission of traits induced by an environmental perturbation have been reported in multiple species, but the molecular mechanisms governing these responses remain obscure. Using *C. elegans* as a model system we demonstrate that high temperature-induced increase in expression from a somatically expressed transgene array persists for multiple generations. This epigenetic memory is governed by transgenerational transmission of two conflicting epigenetic memories: H3K9me3 histone marks are inherited in *cis* and act as the major determinant of expression levels in the next generation, whereas repressive small RNAs are inherited in *trans* and mediate restoration of the repressed state. In addition, epigenetic resetting is reinforced by soma to germline communication mediated by the dsRNA channel SID-1. Finally, we discovered that replication stress during early embryonic development interferes with epigenetic inheritance of a repressed state. These findings contribute to our understanding of the epigenetic inheritance and eventual resetting of environmentally acquired traits.

Resumen

Desde Darwin y Lamarck, a los biólogos les ha intrigado la posibilidad de que rasgos adquiridos debido al ambiente pudieran ser heredados. Se han descrito muchos ejemplos de este tipo debidos a perturbaciones del ambiente y transmitidos durante generaciones en numerosas especies, aunque por el momento no se conoce su regulación a nivel molecular. Usando *C. elegans* como modelo demostramos que el aumento de la expresión de un transgén artificial en células somáticas inducido por altas temperaturas es conservado durante múltiples generaciones. Esta memoria epigenética está regulada por la transmisión entre generaciones de dos memorias epigenéticas: el principal regulador de los niveles de expresión en la siguiente generación es la transmisión en *cis* de la modificación de la histona H3K9me3, mientras que los represores RNA pequeños (dsRNA) se heredan en *trans* y actúan de mediadores en la restitución del estado reprimido de la cromatina. Además, la puesta a cero epigenética es reforzada por la comunicación desde células somáticas a germinales regulada por el canal de dsRNA SID-1. También demostramos finalmente que un estrés en la replicación del DNA durante el desarrollo embrionario interfiere con la transmisión epigenética del estado reprimido de la cromatina. Estos resultados contribuyen a aumentar el conocimiento que tenemos de la herencia epigenética y la posible puesta a cero de los rasgos adquiridos debidos a cambios en el ambiente.

Prologue

In this thesis I describe the use of *C. elegans* as a model system to uncover mechanisms of epigenetic inheritance of environmentally acquired traits. We discovered that raising the temperature elicits a change in expression that persist for multiple generations even after removal of the initial trigger. Recent decades have brought a lot of new insight into the transgenerational processes operating in *C. elegans* and some of the underlying mechanisms and participating genes are now known. This prior knowledge, combined with the availability of methods and resources, allowed us to pinpoint the proteins that regulate this transgenerational effect of temperature. Through hypothesis driven experiments we were able to uncover the mechanism of inheritance and resetting of epigenetic memory induced by an environmental change. These observations are described in the Part I of the Results section.

Moreover, thanks to an unbiased approach of RNAi screening we were able to identify novel putative regulators of epigenetic inheritance. We showed that interfering with DNA replication machinery during *C. elegans* development disrupts transmission of epigenetically repressed state. The second Chapter of the Results describes this phenomenon along with some experimental evidence that points to possible mechanisms. This work provides a deeper understanding of the mechanisms that govern epigenetic inheritance and provide a basis for further studies on non-genetic transmission of environmentally-acquired traits.

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1. Introduction

1.1 Epigenetic inheritance introduction and definitions

1.1.1 What do you mean by 'epigenetic'?

The term 'epigenetics' dates back to Conrad Waddington who in an article published in 1942 used it to bridge the gap between the genotype and the phenotype (Waddington, 1942b). He defined the epigenotype as a regulatory layer that governs genetic activity in time and space and accounts for the whole complex of developmental processes. At that time biologists were realizing that genes and traits do not behave in a one-to-one fashion. This had been exemplified by a study in mice performed by Grüneberg, where he described the pleiotropic effects of a mutated gene called 'gray-lethal' (Gruneberg, 1936). Apart from causing a loss of yellow pigment in the fur, the mutation also resulted in failure of the adsorption of bone, which normally accompanies growth. The latter led to multiple downstream abnormalities in development affecting teeth, skeleton and nervous system. Waddington was also aware that a single trait, such as the shape of a wing of Drosophila melanogaster, is influenced by action of multiple genes (Waddington, 1940). Therefore he emphasized the dynamic and complex nature of the epigenotype, which he refers to as 'the concatenations of processes linked together in a network' (Waddington, 1942b). Nowhere in his early works, does he mention 'inheritance'.

The shift in the definition of 'epigenetics' happened with an article published in 1958 by David Nanney (Nanney, 1958), who focused on persistence of cellular phenotypes observed in the same genetic background. He drew a distinction between 'genetic systems' that depend

on a change in the primary genetic material and 'epigenetic systems' that on one hand rely on the genetic material, yet do not involve any alterations in it. He noted that in *Escherichia coli* adaption to sugar galactoside triggers a response that is sustained even after removal of the galactoside (Novick and Weiner, 1957). As such, adapted and non-adapted bacteria can be maintained indefinitely in the same environment. He postulated that stability is a key feature of epigenetic systems necessary to explain how the various differentiated sates in a developed animal maintain their state throughout the organism's lifetime. Thus, cellular heredity was introduced as a common feature of epigenetic systems. In the following years it became apparent that DNA bases can be enzymatically modified with an addition of methyl group (Gold et al., 1963). The process of DNA methylation was detected in all kingdoms of life (Guz et al., 2010) and became an excellent candidate for the carrier of epigenetic information because the modification did not alter the genetic code. Indeed, many studies showed that methylation of cytosines is associated with changes in gene expression with a more methylated state (of promoters) generally associated with repression (Meehan et al., 1992). Moreover, the modified base can be stably inherited over multiple cell divisions (Saze, 2008). Holliday pointed out (Holliday, 1987) that if such a modification occurred in the germline it could be propagated to the following generation and suggested the term 'epimutation' for that event.

In eukaryotic cells DNA is wrapped around positively charged proteins called histones (Finch et al., 1977), which can be modified in multiple ways by enzymatic activity through addition or removal of chemical moieties such as methyl or acetyl group (Jenuwein and Allis, 2001). The histone modifications can change the activity of genes in their vicinity by limiting their access to the transcriptional machinery (Dillon and Festenstein, 2002). Alternatively, modified histones can help recruit other

proteins that will facilitate either repression or activation in a given locus (Thiel et al., 2004). Due to their physical association with genes and effects on transcriptional activity histone modification where proposed as a likely mean of epigenetic inheritance (Grewal and Elgin, 2002). For long there was little direct evidence that modified histone states are propagated across mitotic division and serve as a true epigenetic mark. On the contrary, many studies pointed to the very dynamic nature of histones with a high rate of recycling (Radman-Livaja et al., 2010). The definition of epigenetics was therefore further expended to include the non-heritable events to the irritation of some of the scientific community (Ptashne, 2013).

Later definitions of epigenetic inheritance incorporated the discovered role of coding and non-coding RNAs (Mattick, 2003), as well as mechanisms based on structural templating such as prions (Serio and Lindquist, 2000). Currently the website of the largest collaborative research project into epigenetics offers a rather non-rigorous definition: 'Epigenetics is an emerging frontier of science that involves the study of changes in the regulation of gene activity and expression that are not dependent on gene sequence' (http://www.roadmapepigenomics.org/overview). During one of the meetings dedicated to the field of epigenetics at the Cold Spring Harbour the following definition was produced: 'An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence' (Berger et al., 2009). It is this definition that I will adhere to in my thesis.

1.1.2 Inheritance of acquired traits

Until the publication of the 'Origin of Species' (Darwin, 1869) the most prevailing model for the evolution of life was one proposed by Jean

Baptiste Lamarck who formulated his theory in his *Philosophie Zoologique* in 1809 (Lamarck, 1809). Lamarck synthetized and gave a more rigorous theoretical framework to views held by most naturalist of his time, reflected for example in the work of Erasmus Darwin (Charles' grandfather) who in his book *Zoonomia* (Darwin, 1794) wrote "that all warm-blooded animals have arisen from one living filament... with the power of acquiring new parts". Lamarck developed these ideas into a theory that comprised the two main elements: 1) changes in environment modify the needs of the organism, which leads to increased use of some organs and a decrease in the use of another; 2) the resulting modification, albeit small, is inherited to the next generation bringing about a gradual change in the phenotype of the organism (Lamarck, 1809). Charles Darwin in his seminal 'Origin of Species' (Darwin, 1869) provided an alternative explanation for the origin of various forms of life: random change and natural selection acting upon it. However, Darwin himself did not entirely discard Lamarck's theory. In one of his works (Darwin, 1868) he even postulated existence of 'gemmules', which were carriers of information that arise in somatic cells, can be modified by environment then passed to the germline, and hence the progeny, conferring it with some selective advantage. In 1893 August Weismann published a paper introducing his 'germ-plasm theory' (Weismann, 1893), which entirely discarded any possibility of inheritance of acquired characteristics. Weismann correctly observed that all cells of a multicellular organism can be divided into somatic cells that perform most of the organism's function that permit it to survive and germ cells that separate from the soma and carry the heritable information. In his theory the germ cells are isolated from the rest of the organism and the environment, therefore any alteration that happens to the body in the life of an organism is lost to the next generation.

For many years naturalist remained doubtful that random mutations and

natural selection could account for traits that seemed so well adapted to the environment. In article published in Nature in 1942 Waddington introduced the term ‘assimilation’ to explain how Darwinian natural selection can lead to fixation of traits that seem to be derived from a direct environmental influence (Waddington, 1942a). For the sake of the argument, he uses a specific example brought up by Robson and Richards (Robson, 1936): “sternal, alar, etc., callosities of the ostrich, which are undoubtedly related to the crouching position of the bird, appear in the embryo. The case is analogous to the thickening of the soles of the feet of the human embryo attributed by Darwin (Darwin, 1869) to ‘the inherited effects of pressure’. As Detlefsen (Detlefsen, 1925) points out, this would have to be explained on selectionist grounds by the assumption that it was of advantage to have callosities, as it were, preformed at the place at which they are required in the adult. But it is a large assumption that variations would arise at this place and nowhere else.” To Robson and Richards the two opposing hypothesis here are the Lamarckian one, that the continual use of an organ causes a heritable change, and the Darwinian, where the callosities appear at the correct place due to a random genetic mutation. Waddington offered a different approach to the problem. He starts with the assumption that an ancestral form of an ostrich had no pre-formed callosities and they would appear in its life as a result of continual organ use. Even though external stimulus causes the appearance of the callosities, a genetically encoded pathway is responsible for the response and build-up of the callous tissue at the location of the stimulus. Variation in this genetic component will lead to selection of the genotype that gives the most optimum level of response. But how is this optimum reached? Waddington realized that invariability and robustness are key qualities of developmental systems subjected to natural selection. He used the term ‘canalization’ and defined it as adjustment ‘to bring about one definite end-result regardless of minor variations in conditions

during the course of the reaction' (Waddington, 1942a). It is clear that to reach this end a developmental process must become insensitive to variations in the environmental stimuli. In the case of the ostrich, this means that selection will favor organisms that form the same amount of callosities regardless of the amount of stimulation. Eventually, the variable environmental stimulus will become superseded by a more reliable internal genetic factor. Such a path of events explains why the callosities would appear already in the embryo and does so without the need to resort to Lamarckian rules of inheritance.

In the 1940s, when Waddington's papers were published, the 'modern evolutionary synthesis' was has already taken it's final shape (Huxley, 1942). Also referred to as 'neo-Darwinian synthesis' or simply 'modern synthesis', it reflected the consensus among biologists about how evolution proceeds. It reconciled Darwinian natural selection and gradual change with the Mendelian genetics through the advancement of population genetics (Fischer, 1918; Fisher and Bennett, 1930; Haldane, 1934; Wright, 1931). The modern synthesis decisively dismissed old Lamarckian concepts, due to lack of any evidence supporting them, and put the natural selection acting on randomly mutating genes at the center of the theory. To date, the synthesis remains the main paradigm in evolutionary biology. It has even become a common knowledge that traits acquired during lifetime cannot be inherited. However, the discovery of epigenetic mechanisms, such as DNA methylation, prompted the scientific community to revisit some of the old ideas of inheritance and adaptation.

1.1.3 Intergenerational, multigenerational, and transgenerational inheritance

When talking about sexually reproducing organisms a distinction should be made between: 1) the epigenetic inheritance that governs somatic development by maintenance and transmission of differentiated state

across mitosis; and 2) epigenetic states that occur in the germline and are transmitted meiotically to the next or multiple subsequent generations. The latter, also referred to as 'transgenerational epigenetic inheritance' will be discussed here and is the focus of my study.

In order to distinguish between possible direct effects of a stimulus on cells of the germline and a 'true' epigenetic inheritance, one that crosses generational boundaries, a dedicated terminology has been proposed (Heard and Martienssen, 2014) (Figure 1). Consider the case of a mammal exposed to an environmental stress. If a pregnant female (F0) experiences a stressful environment, the fetus that she carries (F1) can be considered to be potentially directly affected; any abnormal trait observed in the F1 offspring could simply be a consequence of direct exposure to stress while in the womb. Moreover, the fetus contains a partially developed germline that will give rise to the F2 generation. Those cells could also be affected directly by the stressor. Since they are the founding cells of the F2 generation, a modified phenotype observed in the F2 generation could equally be a result of a direct environmental insult rather than being mediated by an epigenetic mechanism. Such effects are referred to as 'parental' or 'intergenerational' as they do not necessarily require passage of information across generations (Heard and Martienssen, 2014). Only if an effect of an environmental exposure is observed in the F3 generation (or F2 in case of the paternal origin of an environmental insult) does the process merit being called 'transgenerational'. This classification is generally applied to studies in rodents and epidemiological studies on human pathologies, but has also been adopted in studies of invertebrates such as *Drosophila melanogaster* or *Caenorhabditis elegans*.

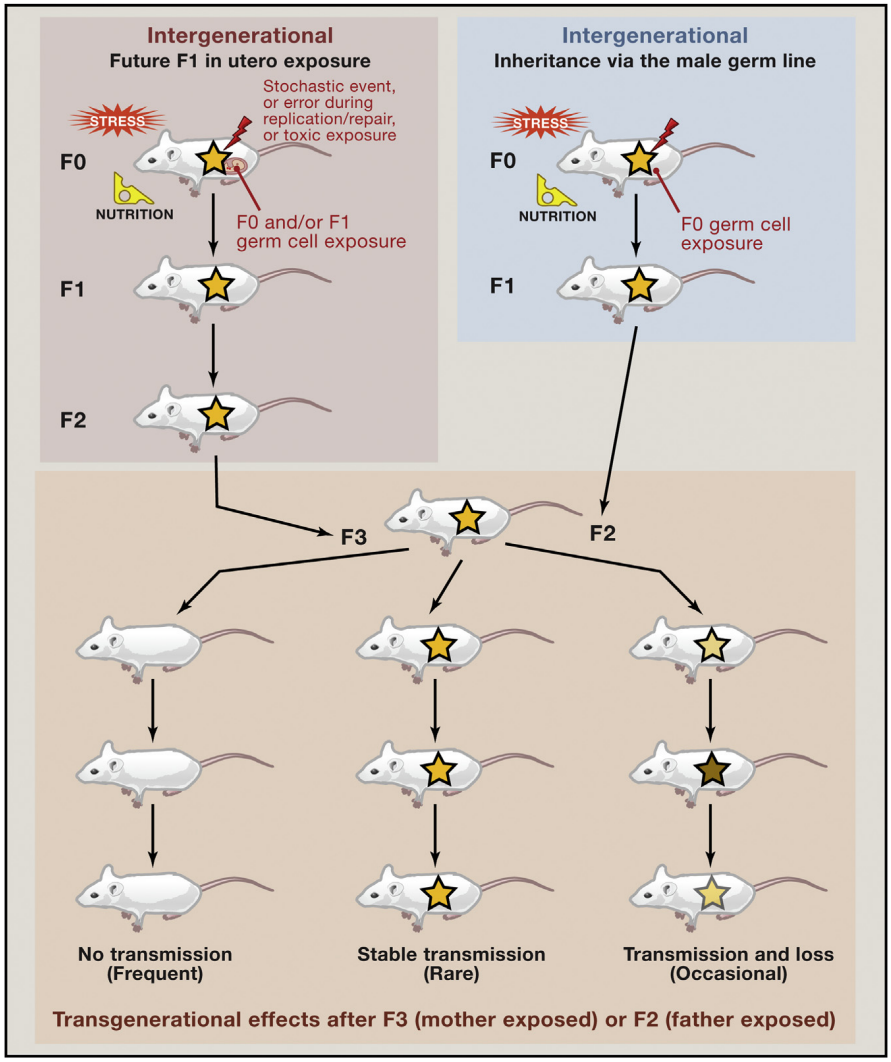

Figure 1. Distinction between Transgenerational and Intergenerational Epigenetic Inheritance. Epigenetic changes can arise stochastically or be triggered by environmental factors such as stress or nutrition. In the case of an affected pregnant mother, the fetus (F1) as well as its germline that will give rise to F2 generation are directly exposed to the trigger. The modified phenotype can be an effect of this direct exposure and if no transmission to the F3 is observed such events are classified as intergenerational or parental effects. If the phenotype persists to F3 generation it can be considered as transgenerational. Similar terminology applies to transmission from the male, only that detection of the modified phenotype in F2 progeny suffices to classify the effect as transgenerational. Figure reprinted from (Heard and Martienssen, 2014).

It is also important to note that the persistence of an acquired trait for more than two generations does not automatically imply the transmission of epigenetic information through gametes. Traits can also be transmitted across generations through a 'gamete independent' route such as through behavior and, in humans, culture. In mice, maternal care has been shown to alter the DNA methylation of a gene encoding estrogen receptor expressed in the brain of the developing pups (Champagne and Curley, 2009). This epigenetic modification induced by maternal behavior is stably maintained to adulthood and promotes a nurturing behavior when they themselves become mothers. This in turn will induce the epigenetic change in their offspring therefore closing the loop that continues indefinitely across generations. Another confounding factor is the microbial flora that inhabits interior and exterior organs of animals and affects multiple traits of the host (Ezenwa et al., 2012). In fruit flies, a certain species of *Acetobateria*, transmitted on eggs from mother to offspring, was found to mediate multigenerational inheritance of stressed induced developmental delay phenotype (Fridmann-Sirkis et al., 2014).

There are many reported cases where the effect of maternal exposure is detected in the F2 but not in the F3 generation (Youngson and Whitelaw, 2008). Although these cases cannot be referred to as 'transgenerational', that does not immediately exclude that epigenetic mechanisms governs the inheritance in the previous generations. It is likely that many environmentally modified traits labeled as 'intergenerational' indeed disappear abruptly from one generation to the next. However, we should also consider the limitation of a non-quantitative approach used in many of the studies, where the phenotype is scored on a binary basis as present or absent (Burggren, 2015). Moreover, many of the applied techniques and methodologies might simply not be sensitive enough to detect a modified trait in the F3 generation.

Here I would like to go back to Nanney's 'Epigenetic Control Systems' (Nanney, 1958) published in 1958, where he noted that epigenetic systems vary greatly in terms of their stability. The same epigenetic circuit can display a different level of stability depending on the growth conditions, such as the already mentioned glucoside adaptation system (Novick and Weiner, 1957). 'Cellular memory' is not an absolute attribute, but rather a quantitative trait. Sorting the epigenetic regulators into categories depending on their stability could sometimes result in an artificial separation between fundamentally similar mechanisms. It might even place the same system in different categories, depending on the conditions of observation.

1.2 Mechanisms

One key question is to determine the molecular mechanisms responsible for intergenerational and transgenerational epigenetic inheritance. Studying the persistence of effects over generations can at least serve as a clue as to which sort of mechanisms might be involved but by directly addressing the mechanisms of epigenetic transmission we can begin to understand how the modified traits flow between generations. Many questions remain open. Which traits can be epigenetically inherited? What determines the stability? Which factors interfere with the process? What impact does it have on the fitness of the organism? Does transgenerational inheritance have any impact on evolution of the species? Below I discuss some potential mechanisms for the inheritance of acquired traits.

1.2.1 Feedback loops and other network motifs

This group of mechanisms consists of dynamic processes that are maintained and propagated over cellular divisions by the sheer nature of the interlinked interactions such as feedback loops and recursive stimulation. It will be best to use an example to illustrate it. Let us

imagine that a transcription factor ‘A’ that is expressed in response to an environmental stimulus. ‘A’ in turn activates expression of another factor ‘B’ which stimulates a downstream response but also binds to the promoter region of ‘A’ activating its transcription. As ‘A’ activates ‘B’ and ‘B’ activates ‘A’ the system will remain active even when the original environmental trigger is removed. Such feedback loops are very common in nature and can govern virtually all cellular processes (Ptashne and Gann, 2002). Interactions between as few as two or three elements can produce various types of responses ranging from stable maintenance of a signal, through oscillations, to a ‘switch-like’ behavior (Alon, 2007). Such mechanisms have not received much consideration in the field of transgenerational inheritance of plants and animals, as they are often deemed insufficient to provide enough stability necessary to propagate the information across generation or even the course of somatic development (Heard and Martienssen, 2014). However they likely deserve more attention.

1.2.2 Prion-like mechanisms and structural templating

Prions are extremely stable misfolded proteins that tend to aggregate and transmit the misfolded state to other proteins (Prusiner, 1998). The mechanism is still not well understood but is proposed to be based on structural templating (Guest et al., 2011). Prions are fairly common in the kingdom of fungi (Jarosz et al., 2014) impacting various facets of cell function (Shorter and Lindquist, 2005) and can be very stably inherited across generations. No evidence has yet appeared that prions can transfer epigenetic information across generations through the germline in plants or animals (Grossniklaus et al., 2013). Another example of template-based inheritance mechanism comes from ciliates such as *Tetrahymena* (Nelsen et al., 1989) and *Paramecium* (Beisson and Sonneborn, 1965*)*. Genetically identical individuals display differences in the patterning of their cilia that

are inherited to their progeny. The pattern in the parent is suggested to serve as a template for the newly built structure in the offspring.

1.2.3 DNA methylation

Covalent modification of the cysteine base is the most established mark of epigenetic inheritance in vertebrates and plants (Feng et al., 2010). In mammals, deposition of the mark is mediated by DNA methyltransferase (DNMT) enzymes that catalyze addition of a methyl group to 5' position of the cytosine ring (Szyf and Detich, 2001). The methyl moiety can be further hydroxylated by an activity of ten-eleven translocation oxidase (TET) proteins (Ito et al., 2010), resulting in a hydroxymetylated cytosine (Tahiliani et al., 2009). Methylated cytosine can be stably transmitted across multiple mitotic divisions (Hashimoto et al., 2012; Wigler et al., 1981). In mammals, the methylated state typically occurs at the 5'-CG-3' dinucleotide (also denoted as CpG) (Gruenbaum et al., 1981), which is a palindromic sequence. After replication a methylated CpG state on one strand will be accompanied by a nascent CpG dinucleotide on the other strand. Such hemi-methylated states generated during DNA replication are recognized by the DNMT1 enzyme that copies the methylated state to the new strand restoring a fully methylated condition (Sharif and Koseki, 2011). New marks can be also deposited on unmethylated di-nucleotides by the action of the *de novo* DNA methylases DNMT3A and DNAMT3B (Chedin, 2011). In plants additional DNA methylation pathways exist and DNA methylation is also common outside of CpG contexts (Saze, 2008). DNA methylation is often found in promoters of genes where it exerts a repressive effect on transcription either by direct interference with the binding of transcription factors or triggering recruitment of histone modifying enzymes that will deposit repressive marks (Boyes and Bird, 1991; Comb and Goodman, 1990). Genome-wide mapping of methylated states revealed large rearrangements during differentiation (Lister et al.,

2013; Lister et al., 2009). Methylated cytosines have also been found on gene bodies where they are suspected to play a positive role in expression through a yet unknown mechanism (Aran et al., 2011; Hellman and Chess, 2007). Cytosine methylation is not a ubiquitous in the animal kingdom. Some animals, such as model organism *C. elegans*, have virtually no detectable cytosine methylation (Hu et al., 2015), whereas others, including Drosophila melanogaster, have very little (Krauss and Reuter, 2011).

For a long time, 5mC methylation was thought to be the only relevant DNA modification in higher eukaryotes. In prokaryotes, 6mA methylation is widespread and has various regulatory functions such as DNA replication, repair and gene expression and defense from viral nucleic acids (Sanchez-Romero et al., 2015). Recent reports demonstrated that 6mA is also found in the genomes of green algae, flies and worms (Fu et al., 2015; Greer et al., 2015; Zhang et al., 2015). The *C. elegans* study (Greer et al., 2015) reported that the delayed loss of fertility that build up over many generations in *spr-5* mutant animals (Katz et al., 2009) is associated with progressive enrichment in the 6mA mark. It is therefore possible that 6mA could act as a true epigenetic mark in the animal kingdom.

1.2.4 Histone modifications

Due to their direct association with DNA, histones have become prime suspects for carriers of epigenetic information. All eukaryotic genomes are assembled into a higher order structure called chromatin. Its main components consists of DNA wrapped around the core histone proteins (the nucleosome), a linker histone that binds the outer side of the core particle and several other accessory proteins as well as RNA (Cutter and Hayes, 2015). The core of the nucleosome is an octameric complex

consisting of four different subunits: H2A, H2B, H3 and H4, each in two copies (Richmond et al., 1984). Biochemical *in vitro* analysis suggested that this complex assembles in a step-wise fashion (Hayes and Lee, 1997). H3 binds to H4 to form a heterodimer which subsequently homodimerizes to create an (H3-H4)2 tetramer. In parallel, the H2A binds to H2B forming another heterodimer. One particle of the (H3-H4)2 tetramer and two particles of H2A-H2B bind concomitantly to DNA to assemble into an octamer and the nucleosome is now assembled. In vivo experiments suggest that the (H3:H4)2 tetramer assembly is suppressed by binding of a histone chaperone ASF1 to the H3-H4 dimer molecule (Liu et al., 2012). In addition to the core histone proteins, a monomeric linker histone H1 also participates in chromatin assembly (Ishimi et al., 1981). H1 binds to the nucleosomes at the point of entrance and exit of the DNA that is wrapped around the core histone octamer (Zhou et al., 2013). Evidence suggests that H1 histone binding to the chromatin has repressive effects on gene expression (Jedrusik and Schulze, 2001) and is proposed to stabilize the higher order structure of the chromatin fiber (Lu et al., 2009; Thomas, 1984).

The terminal ends of core histone proteins (also called histone tails, as they "stick out" of the nucleosome particle) can be chemically modified by the addition of various chemical groups. These modifications affect chromatin activity either by directly changing its properties or by serving as binding sides for other effector proteins (Hublitz et al., 2009; Strahl and Allis, 2000). The most studied histone modifications are acetylation and methylation of lysine (K) residues.

Lysine acetylation neutralizes the positive charge of that amino acid. As DNA is negatively charged this weakens the interaction between the histone and the DNA. (Gavazzo et al., 1997) The nucleosome becomes

loosened and is therefore more accessible to the transcriptional machinery (Hansen, 2006). The association between acetylation and transcription has been confirmed in multiple experiments through the identification of this mark with actively transcribed genes (MacDonald and Howe, 2009) and, most crucially, by the discovery that many transcription co-factors such as CBP are histone lysine acetyltransferases (Kouzarides, 1999). Lysine acetyltransferases, the enzymes that catalyze the deposition of the mark, do not seem to have a preference for a particular site and the same enzyme can modify lysines at different positions within the histone tail (Verdone et al., 2006). Acetylated lysines can serve as docking sites for regulatory proteins through interaction with bromodomains (Filippakopoulos and Knapp, 2014).

Methylation of lysine in histone tails is thought to act predominantly through recruitment of other proteins (Gayatri and Bedford, 2014) and can result in either increased or decreased repression at the affected region (Swygert and Peterson, 2014). Some lysine methyltransferases are capable of adding one, two or three methyl groups (mono-,di-, and tri-methylated moieties) on a single lysine residue (Wang and Jia, 2009). Others work in a step-wise manner, where they need partially mono- or di-methylated substrates to deposit the third methyl group (Towbin et al., 2012). Most methyltransferases exhibit a high degree of specificity towards a single lysine residue within the histone tail (Del Rizzo and Trievel, 2014); that is not to say that histone tails are the only substrates of lysine methyltransferases, other protein can also be affected (Masatsugu and Yamamoto, 2009), as is also true for protein acetylation. The marks can be broadly divided into activating or repressive marks, depending on their association with transcription.

H3K4me2/3 (meaning di-methylation or tri-methylation of histone H3 at

lysine 4 residue) are two of the main epigenetic marks of active chromatin, enriched around the 5' ends of active genes. These marks are generally associated with initiation and maintenance of transcriptional activity (Vakoc et al., 2006). The H3K4me2 mark is more enriched at gene bodies, while the H3K4me3 binding peaks at transcription start sites (Ho et al., 2014). Enzymes that methylate H3K4 residue are found in multi-protein complexes composed of elements that show high conservation among kingdoms of life (Shilatifard, 2012).

H3K36me3 is a mark enriched in the bodies of active genes, especially towards the 3' end (Ho et al., 2014). In yeast, H3K36me3 is deposited on the chromatin co-transcriptionally during the mRNA elongation (Keogh et al., 2005). One function is to recruit histone deacetylase enzyme that removes the acetyl moieties making the chromatin more compact and preventing spurious transcriptional activation in the gene bodies (Carrozza et al., 2005). If the mark is present at the promoter region it will however inhibit transcriptional initiation (Keogh et al., 2005). Therefore it might also be considered a repressive mark with positive or negative effects on transcription, depending on the context (Keogh et al., 2005).

H3K27me3 is a repressive mark deposited by Polycomb complexes (Schuettengruber and Cavalli, 2009)that is a hallmark of 'facultative' heterochromatin (Craig, 2005). It is typically enriched on inactive genes where it blocks transcription likely by multiple mechanisms including by the recruitment of a ubiquitinating enzyme that results in H2AK119Ub (Cooper et al., 2014).

H3K9me2/3 are repressive marks associated with 'constitutive' heterochromatin (Craig, 2005). In most organisms they are highly enriched on silenced, repetitive sequences that comprise much of the genome (Ho et al., 2014).

The ‘old’ division between euchromatin and heterochromatin was based on their relative density during interphase (Heitz, 1928). The name ‘euchromatin’ was given to the brighter regions of the nucleus that contained relatively low concentration of DNA therefore a more relaxed state of chromatin (Heitz, 1928). The dense regions that occupied the outer regions of the nucleus close to the nuclear envelope defined the heterochromatin.

The basic distinction between euchromatin = relaxed & transcriptionally active and heterochromatin = compact and transcriptionally inert has mostly been validated by modern science, but also shown to be a huge simplification, as both active and inactive regions are composed of different states defined by the pattern of histone modifications (Filion et al., 2010). Moreover, some genes located or inserted into heterochromatic regions are robustly expressed (Zhang et al., 2014) and having euchromatic marks does not necessarily mean that a gene is active (Rechtsteiner et al., 2010). It is also unclear to what extend the chromatin environment affects the activity of neighboring genes (Oliver et al., 2002).

There is still a debate in the field about the extent to which do the chromatin marks instruct the expression of the gene, rather than being a product of transcription or reinforcing a transcriptional state determined by inherited upstream factors. Some scientists argued that histone marks should not be referred to as ‘epigenetic’ as there is little evidence that they can be stably inherited across mitotic divisions (Ptashne, 2007). This is particularly true for the active H3K4 methylation marks, as the histones that carry them were shown to be recycled at a high rate (Radman-Livaja et al., 2010). Upstream factors also play a role in the propagation of heterochromatic marks. In yeast, inheritance of centromeric heterochromatin requires small RNAs (Volpe et al., 2002) that guide

reassembly of heterochromatin during DNA replication (Kloc et al., 2008).

1.2.5 Histone inheritance during cell division

To understand how a histone mark could be replicated during mitosis or meiosis we need to consider what happens at the DNA replication fork. Various models have been proposed for how this might be achieved, summarized in Figure 2 which is reprinted from (Greer and Shi, 2012). For DNA to be replicated it must be unwound from the core histones that therefore get displaced from the chromatin fiber (Eggleston et al., 1995). Early studies that relied on *in vitro* reconstituted DNA replication machinery showed that entire histone octameres could be transmitted to the daughter strand without loosing its association to the DNA (Bonne-Andrea et al., 1990; Sogo et al., 1986). A more recent study carried out in a cell culture system (Xu et al., 2010) showed that parental tetramers (H3-H4)2 are reincorporated onto both daughter strands, together with newly synthetized H2A-H2B dimers. Most reports support such a conservative model of histone replication (Masumoto et al., 2011). In an alternative semiconservative mechanism the parental (H3-H4)2 tetramer is split into two dimers and each is deposited on one of the daughter strands. The new nucleosome would therefore contain one 'old' and one 'new' dimer. Several studies detected post replicative nucleosomes composed of such 'mixed' octameres (Katan-Khaykovich and Struhl, 2011), but also noted that this mechanism is restricted to regions that are actively transcribed and therefore experience dynamic histone recycling (Katan-Khaykovich and Struhl, 2011). Whatever the inherited unit, be it an octamer, tetramer, dimer or even a monomer, the redistribution of histones from a single strand to two daughter strands results in dilution of any chemical modifications that they carry. To insure full transmission of chromatin states, histone-modifying enzymes must replenish modifications on the

tails of the histone proteins (Figure 2). This requires the histone modifying enzymes to be recruited to sites of replication. For example, Polycomb proteins remain associated with chromatin during *in vitro* reconstituted DNA replication (Francis et al., 2009).

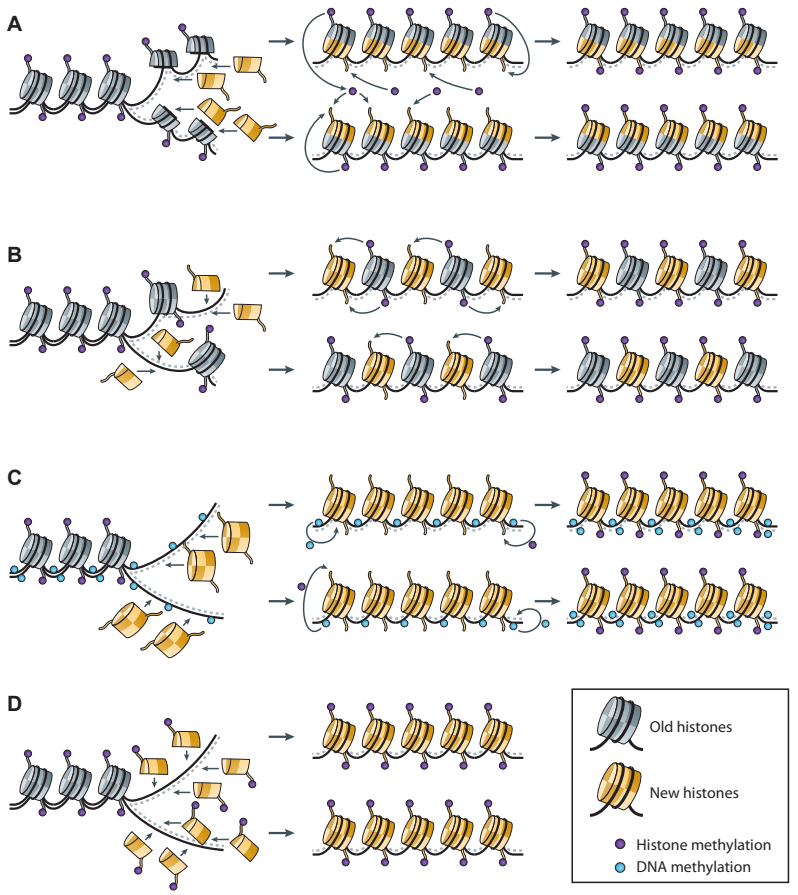

Figure 2. Models for mitotic inheritance of histone methylation marks. (A) Semiconservative model. The parental octamer is split in half between the daughter strands. Marks are then copied from the 'old' halve of the histone octamer to the new one within the same nucleosome (B) Conservative model. Entire octameres are transmitted to the daughter strands and modifications are then copied to the neighboring newly assembled nucleosome. (C) Inherited DNA methylation instructs methylation on specific new histones. (D) Deposition of pre-modified histones. Previously marked free histones are recruited to daughter strands guided by sequence specificity or control of replication timing. Reprinted from (Greer and Shi, 2012).

A study in *C. elegans* showed that during embryogenesis the Polycomb proteins maintain a stable state of the H3K27me3 level across cellular divisions in the early embryo in the absence of transcription activity (Gaydos et al., 2014). Remarkably, a paternal genome inherited from a Polycomb mutant male, hence lacking all H3K27me3 marks, failed to acquire them during embryonic division. This suggests that during *C. elegans* embryonic development, Polycomb complexes act exclusively as maintenance enzymes. The missing marks on the paternally derived genome were restored only later during germline development.

Rather than directly inheriting the histones themselves, transcription factors or methylated DNA could also guide the re-establishment of a proper chromatin environment after replication. This would mean that histones themselves are just secondary to the process. As mentioned before, inheritance of histone states has been documented in *C. elegans* in the absence of transcription and the amount of cytosine methylation in that organism is miniscule; therefore transcription and DNA methylation are unlikely to be responsible for the inheritance. However, the recent discovery of a relatively abundant 6mA DNA methylation in *C. elegans* (Greer et al., 2015) makes it a plausible candidate for a transgenerationally stable epigenetic mark. Small RNAs or long non-coding RNA could also guide deployment of histone marks (Koziol and Rinn, 2010). Inherited RNA molecules resident in the cytoplasm of nucleoplasm could reinstate histone marks after replication by recruiting appropriate histone modifying enzymes to specific regions determined by sequence complementarity (Holoch and Moazed, 2015).

Another alternative is that the methyltransferase enzyme that deposited the mark remains associated with the affected site during DNA replication. As the replication fork displaces the old histones, the enzyme

remains bound to the replication complex and re-establishes the mark on newly incorporated histones. In this case, the methyltransferase enzyme would be the *bona fide* epigenetic marks, not the modifications on the histones. Evidence for such a mechanism was found in the *D. melanogaster* Polycomb/Trithorax system (Petruk et al., 2012). All the above theories do not need to be mutually exclusive and could act in concert to ensure stable transmission of histone modifications across multiple cellular divisions.

1.2.6 Small RNAs

Multiple types of RNA, both coding and non-coding, have been implicated in transgenerational processes (Ashe et al., 2012a; Johnson and Spence, 2011). Small non-coding RNAs can be excellent mediators of long-term inheritance. They can act post-transcriptionally by affecting target mRNA stability and transcriptionally by mediating recruitment of histone modifications (Holoch and Moazed, 2015) and sequence specificity allows them to target specific genes. Importantly, some classes of small RNAs are amplified, allowing maintenance of stable state level across multiple cell divisions and generations (Sapetschnig et al., 2015). A lot of experimental evidence has been amassed in support of their role in the transmission of epigenetic information across generations (Stuwe et al., 2014) and several examples are reviewed in later sections.

1.3 Introduction to *C. elegans*

A small, transparent nematode *Caenorhabditis elegans* has been a champion of developmental and molecular biology for over 30 years. It was introduced as a tool for genetic studies and in 70-ties by Sydney Brenner (Brenner, 1974) and quickly became established as a model system for developmental biology and genetics. Many conserved biological mechanisms where first discovered in *C. elegans* such as

programmed cell death (Sulston, 1976), control of gene regulation by microRNAs (Feinbaum and Ambros, 1999) and RNA interference (Fire et al., 1998). The worm has also helped identify components and mechanisms of cancer-related pathways (Sternberg and Han, 1998) and is one of the prime models in neuroscience (Sengupta and Samuel, 2009), aging (Lapierre and Hansen, 2012) and cell reprogramming (Joshi et al., 2010). It was also the first animal to have its genome completely sequenced (1998), making it a powerful tool to study genomics. More recently, this tiny nematode is also becoming a pioneer in the field of epigenetic inheritance (Padilla et al., 2014).

An adult C. elegans worm is about 1 mm long and entirely transparent, making it a great subject for microscopy analysis.

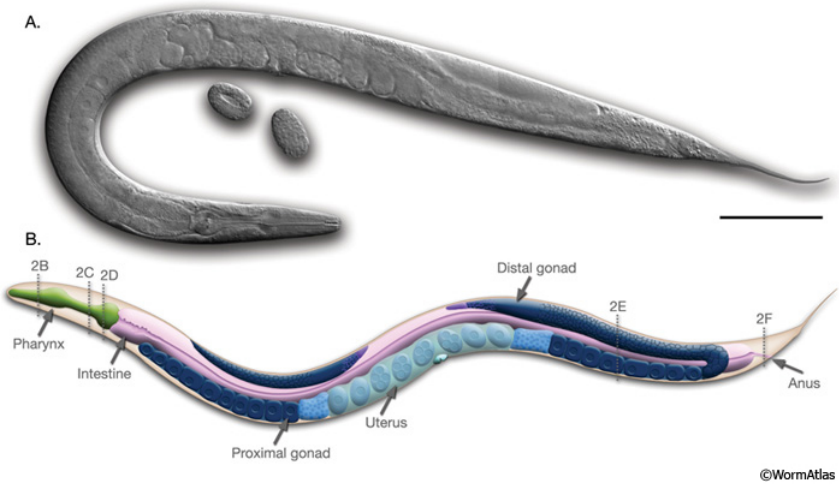

Figure 3. Anatomy of an adult hermaphrodite. (A) DIC image of an adult hermaphrodite, left lateral side. Scale bar 0.1 mm. (B) Schematic drawing of anatomical structures, left lateral side. Source: http://www.wormatlas.org/

The adult body can be generally divided in to the germline, comprised of around 2000 nuclei encapsulated in two syncytial gonad arms, and the soma that consists of 1090 nuclei sub-divided into tissues (Altun, 2015).

The exact position and origin of each somatic cell of the developed organism is known (Sulston, 2003). This was achieved through direct observation (and enormous amounts of patience and perseverance) of cellular divisions from the zygote to the developed larva (Sulston and Horvitz, 1977; Sulston et al., 1983).

C. elegans is a terrestrial, free-living nematode often found on rotting vegetation and fruit. Different isolates of *C. elegans* species have been found across the globe providing the scientists with some genetic diversity (Barriere and Felix, 2005). The strain that is used most commonly was isolated in Bristol, UK, (Nicholas et al., 1959) and later used by Sydney Brenner. This strain is called N2 and is the wild type used in this study. An entire lifecycle of a *C. elegans* lasts about three days, when grown on *E. coli* plates at 20 °C. I will try to describe it briefly and capture the key events important for the narrative of the transgenerational epigenetic phenomenon documented in this work. The specific epigenetic changes that happen during the process are covered later.

During fertilization penetrates through the cell membrane of the oocyte and the two gametes become a zygote. The haploid genome of the sperm nucleus, initially hyper-condensed, becomes relaxed and maternally provided H3.3 histones present in the cytoplasm are rapidly deployed to the paternally derived chromatin (Ooi et al., 2006). Judging from a single image in the supplementary material of (Ooi et al., 2006) it seems as if the paternal histone H3 was lost entirely upon fertilization. However, other studies (Gaydos et al., 2014) that relied on more sensitive techniques provided evidence that some paternal histones are retained on their location after fertilization and the subsequent cell cycles.

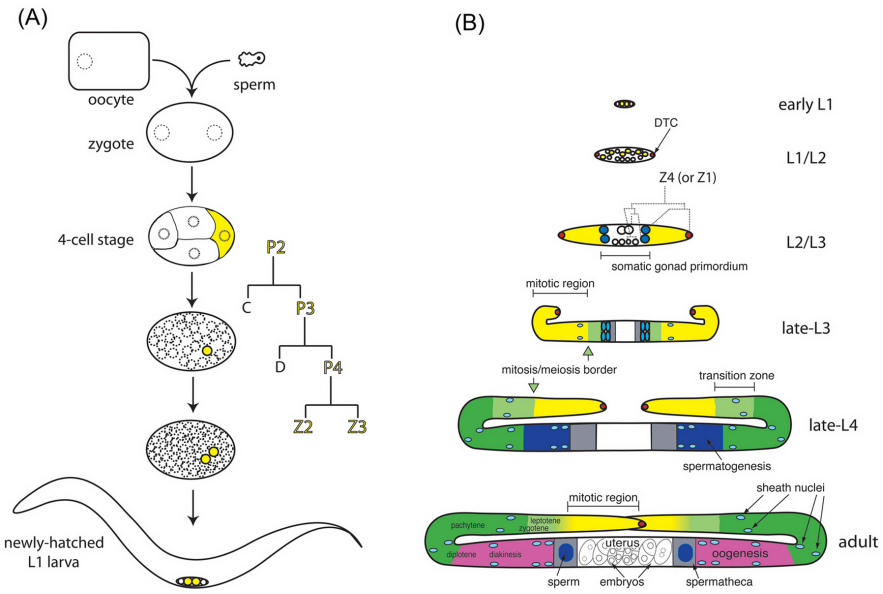

Figure 4. Germline development in *C. elegans*. Reprinted from (Hubbard and Greenstein, 2005).

The oocyte, arrested at prophase of the first meiotic cycle, resumes meiosis right before fertilization (Greenstein, 2005). By the time fertilization is complete it has undergone the two nuclear division cycles of meiosis reducing the maternal genome to a haploid state (Greenstein, 2005). As the two genomes migrate towards each other they both undergo replication, then combine into a single nucleus on the newly formed zygote. From then on the mitotic divisions proceed.

It is important to note here that the cytoplasm of the zygote is derived mostly from the oocyte. All the processes that happen in the first few embryonic divisions are controlled by maternally supplied products including mRNAs and proteins (Begasse and Hyman, 2011). Sperm contributes little mRNA, although recent studies identified a few paternally delivered mRNAs, it is unclear whether they are of any

importance to the development of the animal (Stoeckius et al., 2014). The small RNAs however appear to be contributed by both gametes in roughly the same amount (Stoeckius et al., 2014). Sperm does contribute important proteins to the zygote, such as the paternal centrosome – a multi-protein complex responsible for chromosome separation during mitosis (Oegema and Hyman, 2006). After fertilization the zygote undergoes multiple successive mitotic divisions. In the early embryonic cell cycles, the G1 and G2 phases are skipped (Schierenberg, 2006), instead cells alternate only between DNA synthesis and mitosis leaving little time for checkpoints that could fix possible errors occurring at any stage. The divisions are fast and remarkably coordinated and exhibit what is called a 'stereotypical' development. That means that every embryo will always develop in exactly the same way with respect to the timing of divisions and their eventual fates (Sulston et al., 1983).

Asymmetry in embryonic divisions is apparent form the very first zygotic cell cycle. Soon after fertilization the zygote divides into a larger AB blastomere, and slightly smaller P1, which is the second cell of the germline P lineage (the first one is the zygote). Many cytoplasmic components are divided un-equally between the AB and P1 daughters, most notably the liquid-like particles called the P-granules (Strome and Wood, 1983). Those granules are present in the germline throughout the whole lifecycle of the worm and help maintain germline integrity and totipotency (Gao and Arkov, 2013; Updike et al., 2014). As the P1 cell continues to divide, the germline specific components continue to segregate to only one of the daughter cells, called P2, P3 and P4, respectively. About 160 minutes after fertilization, when the embryo consists of about 100 cells, P4 divides symmetrically to gives rise to Z2 and Z3, which are the primordial germ cells of *C. elegans*. These two cells arrest in development until all the somatic cells differentiate and the worm

hatches from the eggshell as an L1 larva (Kelly, 2014). Such a 'preformistic' mode of germline development, where the germ cells separate from the somatic cells from the very first embryonic divisions, distinguishes *C. elegans* from more complex animals (such as mice), where the germline is specified later in development through an inductive cell-cell interaction mechanism (Seervai and Wessel, 2013).

The worm might arrest as an L1 larva for over 20 days (Lee et al., 2012b) if the conditions for development are unfavorable. If conditions are good and food abundant it resumes growth and development, passing through four molting cycles that separate the intermediate larval stages: L1, L2, L3, L4 and finally develops into an adult (referred to as L5 by some). Alternatively, if conditions are harsh, the worm can enter into a 'dauer' stage where it slows down metabolism, arrests growth and becomes extremely resilient to all sorts of environmental stresses. When the environment improves, it resumes growth and proceeds directly to the L4 stage, from where it continues as normal (Altun, 2015). An adult hermaphrodite worms contains two symmetrically positioned gonads that originated from the two primordial germ cells. The germline develops continuously during growth but in several distinct stages (Hubbard and Greenstein, 2005). Between the L1 and L3 stages, the germ cells undergo mitotic proliferation. Towards the end of the L3 stage, many germ cells start to undergo meiotic differentiation towards sperm. In total, each gonad arm will produce between 100 – 150 functional sperm cells, which are stored in spermatheca and used for self-fertilization. At late L4 stage the gonad switches from spermatogenesis to oogenesis, which then continues throughout the adult lifetime. Mature oocytes are arrested at prophase I of meiosis, MSP protein secreted by sperm triggers meiotic activation in the '-1' oocyte (one closest to the spermatheca but not yet fertilized) which then ovulates entering the spermatheca where it becomes

fertilized by the sperm, therefore completing the cycle (Kim et al., 2013). During much of germline development the germline nuclei are part of a one large syncytium and therefore share a common cytoplasm. Only towards the final stage of meiotic differentiation does cellularization occur, enclosing each gamete in a cell membrane.

Although they are not required for successful reproduction, male worms are sired from the hermaphrodite population at a rate of about 0.1 % (measured in standard laboratory conditions; this number can vary depending on age of the mother, temperature, genetic background, etc., but it will be always quite low) (Altun, 2015). In *C. elegans* is the number of sex chromosomes determines the sex of the animal (Zanetti and Puoti, 2013). The genome of a hermaphrodite worm is comprised of five autosomes and one sex chromosome (called X), each in 2 copies. Occasionally, during meiotic progression of one of the gametes, the X chromosome will be lost due to a non-disjunction event. After fertilization, the genome of the organism will contain only a single copy of the X chromosome (X0 instead of XX), making it a male. Males differ morphologically from hermaphrodites and produce only sperm. Therefore *C. elegans* worms can be expanded clonally by the virtue of self-fertilization, but at the same time can produce males which greatly facilitates mixing of different genotypes (as well as 'epi-genotypes'). Combined with powerful tools and immense knowledge gathered about its biology and genetics, *C. elegans* is arguably the best choice on the market for studying mechanisms of epigenetic inheritance.

1.4 Histone methylation dynamics during the germline cycle of *C. elegans*

The germline lineage, unlike the somatic one, contributes directly to the

subsequent generations forming an uninterrupted chain of divisions and is therefore often referred to as an 'immortal' lineage. All epigenetic information passed transgenerationally must flow through the germline cycle and be stably maintained throughout all of its stages. This includes a hypothetical epigenetic signal originating in the soma that would need to be communicated to and stabilized in the germline to be passed down to the offspring. Among the known and studied histone modifications, the transgenerational behavior of methylations on lysine 4, 9, 27 and 36 have been analyzed with most scrutiny. The methylation state, as described above, can be maintained by replenishing marks lost after DNA replication using the retained histones as templates (Gaydos et al., 2014). Methyl marks are also deposited co-transcriptionally upon passage of RNA polymerase (Rechtsteiner et al., 2010) and some can be targeted by the activity of small RNAs (Gu et al., 2012). Moreover, histone methylation on one residue can affect the state on a different residue within the same or amongst neighboring nucleosomes, forming an interconnected network of positive and negative associations (Greer et al., 2014).

1.4.1 H3K4 methylation

Methylation of H3 on lysine 4 is a well-established mark of transcriptional activity (Ruthenburg et al., 2007). Studies in *C. elegans* showed that the global level of methylation at this mark can depend on its state in the previous generation (Arico et al., 2011; Greer et al., 2011). Mutation in *spr-5*, an H3K4 de-methylase homologous to human LSD1, results in the progressive accumulation of H3K4me2 over several generations (Katz et al., 2009). This was accompanied by a progressive increase in transcriptional activity of multiple spermatogenesis genes that correlated with enrichment in H3K4me2 at their promoters. The *spr-5* mutant worms have a 'mortal germline' phenotype characterized by increased penetrance

of sterility in later generations, likely caused by an additive increase in H3K4me2 with each germline cycle. The enzymes that catalyze deposition of H3K4 methylation are part of protein complexes referred to as COMPASS (complex of proteins associated with Set1p) in yeast and MLL (mixed-lineage leukemia) in humans. Orthologs of subunits of this complex are found in *C. elegans* and include WDR-5.1, ASH-2, SET-2 (Shilatifard, 2012). If any of these proteins is transiently lost for only a single generation, the lifespan of the descendant animals is increased for four subsequent generations (Greer et al., 2011). The lifespan extension phenotype is dependent on the presence of germline and the activity of the H3K4 demethylase RBR-2. This demonstrates that the lack of activity of histone methyltransferases in the germline can have consequences for the soma.

The H3K4me2 and me3 marks appear to be largely depleted in the Z2/Z3 germ cell precursors in the developing embryo (Schaner et al., 2003) and only start to accumulate after hatching, as the germline starts proliferating. In mitotic and meiotic germline the mark is associated with the transcription start sites of actively transcribed genes (Xiao et al., 2011). Convincing evidence for heritability of H3K4 methylation states comes from the observation of sex-specific differences in the methylation pattern. The X chromosomes are largely depleted of genes expressed during germline development (Reinke et al., 2004). But during oocyte maturation, many genes on the X chromosome become expressed. This is not the case during male gamete development, where the X is maintained in quiescence (Kelly et al., 2002). The differences are mirrored by the coverage of the H3K4me2 histone modification. In the male germline the transcriptionally inactive X is strongly depleted of H3K4me2 compared to the autosomes; this is true for spermatogenesis in hermaphrodites as well as in males. In maturing oocytes the marks are restored on the X and the

overall levels become indistinguishable from the autosomes. Interestingly, the sex-specific differential marking of the X chromosome can still be observed in the early embryonic cells after the third round of cellular divisions, suggesting that the states established in the germline are propagated in the zygote (Bean et al., 2004).

H3K4me2 and me3 marks were detected using monoclonal antibodies in embryos depleted of RNA polymerase *via* RNAi (Li and Kelly, 2011). Staining for both marks was as strong as in wild-type embryos up until the point of embryonic arrest (50 -100 cell stage). This suggests a transcription-independent mechanism of propagation of these two marks in the early embryos (Li and Kelly, 2011). In the transcriptionally quiescent P lineage, the H3K4me2 and H3K4me3 signal is maintained through the first three division. During the next two division the antibody signal for both marks starts to diminish i becomes almost undetectable at the Z2/Z3 stage (Li and Kelly, 2011; Xiao et al., 2011). The removal of H3K4me2 in Z2/Z3 germline progenitors could be analogous to the wave of epigenetic reprogramming observed during specification of primordial germ cell in mice (Abe et al., 2011). The birth of primordial germ cells Z2 and Z3 is accompanied by a transient appearance of Ser2 hyperphosphorylated RNA Pol II signal (Furuhashi et al., 2010), which is a hallmark of a transcriptionally active RNA Polymerase (Saunders et al., 2006). This mark is not maintained in the later stages and becomes greatly reduced by the 1.5-fold stage (Furuhashi et al., 2010). This suggests that in primordial germ cells RNA Pol II is transcriptionally engaged for a brief window of time, but in later stages remains inactive.

Repetitive transgenic arrays that contain reporters with regulatory regions of germline active genes are subjected to silencing in the germ cells of *C. elegans* (Kelly et al., 1997). This silencing appears to be mediated by

heterochromatin based processes involving H3K27me3 (Kelly and Fire, 1998), H1.1 linker histone (Jedrusik and Schulze, 2001) and heterochromatin protein 1 (Couteau et al., 2002). Using a set of integrated and extra-chromosomal GFP reporters (some expressed and others silent in the germline) Arico et al. demonstrated that germline expression of a transgenic reporter is accompanied by acquisition of H3K4me2 on the its chromatin (Arico et al., 2011). Moreover, that mark was also detected in 1 and 2-cell embryos that came form parents with germline-expressed transgenes. Germline has an inherent tendency to silence foreign sequences (Kelly, 2014). In one case a non-repetitive array containing translational *let-858::gfp* reporter was maintained in germline-expressed state by the presence of *let-858* mutation in the background. Transgene expression rescues the mutant and therefore expression is maintained through selection, as *let-858* gene is essential and causes sterility when lost or mutated. When the *let-858* mutation was crossed out, the *let-858::gfp* reporter remained expressed in the germline for two generations, but within the following two became silenced. The loss of germline expression was accompanied by a decrease in H3K4me2 mark detected in early embryos on transgene chromatin and in the germline (Arico et al., 2011). Interestingly, progeny of germline-expressed *let-858::gfp* animals exhibited higher somatic expression of the reporter (Arico et al., 2011). Thus, the methylation state of germline-silenced transgene can be inherited to the zygote and is associated with quantitative differences in the expression of the transgene in somatic cells. Other histone marks tested, such as H3K27me3 and H3K9me3, showed no obvious correlation to germline expression. For example H3K9me3 was enriched equally on germline-expressed and germline-silenced transgenes (Kelly, 2014).

1.4.2 H3K36me3

Another histone methylation that has received a considerable amount of

investigation in *C. elegans* is H3K36me3. Two enzymes that deposit this mark have been identified: MES-4 and MET-1. In a mutant lacking both genes no H3K36me3 modification was detected (Rechtsteiner et al., 2010), suggesting they are the only two enzymes that mediate that mark. H3K36me3 marks are maintained during the early embryonic division cycles by the activity of MES-4 that acts in a transcription-independent manner (Rechtsteiner et al., 2010). Immunofluorescence studies showed that, contrary to H3K4me2, H3K36me3 staining remains strong in the Z2/Z3 germ cells. In fact, its level remains stable throughout the whole germline cycle (Furuhashi and Kelly, 2010) making it an attractive candidate for the carrier of epigenetic information. In the absence of MES-4, worms become sterile, demonstrating its essential role in fertility. Maternal provisioning of the MES-4 protein is necessary and sufficient to rescue the phenotype highlighting the importance of this protein in chromatin maintenance in early embryonic cycles (Capowski et al., 1991). Why is MES-4 activity so important for the germ cells? One model suggests that it participates in epigenetic propagation of the germline expression program (Furuhashi et al., 2010; Rechtsteiner et al., 2010). In its absence, the memory of which genes should be transcribed in the germ cells is lost leading to aberrant expression pattern and loss of fertility.

The second enzyme, MET-1, acts co-transcriptionally (Rechtsteiner et al., 2010) and in its mode of activity is proposed to be very similar to yeast Set1p (Strahl et al., 2002). The *met-1* null mutant does not have any obvious phenotypes but combined with a mutation in the H3K9 methyltransferase *met-2* worms exhibit severe defects in vulva development and a mortal germline phenotype (Andersen and Horvitz, 2007).

Genomic studies performed in recent years using chromatin

immunoprecipitation followed by microarray hybridization (ChIP-chip) or sequencing (ChIP-seq) technologies provided genome wide maps of chromatin domains (Gerstein et al., 2010). The data revealed a strong anti-correlation between H3K36me3 and H3K27me3 that were shown to partition the genome into broad domains of one of the two marks (Gaydos et al., 2012). The H3K36me3 mark correlated with germline expression, while H3K27me3 domains contained mostly silenced regions. The domains appear to exist in dynamic opposition. The H3K27me3 domains were shown to encroach onto the germline genes if the H3K36me3 mark was lost due to *mes-4* depletion. This could potentially explain the *mes-4* sterility phenotype, as the H3K27me3 marks have repressive properties and could silence genes normally active in the germline.

The H3K36me3 domains were also shown to be anti-correlated to regions containing centromeric histone variant CeCENP-A as well as kinetochore protein KNL-2 (Gassmann et al., 2012). The model proposed in this paper suggests that germline transcription prevents recruitment of the centromeric proteins splitting dividing the genome roughly in half between centrosome bound and unbound regions, which allows efficient execution of holocentric division of chromosomes during mitosis.

1.4.3 H3K27me3

Tri-methylation on H3 lysine 27 is mediated by a complex that contains MES-2, MES-3 and MES-6 proteins (Bender et al., 2004). MES-2 and MES-6 are homologues of a conserved group of proteins belonging to the Polycomb Group Repression Complex 2 (PRC2); MES-3 is a worm specific protein. Similarly to *mes-4*, mutation in any of these components results in 'maternal effect sterility' (Bender et al., 2004; Capowski et al., 1991). H3K27me3 domains are spread out through entire length on the autosomes (showing no enrichment at chromosomal ends) and cover

much of the X chromosome, where they are slightly enriched relative to the autosomes (Gaydos et al., 2012). MES-2/3/6 proteins are expressed in the germline and during embryonic development proteins become enriched in the germ cells while gradually disappearing from the somatic cells (Holdeman et al., 1998; Xu et al., 2001). In the adult worms the only somatic cells where they were detected were those of the intestine. As mentioned before, the PRC2 complex in *C. elegans* participates in the repression of multicopy transgenes in the germline of the worm (Kelly and Fire, 1998). In somatic cells, during embryonic development, the repression is alleviated, consistent with the disappearance of the PRC2 components. In a paper published in 2014, Gaydos et al. elegantly demonstrated heritability of H3K27me3 modified histones during the early embryonic divisions of *C. elegans* (Gaydos et al., 2014). They crossed a PRC2 mutant mother to a wild-type male and observed that the paternal genome in a 1-cell zygote retained the modified histone marks. Importantly authors also showed that the deleted PRC2 component was not present in sperm. Therefore PRC2 was non-functional in early embryonic development. The modified mark persisted through subsequent embryonic divisions maintaining association with just one half of the genome and reducing intensity in each round until about the 24-cell stage where it eventually became undetectable. Similar dynamics were observed in an analogous experiment that used the H3K9me2 mark delivered through sperm to a mutant egg lacking the enzymes necessary for deployment of this mark. Therefore it appears that parental histones are indeed passed to the daughter strands after replication, at least within some heterochromatin (Gaydos et al., 2014). The same study showed that if a PRC2 mutant male was crossed to a wild-type mother no dilution was observed and the mark present on the maternal genome was efficiently maintained at high level throughout the embryonic cycles. The paternal genome delivered from a mutant male (and therefore lacking the

H3K27me3 mark) failed to acquire the mark throughout the course of embryonic development. This shows that during early embryonic divisions, PRC2 acts as a maintenance enzyme that 'fills in' the gaps in the chromatin domains after each round of replication. However, it is unable to target 'naïve' regions.

This, however, was not true for the other heterochromatin mark investigated. In an analogous experiment, a genome inherited from *met-2, set-25* parents that lacked any H3K9me2, re-acquired the mark already after the first cell division. It suggests that either pre-modified H3K9me2 histones were incorporated into the newly synthetized heterochromatin or the mark was established on unmodified histones incorporated during replication.

1.4.4 H3K9me2/3

H3K9 can exist in unmethylated, mono-, di- or tri-methyl states (Towbin et al., 2012). All methylated H3K9 states are enriched on chromosomal arms of the autosomes (Gerstein et al., 2010). Transposable elements and repetitive sequences show a high occupancy of methylated H3K9 consistent with their role in repressing these elements (Gerstein et al., 2010). Immunofluorescence analysis performed on dissected germlines revealed that H3K9me2 is present on the chromatin at all stages of germ cell development and that during meiosis it becomes highly enriched on unpaired X chromosomes (Bessler et al., 2010). H3K9me3 is detected in all regions of an adult male and hermaphrodite germlines but does not exhibit any sex chromosome enrichment (Bessler et al., 2010). However, high copy transgenes are highly enriched for H3K9me3, particularly if they contain soma-specific promoters, whereas H3K9me2 showed no enrichment on transgenes with somatic promoters and a strong enrichment on a transgene containing a ubiquitous *let-858* promoter, which also exhibited a mild enrichment in H3K9me3 (Bessler et al., 2010). In the

absence of MET-2, most of the H3K9me2 staining is lost along with the enrichment on chromosomes, but H3K9me3 levels are unaffected. Conversely, inactivation of *mes-2* reduced the level of H3K9me3 but not H3K9me2. The differential genetic requirement and localization of the H3K9me2 and H3K9me3 marks suggests that these two modifications are acquired independently (Bessler et al., 2010).

However, a study of H3K9 methyltransferases in embryonic development suggests a slightly different model of H3K9me2/3 acquisition (Towbin et al., 2012). The authors investigated the behavior of repetitive transgenic arrays in 50- to 100-cell stage embryos, where they are located at the nuclear lamina. They established that this positioning is dependent on redundant activity of the two H3K9 methyltransferases MET-2 and SET-25. Mutation of each alone caused an increase in expression of genes located at the array but the positioning to the lamina was not affected. The *met*-2, set-25 double mutant, however, exhibited a very penetrant loss of array lamina localization and an even greater increase in expression. Quantitative mass spectrometry analysis revealed that in *set-25* mutants all of the H3K9me3 is lost; this result was confirmed by immunofluorescence. The H3K9me1 and me2 marks were unaffected. In *met-2* mutants, the levels of all three H3K9 methyl marks were reduced and in *met-2, set-25* double they were lost entirely. The authors suggested that the two methyltransferases work in a stepwise manner. MET-2 was found to localize to the cytoplasm and was therefore proposed to add mono-, and di-methyl groups to lysine 9 of free H3 histones outside of the nucleus. The modified histones then shuttle into the nucleus and are incorporated into the heterochromatic regions, where the H3K9 becomes tri-methylated by SET-25. Consistent with this hypothesis, SET-25 was found to localize to the chromatin with a strong enrichment at the H3K9me3 rich transgene array. Using a DamID-based assay authors also

showed that in *met-2, set-25* mutants endogenous chromosome regions bound to the nuclear lamina decrease the strength of this association. Hence, MET-2 and SET-25 mediated methylation guides peripheral positioning of endogenous heterochromatin (Towbin et al., 2012).

met-2 as well as *set-25* single mutant worms are fertile with no obvious phenotypes. The double mutants are also fertile, but exhibits some degree of sterility at 25 °C (Garrigues et al., 2015). Similar to the balance between H3K36me3 and H3K27me3 methylation described above, the H3K9me2 mark is highly anti-correlated to H3K4me2 when measured at 1kb scale across the genome (Liu et al., 2011). Moreover, mutations that cause reduction in H3K4 methylation also result in increased H3K9me2 (Kerr et al., 2014) and the phenotype associated with *spr-5* (the gradual transgenerational increase of H3K4me2) was accompanied by a loss of H3K9me2 (Greer et al., 2014). If the *spr-5* mutation is combined with a *met-2* mutation, the fertility is lost immediately, instead of gradually over many generations (Kerr et al., 2014). This highlights that the interaction and balance between different methylation states is of vital importance to the physiology.

Apart from the antagonism between active and repressive chromatin states, histone marks can also complement and replace each other in some functions. One of the main roles of H3K27me3 repression is to repress genes on the X chromosome (Pirrotta, 2002). In the absence of PRC2 that deposits this mark, hermaphrodite and male worms fail to maintain this repression and become infertile. Interestingly, if a male worm inherited its single X chromosome from a male, the requirement for PRC2 in X repression is bypassed by the alternative mode of repression – through di-methylation of H3K9 (Gaydos et al., 2014). Recently, the H3K9me3 mark was shown to participate in a nuclear RNAi pathway (Buckley et al.,

2012; Burkhart et al., 2011; Gu et al., 2012). Genes targeted by exogenous and endogenous RNAi mechanisms were shown to acquire this mark concomitant with a decrease in expression (Buckley et al., 2012), suggesting that it mediates transcriptional silencing of those genes. This discovery is of great importance to the field of epigenetic inheritance as it links together two mechanisms, diffusible small RNA molecules and histone modifications. But to understand how it is integrated with the RNAi mechanism we must first explain a little bit about the RNA interference.

1.5 RNAi inheritance in C. elegans

In 1998 Mello and Fire reported that exogenously supplied double stranded RNA (dsRNA) triggers sequence-specific gene silencing in *C. elegans*, a phenomenon referred to as RNA interference (RNAi) (Fire et al., 1998). The process was soon observed to function in both plants and animals and gave biologists a powerful tool to study gene function (Lu et al., 2005). RNAi is believed to function as a defense system that prevents the spread of foreign nucleic acids derived from transposons and viruses (Rechavi et al., 2011; Robert et al., 2005) (Wilkins et al., 2005) and multiple genes required for RNAi are also required for efficient repression of multi-copy transgenes (Fischer et al., 2013; Grishok et al., 2005). Mutations in genes that function in RNAi also affect the expression of endogenous genes controlling proper development, fertility and aging (Billi et al., 2014). Soon after the discovery of RNAi it became apparent that the silencing of the targeted gene could – in some cases – persist for multiple generations in the absence of the initial dsRNA trigger that initiated the response (Grishok et al., 2000). Such long lasting effects (Vastenhouw et al. reported repression of a GFP transgene 80 generations post-injection) are more of an exception than a rule; only 13 out of 171 tested genes showed a heritable silencing RNAi response, in most other

cases the effect was observed in the F1 progeny, but not in the following generations (Vastenhouw et al., 2006). It is still unclear what determines that gene should experience a multigenerational heritable response to externally supplied RNAi, but germline expression of the affected gene seems to be a pre-requisite as all of the 13 genes mentioned above were germline-expressed (Vastenhouw et al., 2006).

1.6 Classes of small RNAs

C. elegans produces thousands of small RNAs that target coding genes, transposons, pseudo-genes as well as other non-coding RNAs (Billi et al., 2014). The small RNAs can be classified into distinct groups based on their biogenesis, expression pattern, structural features (such as length, sequence features and specific modifications) and function of the molecules.

1.6.1 miRNAs

MicroRNAs (miRNAs) were the first class of small regulatory RNAs to be discovered (Lee et al., 1993). They were originally identified in *C. elegans*, but were soon found to be present in both plant and animals, including humans (Pasquinelli et al., 2000). miRNAs are encoded endogenously and control multiple developmental process (Reinhart et al., 2000; Wienholds et al., 2005). Most of them derive from long precursors with a hairpin structure that is transcribed by Polymerase II (Lee et al., 2004). The pre-miRNA is processed in the nucleus, exported to the cytoplasm where it is cleaved by Dicer and then split into single stranded molecules that are mature miRNA molecules, typically about 22 nucleotides long (Ha and Kim, 2014). miRNAs are bound by Argonaute proteins and recognize their target mRNAs through imperfect base pairing (Bartel, 2009), this allows a single miRNA to regulate multiple targets (Jackson and Standart, 2007). Binding of miRNAs results in target

repression through one or many of the possible mechanisms: inhibition of translation initiation or elongation, premature translation termination, sequestration in P-bodies, mRNA decay or direct mRNA cleavage (Valinezhad Orang et al., 2014). Regulatory regions containing miRNA-binding sites are most commonly found in 3' untranslated regions (3' UTRs) (de Moor et al., 2005).

1.6.2 piRNAs

In *C. elegans*, piRNAs (also referred to as 21U RNAs) are characterized by a length of 21 nucleotides, a 5' uridine and association with two PIWI clade Argonautes, PRG-1 and PRG-2 (Bagijn et al., 2012; Batista et al., 2008). Unlike siRNAs whose biogenesis requires RNA as a template, *C. elegans* piRNAs are transcribed directly from genomic loci in the germline and are proposed to serve as a surveillance system against foreign transcripts (Bagijn et al., 2012). piRNAs are expressed in the germline and PRG-1 is required for normal fertility, particularly in the male gonad (Batista et al., 2008). Target recognition, although very poorly understood, appears not to require perfect sequence complementary allowing up to three mismatched nucleotides (Bagijn et al., 2012; Lee et al., 2012a). The function of piRNAs appears to be to survey the genome and silence expression of foreign elements such as transposons or viral particles in the germline (Lee et al., 2012a). In *Drosophila melanogaster*, piRNAs are amplified through a 'ping pong' mechanism (Brennecke et al., 2007). No evidence of such an amplification cycle has been found in *C. elegans* (Das et al., 2008). Instead, piRNAs trigger production of siRNAs that induce transcriptional and post-transcriptinal silencing of the targets (Das et al., 2008; Lee et al., 2012a; Luteijn et al., 2012).

1.6.3 26G endo-siRNAs

This class of endogenous small RNAs is defined by a characteristic length

of 26 nucleotides and a strong 5' guanosine bias (hence, 26G- RNAs) (Ruby et al., 2006). They have a 5' mono-phosphate and are enriched in the male and female germlines (Han et al., 2009). The biogenesis of 26G-RNAs is initiated by activity of the ERI (enhanced RNAi) complex that includes helicase, exonuclease, RNA dependent RNA polymerase and Dicer (Pavelec et al., 2009). The complex uses mRNA as a template to synthesize antisense molecules that are then loaded into a primary Argonaute (Vasale et al., 2010). In the female germline, this Argonaute is ERGO-1, while in the male germline the 26G RNAs are loaded to ALG-3 and ALG-4. The ERGO-1 class 26G RNAs are also highly abundant in embryos (Han et al., 2009; Stoeckius et al., 2014) and can be still detected during early larval development (Gent et al., 2010). Most targets of ERGO-1 bound 26G RNAs are genes expressed during embryonic and early larval development, suggesting a role in the regulation of these processes (Han et al., 2009). Many long non-coding RNAs, pseudogenes and non-annotated transcripts are also found among targets of ERGO-1 associated 26G RNAs (Han et al., 2009). The ALG-3/4 bound 26-G RNAs are essential for fertility at 25 °C and target genes involved in spermatogenesis (Conine et al., 2010; Conine et al., 2013). Both ERGO-1 and ALG-3/4 bound 26G endo-siRNAs initiate downstream production of much more abundant secondary RNAs (22G endo-siRNAs) (Vasale et al., 2010). The endo-siRNA pathway has only recently been discovered and many question remain unanswered. It is currently not clear what determines if a gene is targeted by the 26Gs or not. While the role of ALG-3/4 in maintenance of sperm gene expression across generations has been show, it is unclear if this mechanism can confer adaptation to the changing environment or mediate fine-tuning of gene expression in response to external stimuli.

1.6.4 Secondary siRNAs

This class of small interfering RNAs (siRNAs) encompasses various types of molecules that can be derived endogenously or originate from processing of externally supplied dsRNA. siRNAs associate with secondary Ago proteins and silence target mRNA post-transcriptionally in the cytoplasm (Doench et al., 2003; Gu et al., 2009; Tsai et al., 2015) and transcriptionally in the nucleus (Buckley et al., 2012).

1.6.5 WAGO 22G siRNAs

siRNAs are the most abundant class of small RNAs in *C. elegans* (Ruby et al., 2006) and they form a pathway on which other RNAi pathways converge. They are products of non-primed RdRP mediated synthesis triggered by primary siRNAs (Sijen et al., 2001), piRNAs (Shirayama et al., 2012), 26G-siRNAs (Sijen et al., 2007) and sometimes miRNAs (Correa et al., 2010). The name '22G-RNAs' is due to that they have a strong bias for guanosine nucleotide at the 5' end, have a typical length of 22 nucleotides and 5' triphosphate modification. Multiple endogenous siRNAs have been identified in worms through deep sequencing (Ruby et al., 2006). As mentioned above, 26Gs RNAs trigger production of siRNAs, but they are not their only source, as loss of 26G RNAs does not deplete all siRNAs (Gent et al., 2010; Vasale et al., 2010). Production of siRNAs is proposed to take place in perinuclear processing compartments called 'mutator foci' (Phillips et al., 2012). As the vast majority of siRNAs are complementary to the exonic regions, it has been suggested that only processed mRNA is used as template for amplification (Asikainen et al., 2008). WAGO 22G RNAs have been found associated to 12 secondary Argonautes (called WAGOs), which bind and stabilize the 22G RNAs and function redundantly to mediate silencing (Yigit et al., 2006). Simultaneous loss of all 12 Argonautes results in temperature sensitive sterility, high incidence of males and RNAi insensitivity (Yigit

et al., 2006). Two of these WAGOs, NRDE-3 and HRDE-1 shuttle siRNAs to the nucleus where they function in a nuclear RNAi pathway (Ashe et al., 2012b; Burton et al., 2011), covered in more detail below.

1.6.6 CSR-1 22G RNAs

Another class of 22G RNAs was described in *C. elegans* and is defined by their association with an Argonaute protein called CSR-1 (Claycomb et al., 2009). These siRNAs are of the same kind as the WAGO siRNAs: they have strong bias for a guanosine nucleotide at the 5' end, have a typical length of 22 nucleotides, 5' triphosphate modification and display perfect base pairing when compared to their putative target mRNAs (Claycomb et al., 2009). The CSR-1 22G-RNAs are antisense to most of the genes expressed in the germline (Claycomb et al., 2009). CSR-1 is one of the few *C. elegans* Argonautes that contains a catalytically active RNase H domain, which displayed an endonuclease activity in an *in vitro* assay (Aoki et al., 2007). Surprisingly, loss of CSR-1 did not lead to a significant decrease in the levels of target transcripts (Claycomb et al., 2009). Rather, in the absence of CSR-1, chromosomes fail to segregate properly during mitosis resulting in embryonic lethality (Claycomb et al., 2009). A similar phenotype was observed in *drh-3*, *ekl-1* and *ego-1* mutants, which all encode factors participating in biosynthesis of CSR-1 associated 22G-RNAs (Claycomb et al., 2009). The phenotype manifests at the formation of the metaphase plate, which becomes disordered when CSR-1 is compromised. One proposed explanation for this is that CSR-1 participates in maintenance of the broad heterochromatic and euchromatic domains (Cecere et al., 2014) in the germline, which were found to correlate with the attachment of centromeric histone variants (Gassmann et al., 2012). The CSR-1 pathway was also found to be required for maturation of replication-dependent histone mRNAs (Avgousti et al., 2012). Depleted histone pools in worms with a compromised CRS-1

pathway could explain the disordered metaphase phenotype.

CSR-1 protein and the associated 22G-RNAs are very abundant in sperm and are transferred to the zygote during fertilization (Conine et al., 2013). The CSR-1 22G RNAs present in sperm target spermatogenesis genes and also some oogenesis specific genes. Inheritance of the CSR-1 bound 22Gs from the father is essential for normally high expression of spermatogenesis and oogenesis genes in the offspring and in its depletion leads to sterility at high temperature (Conine et al., 2013). Therefore CSR-1 pathway mediate intergenerational transfer of germline expression program (Conine et al., 2013). The molecular mechanism through which CSR-1 exerts its functions is still unclear. We do know that CSR-1 associates with Pol II and promotes sense-oriented transcription at the target genes (Cecere et al., 2014). The CSR-1 22G RNA pathway also affects H3K9me2 distribution in the germline nuclei (She et al., 2009). Wild-type pachytene nuclei are characterized by strong enrichment of H3K9me2 on unpaired chromosomes and transgenes. In *csr-1*, *ego-1*, *drh-3* and *ekl-1* mutant worms, this enrichment disappears and the H3K9me2 becomes dispersed (She et al., 2009). Finally, recruitment of CSR-1 to mRNA transcripts protects them form being silenced by the nuclear HRDE-1 pathway (Seth et al., 2013; Wedeles et al., 2013) and can also activate them in *trans*, hence was named RNAa for 'RNAi induced epigenetic gene activation' (Seth et al., 2013). This property and evidence for it is described later.

1.7 Mechanism of RNA interference

Most of our knowledge about the RNAi pathway comes from studying the response to exogenously provided double stranded RNA. Various routes can be used to introduce the dsRNA into the organism. The most direct way is through microinjection directly into the gonad (Fire et al., 1998).

Alternatively, the dsRNA can be expressed in a bacterial host, which is then fed to the worm (Timmons et al., 2001). From the intestine, the double stranded RNA can spread to other tissues and induce a systemic silencing response (Winston et al., 2002). Cells rely on a transmembrane protein SID-1 to import dsRNA and trigger sequence specific silencing (Winston et al., 2002), whereas dsRNA export is a SID-1 independent process (Jose et al., 2009). Interestingly, import of extracellular dsRNA into the intestinal lumen does not require SID-1; instead, it requires a pH sensitive transmembrane protein SID-2 and is proposed to involve vesicular transport (McEwan et al., 2012).

Upon delivery to the germline, dsRNA becomes bound by RDE-4 that guides it to a complex that contains an RNAse III enzyme Dicer and an Argonaute protein RDE-1 (Tabara et al., 2002). Early experiments suggested that RDE-4 is essential for RNA interference, however a subsequent study showed that the requirement for RDE-4 could be overcome if the supplied trigger dsRNA was at a high concentration (Guo et al., 2013). Dicer cleaves the dsRNA into small 21-25 nt fragments that are called primary small interfering RNAs (siRNAs) and are characterized by two nucleotide long overhangs at each 3' end and a 5' monophosphate (Hammond et al., 2000; Tabara et al., 2002). The primary siRNAs bind to RDE-1, the primary worm Argonaute. One of the strands of the primary siRNA (called the passenger strand) is degraded through the RNAse H activity of RDE-1 (Steiner et al., 2009). The remaining 'guide strand' remains associated with RDE-1 and allows it to bind to an mRNA that contains a complementary sequence. A number of mechanisms for RNAi triggered post-transcriptional silencing have been described (Verdel et al., 2009). In flies and mammals the equivalent primary Argonaute to RDE-1 displays a strong slicer activity that results in cleavage of the target mRNA, which is sufficient to down-regulate the total expression level

(Miyoshi et al., 2005). Although *C. elegans* RDE-1 contains a slicer domain, it does not induce cleavage of the target mRNA (Steiner et al., 2009). The only function of the slicer domain in RDE-1 seems to be the maturation of primary siRNA through cleavage of the passenger strand within the RISC complex (Steiner et al., 2009). RDE-1 recruits an endoribonuclease RDE-8 to the target mRNA that might be involved in silencing (Tsai et al., 2015). In addition, upon binding to the mRNA target, RDE-1 engages an RNA-dependent RNA polymerase (RdRP) that catalyzes the production of secondary siRNAs (Sijen et al., 2007). Through unprimed RNA synthesis, using mRNA as a template, secondary siRNAs are produced (Sijen et al., 2007). These are the WAGO 22G secondary siRNAs described above. They are much more abundant than primary siRNAs and differ from them in that they are only of antisense polarity and have a triphosphate at the 5' end (Sijen et al., 2007). In *C. elegans,* secondary siRNAs as well as secondary Argonautes are essential to instill a silencing response suggesting that they are the true effectors of RNA interference mediated silencing in this organism (Yigit et al., 2006). How do the secondary siRNAs achieve the silencing?

1.7.1 Post-transcriptional gene silencing

The early studies on RNAi in *C. elegans* suggested that silencing is achieved through a post-transcriptional mechanism (Fire et al., 1998), since injection of double stranded RNA fragments complementary to the promoter and intron sequences did not result in interference (Fire et al., 1998). It is still unclear how the siRNA-mediated post-transcriptional silencing actually works. Direct mRNA cleavage by the secondary Argonautes is unlikely as most of them lack the residues required for slicer activity (Yigit et al., 2006). In fact, the only secondary Argonaute with demonstrated slicer activity is CSR-1 (Aoki et al., 2007), which does not participate in the silencing process. As mentioned above, a recently

discovered RDE-8 endoribonuclease that is recruited by RDE-1 to the target mRNA could be directly cleaving the target molecules (Tsai et al., 2015). Reducing mRNA stability through RDE-10/RDE-11 mediated de-adenylation could be another major mechanism through which the target mRNA is depleted (Yang et al., 2012).

1.7.2 Transcriptional gene silencing and heritable RNAi

The connection between RNAi and transcriptional repression was discovered in plants (Mette et al., 2000) as subsequently in fission yeast (Reinhart and Bartel, 2002; Volpe et al., 2002). RNAi induced transcriptional silencing was also suspected to occur in *C. elegans*. Many components of RNAi pathway (but not a core component: RDE-1) were shown to be required for silencing of transposons (Ketting et al., 1999) and other repetitive elements, including transgenes (Dernburg et al., 2000; Ketting and Plasterk, 2000). But this silencing was thought to occur at a post-transcriptional level based on measurements of pre-RNA and mRNA levels (Sijen and Plasterk, 2003).

Silencing of repetitive transgenes in the germline of *C. elegans* was shown to be stably transmitted to future generations (Kelly et al., 1997; Strome et al., 2001) and to require PRC2 complex proteins (Kelly and Fire, 1998) suggesting chromatin-based repression. The germline silencing process was also observed to be temperature-dependent with stronger repression observed at lower temperatures (Strome et al., 2001). Some silenced transgenes were shown to be enriched in H3K9me3 methylation (Bessler et al., 2010), a mark that was later linked to nuclear RNAi (Gu et al., 2012). In 2005 (Robert et al., 2005) found that germline silencing is lost in the absence of multiple chromatin and RNAi components, suggesting an RNAi-triggered mechanism of repression.

Somatic transgene silencing triggered by RNAi was documented by (Grishok et al., 2005) in response to feeding worms with dsRNA homologous either to the transgene or to the vector backbone of the transgene construct. The silencing was concluded to be transcriptional, as it resulted in reduced pre-mRNA levels, decreased association of RNA polymerase II and reduced histone acetylation at the transgene locus. The process requires exo-RNAi pathway components RDE-1, RDE-4 and RFF-1, as well as the heterochromatin protein 1 (HPL-2), PRC-2 components and several RNA binding porteins (Grishok et al., 2005). Unlike the heritable germline silencing, this RNAi triggered transcriptional gene silencing was not transmitted across generations.

An important link between RNAi and chromatin was established with the discovery of a nuclear RNAi pathway (Burkhart et al., 2011; Gu et al., 2012; Guang et al., 2010). This pathway consists of nuclear factors NRDE-1, 2 and 4 (Gu et al., 2012; Guang et al., 2010) and a secondary nuclear Argonaute protein which is represented by NRDE-3 in somatic cells (Burton et al., 2011) and HRDE-1 in the germline lineage (Buckley et al., 2012). Secondary siRNAs bound by the nuclear Argonautes NRDE-3 and HRDE-1 are shuttled from the cytoplasm to the nucleus (Buckley et al., 2012; Burton et al., 2011). There, guided by the sequence of the 22G RNAs, they induce transcriptional silencing at the locus where the complementary mRNA is being transcribed (Burkhart et al., 2011; Gu et al., 2012). Three hallmarks of transcriptional silencing have been reported: an increase in the repressive H3K9me3 mark at the chromatin locus (Burkhart et al., 2011; Gu et al., 2012), inhibition of RNA Polymerase II transcriptional elongation (Guang et al., 2010) and a decreased pre-mRNA levels of the target gene (Burkhart et al., 2011; Gu et al., 2012). It is currently not clear whether RNA Pol II inhibition is simply a result of H3K9me3 deposition at the locus, or if these two

processes occur independently.

Four routes for triggering nuclear RNAi-mediated transcription silencing have been described: endo-siRNAs, exo-siRNAs, piRNAs and mobile dsRNAs (Ashe et al., 2012b; Buckley et al., 2012; Burkhart et al., 2011; Devanapally et al., 2015; Shirayama et al., 2012). The first route for triggering the nuclear RNAi pathway is via endogenous siRNAs (Burkhart et al., 2011). The evidence comes from depletion of the H3K9me3 mark on several endo-siRNA target genes in the *nrde-1/2/3/4* mutants (Burkhart et al., 2011) as well as in *hrde-1* mutant (Buckley et al., 2012).

The second route for triggering the nuclear RNAi pathway is through the supply of exogenous dsRNA by injection or feeding (Burton et al., 2011; Gu et al., 2012). The H3K9me3 footprint was observed on multiple loci targeted by dsRNA. Depletion of nuclear RNAi does not impair the RNAi response in the animals directly exposed to dsRNA and they experience a penetrant knockdown (Buckley et al., 2012; Burton et al., 2011). However, the heritability of gene silencing is lost in nuclear RNAi mutants *nrde-1/2/4* and *hrde-1* (Buckley et al., 2012; Burton et al., 2011). Somatic Argonaute NRDE-3 is required for the silencing effect of the inherited RNAi detected in the soma of the F1 generation (Burton et al., 2011), but it was shown to be dispensable for long-term silencing in the germline (Buckley et al., 2012). It is unclear whether NRDE-3 is directly involved in inheriting the siRNAs or is simply a somatic effector Argonaute of siRNAs delivered from the previous generation by another Argonaute protein (Zhuang et al., 2013b).

An interesting feature of the nuclear RNAi pathway is the delay observed between the appearance of the siRNAs and the H3K9me3 marks (Burton et al., 2011; Gu et al., 2012). In the first generation exposed to dsRNA through feeding a build up of the antisense 22G RNAs can be detected

already after 4 hours and 24 hours later it reaches a maximum level (Gu et al., 2012). Meanwhile, virtually no H3K9me3 is observed in the first generation, even after 24 hours post feeding initiation. A robust increase in H3K9me3 is only observed in the following generations. Since the H3K9me3 modification does not appear until the F1 generation, it suggested that the siRNAs are the true carriers of the transgenerational RNAi silencing and that the H3K9me3 is reinstated in each generation (Gu et al., 2012). A delay in the two signals is also observed when the externally supplied dsRNA trigger is removed (Gu et al., 2012). The antisense small RNA pools are reduced already in the following generation and continue to drop each generation until the F3 when the levels are barely higher than in the naïve N2 worms. The H3K9me3 marks remain at the same level as in the previous generation and only start to drop in the F2 generation and at the F3 reach baseline level (Gu et al., 2012).

A similar phenomenon was observed by in the analysis H3K9me3 occupancy on several endogenous targets of HRDE-1 pathway (Buckley et al., 2012). When HRDE-1 was lost, the level of H3K9me3 did not drop abruptly, rather decreased progressively over the 6 subsequent generations. Simultaneously expression of those genes gradually increased at each generation. This demonstrates that on certain loci H3K9me3 can be inherited for a limited number of generations in the absence of HRDE-1. Therefore it is possible that the H3K9me3 modified histones and 22G RNAs are both inherited and regulate target gene expression across generations. Another experiment documented in the same study supports such a model. The authors crossed males with a robust *gfp* germline expression to hermaphrodites that carried the same *gfp* allele (linked to a recessive phenotypic marker) but that had been silenced by dsRNA supplied through feeding. In the F1 cross-progeny no GFP expression was

detected, suggesting that the paternal transgene was silenced in *trans*. In the F2 self-progeny, the worms that contained two copies of the maternally supplied *gfp* allele remained silenced. However, 100 % of the F2 worms that inherited at least one copy of the ancestrally active, paternal *gfp* allele showed expression (Buckley et al., 2012). One explanation for this is that epigenetic 'memory' of expression was maintained in *cis* on that allele.

A third route for triggering the HRDE-1 nuclear RNAi/chromatin pathway was discovered through the study of single copy transgenes and their expression in the *C. elegans* germline (Ashe et al., 2012b; Luteijn et al., 2012; Shirayama et al., 2012). The MOSCI technology (Frøkjær-Jensen et al., 2008) allows the insertion of single copy transcriptional or translational reporter at a precise position in the genome. Single copy reporters are thought to mimic very closely endogenous genes. They are not subject to the germline defense mechanisms targeted to repetitive sequences and therefore can be robustly expressed in germ cells (Frøkjær-Jensen et al., 2008). Despite this, some single copy transgenes, although initially expressed, become silenced after a number of passages (Shirayama et al., 2012). Recent studies found that this is due to the activity of a nuclear RNAi pathway triggered by the action of Piwi-interacting small RNAs (piRNAs) (Bagijn et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012). The pathway is triggered by the PRG-1 Argonaute/PIWI protein, which guided by a piRNA molecule, binds to a target mRNA (Ashe et al., 2012b; Luteijn et al., 2012; Shirayama et al., 2012). As mentioned before, piRNA target recognition does not require perfect base pairing, which combined with their immense variety allows them to target virtually any possible sequence (Bagijn et al., 2012). Binding of the PRG-1 is proposed to recruit RdRPs and trigger production of siRNAs. These bind to the HRDE-1 nuclear Argonaute that shuttles

them to nucleus instigating the transcriptional silencing pathway (Ashe et al., 2012b; Luteijn et al., 2012; Shirayama et al., 2012). This epigenetic process of germline silencing was named RNAe (Luteijn et al., 2012).

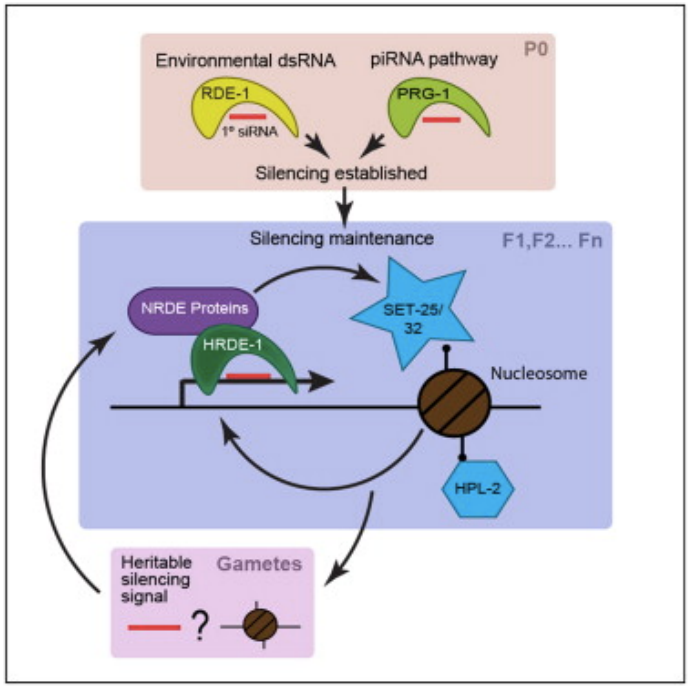

Figure 5. Nuclear RNAi and chromatin components control heritable epigenetic silencing in the germline. Silencing can be triggered either by ingested or injected double stranded RNA or through activity of piRNA pathway. Maintenance of the silenced state requires germline-specific nuclear Argonaute HRDE-1, nuclear RNAi components NRDE-1/2/4 and chromatin proteins SET-25, SET-32 (putative histone methyl-transferases) and HP1 homologue HPL-2. Reprinted from (Ashe et al., 2012).

An important feature of this pathway is that once epigenetic silencing is established, it can become independent of the 21U or dsRNA trigger (Ashe et al., 2012b; Devanapally et al., 2015). The maintenance of this repressed state across generations relies on putative H3K9me3 histone methyltransferases SET-25 and SET-32, heterochromatin binding protein 1 (HPL-2) (Ashe et al., 2012b) and the nuclear RNAi pathway

components NRDE-1/2/4 and HRDE-1 (Buckley et al., 2012; Gu et al., 2012). The maintenance of a silenced state is associated with continual production of small RNAs antisense to the reporter mRNA as well as enrichment in H3K9me3 at the transgene (Figure 5) (Ashe et al., 2012b; Luteijn et al., 2012; Shirayama et al., 2012).

A recent study showed that the nuclear RNAi components not only act as effectors of silencing but also mediate the production of 'tertiary' 22G RNAs (Sapetschnig et al., 2015). These tertiary 22G RNAs are biochemically indistinguishable from the secondary siRNAs that instigate their production. Furthermore, they can act themselves as triggers and initiate silencing on a complementary locus forming a positive feed-forward amplification loop (Sapetschnig et al., 2015).

Apart from the nuclear Argonaute HRDE-1, cytoplasmic Argonautes WAGO-1, WAGO-10 and WAGO-11 were shown to participate in silencing single copy transgenes in the germline (Shirayama et al., 2012). Other chromatin components, apart from SET-25, SET-32 and HPL-2, were found to be essential for the RNAe, such as the H3K36me3 enzyme MES-4 (Shirayama et al., 2012).

Finally, long-term germline gene silencing in *C. elegans* can also be triggered by production of dsRNA in somatic cells (Devanapally et al., 2015). Silencing of genes in the germline in response to somatic dsRNA requires the dsRNA channel SID-1 (Winston et al., 2002) and results in HRDE-1-dependent silencing of the region complementary to the dsRNA trigger (Devanapally et al., 2015). It is still unclear whether dsRNA is processed before transport to the germline. The transported molecules that putatively travel to the germline and induce the silencing are called mobile dsRNAs (Devanapally et al., 2015).

1.7.3 What is the inherited molecule?

Both small RNAs and chromatin marks are involved in the transgenerational maintenance of the repressed state in the germline. The key question is which of these is the inherited epigenetic signal that carries the memory of repression through the gametes to the next generation (Figure 5)? In a recent study (Sapetschnig et al., 2015) describe a genetic cross between mothers heterozygous for a silenced epi-allele, and fathers homozygous for an active transgene with a homologous sequence. In the resulting F1 progeny the paternally derived locus became silenced regardless of whether the maternal silenced epi-allele was inherited or not. This demonstrates that inheriting only the small RNAs is sufficient to mount the silencing response in the next generation (Sapetschnig et al., 2015). However, it does not exclude the possibility, that the chromatin state is also inherited and has influence on the expression of the associated allele.

1.7.4 Arms race of 22G RNAs

The vast diversity of genomically encoded piRNAs combined with their tolerance of up to three mismatching nucleotides allows them to target virtually any encountered sequence (Bagijn et al., 2012). This raises an important question: how do the endogenous genes evade PRG-1/2 triggered silencing? It appears that a parallel siRNA pathway acts to protect germline-expressed genes from the silencing activity of piRNA triggered nuclear RNAi pathway (Wedeles et al., 2014). At the heart of this 'protective' pathway lies the CSR-1 Argonaute which binds 22G-RNAs that guide it to the chromatin of germline expressed genes (Claycomb et al., 2009). Recent evidence suggests that 'CSR-1 has a direct effect on the Pol II complex and promotes its association with CSR-1 target loci through interactions of 22G-RNAs with nascent transcripts' (Cecere et al., 2014). Accumulation of CSR-1 associated 22G-RNAs

correlates with resistance of single-copy transgenes to silencing (Seth et al., 2013; Wedeles et al., 2013).

The CSR-1-associated 22G RNAs have been named 'licensing' RNAs and were shown to act in *trans* and induce de-repression of silenced constructs with a complementary sequence in a process called RNAa (Seth et al., 2013). The WAGO-associated 22G RNAs also act in *trans* to silence other transgenes (Sapetschnig et al., 2015; Shirayama et al., 2012). Therefore, after combining a silenced transgene with an active transgene with large stretch of sequence complementarity, either one becomes activated, or the other silenced after a few generations of co-inhabitance (Seth et al., 2013; Shirayama et al., 2012). It is unclear what determines the outcome. The 'licensing' and the 'silencing' populations are proposed to engage in 'arms race', where their relative abundance determines which way the balance will tip (Wedeles et al., 2014).

1.7.5 Competition between the RNAi pathways

Endogenous and exogenous RNA interference pathways operate through parallel mechanism making use of overlapping sets of protein components (Billi et al., 2014). This necessity to share common components was shown to result in 'competition' between the pathways for the shared elements such as the secondary Argonautes, Dicer or RdRPs (Sarkies et al., 2013; Zhuang and Hunter, 2012). In this way, the level of activity of one pathway can affect efficiency of another. Worms that are deficient in producing endogenous siRNAs experience an increased sensitivity to exogenously supplied dsRNA trigger (Kennedy et al., 2004; Pavelec et al., 2009; Simmer et al., 2002) and have enhanced miRNA efficacy (Zhuang and Hunter, 2012). On the other hand, subjecting animals to exo-RNAi results in increased expression of miRNA-regulated stage-specific developmental genes (Zhuang and Hunter, 2012). Similarly, in response to

viral infection that engages the RDE-1 Argonaute, the endogenous targets of the RDE-1 became partially de-repressed (Sarkies et al., 2013). Thus, a cross talk between the exo- and different endo-RNAi pathways is an inherent property of the system and might serve to coordinate different responses.

1.7.6 Small RNAs and multi copy transgene silencing

A deep-sequencing map of small RNAs extracted from worms carrying an integrated multi-copy transgene revealed abundant siRNAs targeting all segments of the integrated construct (Fischer et al., 2013). In the same study, a genome-wide screen identified 69 genes whose inactivation results in a stronger repression of the transgene (Fischer et al., 2013). Interestingly, about half of the hits encode proteins involved in endogenous RNAi pathways such as ERI-6, RDE-4, ERGO-1, DRH-3, and CSR-1. The majority of the tested hits also exhibited enhanced response to exogenous RNAi, which suggests that the exo-RNAi pathway has important role in silencing repetitive DNA fragments in somatic tissues. ERI-6 encodes a helicase required in the first step of production of endo-siRNAs that mostly targets recently duplicated genes (Fischer et al., 2011). Immunoprecipitation revealed that in *eri-6* mutants transgene chromatin is enriched in H3K9me3 and depleted in H3K4me3, both consistent with its lower expression compared to wild type. Paradoxically *eri-6* mutants also had a reduced pool of siRNAs targeting the multicopy transgene. To explain this, authors suggested that the transgene might be targeted by both CSR-1 and NRDE-3 associated siRNAs. The relative partitioning of siRNAs between the activating CSR-1 and silencing NRDE-3 pathway would determine the level of transgene repression, not the absolute levels of siRNAs. This balance might be distorted in *eri-6* mutants, which would explain the enhanced silencing of the transgene despite the overall lower level of siRNAs. According to this hypothesis,

the reduced level of transgene expression after depletion of CSR-1 is due to 'freeing up' the siRNAs from the CSR-1 pathway, which are reutilized by NRDE-3 pathway to enhance somatic silencing (Fischer et al., 2013). However, there is no direct evidence that transgene specific siRNAs associate with CSR-1, neither that siRNAs can be transferred form one secondary Argonaute to another.

1.8 Studies in yeast on heterochromatin inheritance

Much of our understanding of epigenetic inheritance comes from studies on heterochromatin dynamics in two yeast model systems: budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*. Below I summarize the current knowledge on establishment, maintenance and inheritance of heterochromatin domains in these two model organisms.

1.8.1 Heterochromatin dynamics in *Saccharomyces cerevisiae*

In budding yeast *S. cerevisiae* heterochromatin is present on silent mating-type loci, telomeres and rDNA repeats (Rusche et al., 2003). Three key components are required for the formation of silenced heterochromatin regions. First, DNA regions called 'silencers' present at the silenced locus constitute binding sites for two transcription factors such as Rap1 and Abf1 (Brand et al., 1985; Shore and Nasmyth, 1987). These in turn recruit the second class of regulators: the Silent Information Regulator proteins Sir1, Sir2, Sir3 and Sir4, that spread along the chromatin and inhibit transcriptional activity (Moazed, 2001). SIR proteins are directly involved in compacting the chromatin through self-association and histone deacetylation (Kueng et al., 2013). The third component required for silencing is the histone protein H4 whose acetylation state at lysine 16 is regulated by Sir2 (Imai et al., 2000; Johnson et al., 1992). Loss of any of these three elements leads to loss of heterochromatin and de-silencing of

the locus (Kueng et al., 2013). Deleting silencer region using site-specific recombination resulted in loss of silent state after a single cellular division (Cheng and Gartenberg, 2000) demonstrating that the state of chromatin cannot be replicated autonomously. Could the establishment of heterochromatin happen *de novo* after every cellular division with the silencers nucleating the process? This scenario is unlikely due to the observed epigenetic stability of the silenced domains as evidenced by the position effect variegation experiments (Yankulov, 2013), which showed that a reporter gene positioned near a silenced domain (such as telomeric region) can become silenced through the spreading of the neighboring heterochromatin domain (Aparicio et al., 1991). Both silenced and active states are stable over multiple generations with occasional switching. Therefore epigenetic state of the mother is copied to its daughters. A model that reconciles the requirement for the silencers and epigenetic memory was proposed (Moazed, 2011). During replication of the silenced state, deacetylated histones are distributed randomly to the daughter strands. Assembly of the heterochromatin state is nucleated at the silencer region and assisted by the deacetylated state of the histone tails, which was shown to increase affinity for binding SIR proteins (Liou et al., 2005; Onishi et al., 2007). In the active epigenetic state the histone tails are acetylated and have low affinity for SIR proteins. Therefore even if the silencer binding proteins are recruited, it is insufficient (with exception of rare 'stochastic' events) to establish a heterochromatic domain (Moazed, 2011).

1.8.2 Heterochromatin dynamics in *Schizosaccharomyces pombe*

The fission yeast *Schizosaccharomyces pombe* has played a pioneering role in understanding the biology of heterochromatin dynamics. In *S. pombe,* as in budding yeast, DNA binding proteins participate in the assembly of heterochromatin at telomeres and mating type loci (Kanoh et

al., 2005; Kim et al., 2004). Heterochromatin is also present at pericentromeric DNA repeats where small interfering RNAs (siRNAs) play the role of specificity factors (Volpe et al., 2002). The process of RNAi mediated transcriptional silencing has been a subject of intense studies leading to the identification of multiple proteins and bringing us closer to understanding the mechanism of heterochromatin inheritance (Allshire and Ekwall, 2015).

Contrary to the situation in *C. elegans*, where many RNAi factors are encoded by many, often redundant, paralogues, the genome of *S. pombe* encodes a single copy of Argonaute (*ago1*), RNA-dependent RNA Polymerase (*rdp1*) and Dicer (*dcr1*). Deletion of any of these genes results in loss of silencing at the pericentromeric repeats accompanied by a large reduction of the heterochromatin mark H3K9me3 (Volpe et al., 2002). Components required for directing RNAi-mediated heterochromatin formation assemble into three distinct complexes: the RNA-induced transcriptional silencing complex (RITS), the RNA-directed RNA polymerase complex (RDRC) (Motamedi et al., 2004) and Clr4-Rik1-Cul4 complex (CLRC) (Hong et al., 2005). The RITS complex is composed of the Argonaute Ago1, the chromodomain protein Chp1 and the GW domain protein Tas3 (Till et al., 2007; Verdel et al., 2004). Non-coding RNA transcribed from pericentromeric repeats act as a recruitment site for the RITS complex (Kato et al., 2005). This is mediated by sequence specific interaction with Ago1 bound small RNAs (Motamedi et al., 2004; Reinhart and Bartel, 2002). Chp1 stabilizes the interaction with the chromatin by binding to the methylated H3K9me histone tail (Verdel et al., 2004). The Ago1-bound long non-coding RNA becomes a template for the synthesis of double stranded RNA by the RDRC complex, which includes an RNA-dependent RNA Polymerase Rdp1, an RNA helicase Hrr1 and a member of the polyA polymerase family Cid12 (Motamedi et

al., 2004). Dicer cleaves the resulting dsRNA into siRNAs that are loaded into Ago1 (Noma et al., 2004), which then associates with other RITS components. This feed-forward loop provides a stable level of small RNAs that keep the heterochromatin silenced. Importantly, the RITS complex, once bound to the chromatin, recruits the multi-protein CLRC complex (Noma et al., 2004). The key element of that complex is Clr4, which deposits a heterochromatic mark on the chromatin by trimethylating H3K9. This mark serves as a binding site for the HP1 proteins Swi6 and Chp2 that promote heterochromatin formation through recruitment of additional chromatin modifying enzymes (Fischer et al., 2009; Sadaie et al., 2008). Whereas deletion of the Clr4 gene abolishes formation of heterochromatin, genetic removal of RNAi components Ago1 or Dcr1 causes an incomplete loss H3K9 marks at centromeric regions (Shanker et al., 2010). This supports the existence of an additional RNAi-independent mechanism for the establishment of centromeric heterochromatin. Because deletion of Clr4 impairs both heterochromatin formation and siRNA production (Motamedi et al., 2004; Noma et al., 2004; Shanker et al., 2010) it is believed that centromeric heterochromatin is maintained by a positive feedback loop between the methylation on H3K9 and the RNAi pathway.

Two recent studies (Audergon et al., 2015; Ragunathan et al., 2015) demonstrated that ectopically induced heterochromatin could be inherited across multiple mitotic divisions even after removal of the sequence-specific initiator. In one experiment, 10 % of cells retained repression 20 divisions after removal of the heterochromatin initiator (Ragunathan et al., 2015). This maintenance requires Clr4 with an intact chromodomain, HP1 proteins and the histone deacetylases Clr3 and Sir2 but not Ago1 or Dcr1. Moreover, deletion of a putative histone deacetylases Epe1 stabilizes the heterochromatin state and allows its transmission for over 50 generations

(Audergon et al., 2015; Ragunathan et al., 2015). These data suggest a read-write model of inheritance mediated by Clr4, which is able to bind as well as deposit H3K9me2/3 marks. Therefore, in yeast, histones can act as carriers of epigenetic memory, although the active removal of these modifications means that the memory is only retained over a limited number of generations in wild type organisms.

1.9 Epigenetic inheritance in animals and plants

1.9.1 Germline reprogramming in mammals

In plants and animals, that separate the germline cells from the somatic cells, an epigenetic change acquired during an organism's lifetime needs to be present in the germline, passed down to gametes through meiosis and after fertilization to resist epigenetic reprogramming processes that erase DNA and histone methylation marks from the two parental genomes. In mice and humans two major waves of epigenetic resetting take place during embryonic development, first one starts in the zygote right after fertilization and the second one is specific to primordial germ cells and happened few weeks later when they are specified and start to proliferate (Feng et al., 2010). During the preimplantation period that starts the first wave removes the epigenetic modifications acquired during the differentiation of gametes (Guo et al., 2014; Smith et al., 2014). Active demethylation strips the methylated cytosines form the paternal genome. Maternal genome methylation is lost more gradually in a passive, replication dependent process (Seisenberger et al., 2012). This epigenetic resetting is essential for restoration of pluripotency that is needed for the development of different tissues (Burton and Torres-Padilla, 2014). In both mice and man, some regions that evade this wave of demethylation have been identified, mostly in gene bodies (Guo et al., 2014; Smith et al.,

2014). In some cases these regions have been linked to intergenerationally transmitted phenotypes discussed later (Radford et al., 2014).

Studies in mice revealed a large asymmetry between the oocyte and sperm in terms of their chromatin content (Cantone and Fisher, 2013). Oocyte chromatin is rich in histone modifications acquired during oocyte growth such as H3K9me2 and H3K27me3. These marks are maintained in the zygote and the subsequent cell divisions (Cantone and Fisher, 2013). In sperm, most of the histones are replaced by protamines, which greatly increases compaction of the genome (Wykes and Krawetz, 2003). However, there are regions that resist this exchange and retain histones, including some promoters of genes expressed in development (Hammoud et al., 2009), allowing the potential transmission of epigenetic information encoded on the histone tails.

In humans, histones carrying the canonical constitutive heterochromatin mark H3K9me3 are retained on sperm chromatin and contribute to the formation of paternal embryonic heterochromatin (van de Werken et al., 2014). Maternally supplied chromatin modifiers (HP1 and histone methyltransferases Suv39h1 and Suv39h2) propagate the marks across early embryonic divisions (van de Werken et al., 2014). In mouse embryos, following fertilization, the paternal heterochromatin lacks the H3K9me3 modification (Puschendorf et al., 2008). Instead, Polycomb Group proteins reconstitute paternal heterochromatin through deposition of H3K27me3. The asymmetry between the H3K27me3-rich paternal genome and H3K9me3 rich maternal genome gradually disappears and around 8-cell stage H3K9me3 replaces H3K27me3 on paternal genome (Puschendorf et al., 2008). Thus, humans and mice appear to differ in this early stage of development.

Primordial germ cells (PGCs), which in mice form between the 6th and 7th day of embryonic development, underdo a second wave of genome demethylation as they migrate and colonize the genital ridge between 8th and 13th day of embryonic development (Guibert et al., 2012). Repression of DNA methylation and continuous DNA replication results in dilution of the mark (Seisenberger et al., 2012). This loss is accompanied by the activity of TET enzymes that hydroxidize methyl groups (Hackett et al., 2013; Yamaguchi et al., 2013) allowing their subsequent removal. Meanwhile, chromatin undergoes reorganization through depletion of H3K9me2, increase in H3K27me3 and X-chromosome reactivation, which brings the germ cells to the 'basal' epigenetic state at approximately 14th day of embryonic development. Some regulatory elements were shown to escape the 2 reprogramming waves and remain methylated in PGCs, therefore are potential carriers of transgenerational epigenetic inheritance (Hackett et al., 2013). These include so-called imprinted genes, which are differentially methylated depending on the gamete of origin (DeChiara et al., 1991; Wood and Oakey, 2006). This methylation is maintained at the locus throughout the development keeps it repressed. As a result, the gene is expressed from only one parental allele (Wood and Oakey, 2006). The majority of differentially imprinted genes in mammals function during embryonic development, but some are expressed also in adult tissues, including brain (Wood and Oakey, 2006). The mechanism through which the imprinted regions evade demethylation remains poorly understood. Some studies found evidence that histone modifications and maternally supplied *trans* factors cooperate to protect the imprinter regions from demethylation (Li et al., 2008; Quenneville et al., 2011). In mature sperm, some of the imprinted genes are enriched for H3K9me2 (Nakamura et al., 2012), suggesting that inherited histone modifications might be involved in shielding the region from demethylation. Other regions that resist methylation in both maternal and

paternal regions include retrotransposons (Lane et al., 2003), and repetitive regions at the centromeric heterochromatin (Santos et al., 2002).

1.9.2 Germline reprogramming in Plants

In flowering plants germline is formed from post-embryonic stem cells in shoot and floral meristems. These progenitor cells can be considered as part of the soma as they also give rise to somatic cells of leaves and branches, unlike the germline in most animals that becomes clearly separated form the soma during embryonic development. For this reason epigenetic modifications acquired during plant growth can easily make their way to the germline. DNA demethylation takes place during germline development, but it is limited only to asymmetric cytosines in sperm cells, while methylation on symmetric sequences, such as CpG, remain mostly intact (Calarco et al., 2012; Ibarra et al., 2012). After fertilization, small RNAs guide restoration of of DNA methylation on the asymmetric cytosines (Calarco et al., 2012; Ibarra et al., 2012). Chromatin is extensively remodeled in the pollen with special H3 histone variants and loss of many methylation marks (Ingouff et al., 2007; Schoft et al., 2009). Both gamete types in plants contain companion cells that do not contribute genetically to the next generation but act as vegetative support for the gametes and zygote. Epigenetic reprogramming in the companion cells leads to reactivation of transposons. This results in accumulation of small RNA in the gametes that reinforce imprinting and silencing of transposons in the germline genome (Slotkin and Martienssen, 2007).

1.9.3 Transgenerational Epigenetic Inheritance in Plants

Barbara McClintock discovered the first example of epigenetic inheritance during her work on transposons in plants. She observed that in maize some transposons can switch between an active and silent state and that this state exhibits remarkable transgenerational stability and a potential to

affect expression of nearby genes (McClintock, 1961). It took several decades to discover that DNA methylation is responsible for the expression state and its inheritance (Chandler and Walbot, 1986; Dennis and Brettell, 1990; Lisch, 2012). A trans-acting silencing mechanism was also first observed in plants, although it was not known at the time that RNAi mediates it. Jorgensen and colleagues introduced extra copies of a gene responsible for dark pigmentation in petunia attempting to make the flowers darker. Instead, they obtained bright white flowers and detected that the endogenous pigmentation gene was silenced (Napoli et al., 1990). The process was named co-suppression and we now know that it is be mediated by small RNAs (De Paoli et al., 2009). Another example of transgenerational gene silencing in plants is a process called 'paramutation'. It was observed in the 1950s in maize (Brink, 1956; Coe, 1959) in studies on three loci that determine the pigment of corn kernels. A silenced state of these alleles can affect and silence active alleles when they are introduced into the same organism through breeding. A trans-silenced allele remains silent for many generations, and can itself silence a homologous active allele (Brzeski and Brzeska, 2011). Various other examples of paramutation have been discovered in plants (Arteaga-Vazquez and Chandler, 2010). Genetic screens in maize and *Arabidopsis* identified multiple RNAi and chromatin related genes required for the process of paramutation including RNA-meditated RNA Polymerase (*mop1)*, DNA methyltransferase (*met1*) and histone deacetylases (*hda6*) (Eun et al., 2012). A powerful demonstration of the importance of epigenetic inheritance in plants comes from studies on epigenetic recombinant inbred lines (epi RILs) (Cortijo et al., 2014; Johannes et al., 2009; Mirouze et al., 2012). These are made through genetic crosses between genetically identical (or extremely similar) plants that have a very different epigenetic state. First, a plant carrying a mutation in a DNA methylation enzyme *ddm1* (hence a severely hypomethylated genome) is

crossed to a wild-type plant with a normally methylated genome. The resulting F1 'epi-heterozygous' is backcrossed to a wild type to remove the *ddm1* mutation and then inbred for six generations to generate the epiRILs. Phenotyping and epigenetic profiling of those lines revealed stable inheritance of many epigenetic states affecting various traits such as root length or flowering time (Johannes et al., 2009). Through a quantitative genetics approach, it is estimated that differentially methylated regions account for up to 90 % of the observed heritability of gene expression (Cortijo et al., 2014).

1.9.4 Studies in flies

One of the first evidences for epigenetic control of gene expression came from studies in flies performed by Muller in 1930 (Muller, 1930). He described an X-ray induced chromosomal rearrangement that changed the position of the *white* allele, which affects the color of the eyes. The resulting phenotype was a fly with 'mottled' eye color with some white and some red patches, different in each eye and each individual. This, apparently stochastic effect on the gene activity that depended on its location in the chromosome was called position effect variegation (PEV) (Muller, 1930). Subsequent studies showed that the effect is due to 'spreading' of silencing heterochromatic domain to the neighboring euchromatic domain (Lewis, 1950). The strength of the effect on the allele depended on its distance to the heterochromatic domain (Demerec and Slizynska, 1937). Temperature at which the flies were reared was shown to have a large impact on the variegation strength (Gowen and Gay, 1934). Higher temperature suppressed variegation and lower temperature enhanced it (increased phenotype severity). The penetrance of PEV phenotype is also dependent on the total amount of heterochromatin in the genome (Spofford, 1967). Increasing global amount of heterochromatin in the genome suppressed the PEV phenotype, which suggested that there is

a limit to how much heterochromatin can be maintained in a cell. Screens for mutations that either enhance (*e(var)* - *enhanced variegation*) or suppress (*su(var)* - *suppressed variegation*) the PEV phenotype identified large number of genes involved in chromatin organization (Girton and Johansen, 2008). These include H3K9 methyltransferase Su(var)3-9 (Schotta et al., 2003), Su(var)2-5/HP1 H3K9me-binding protein (Eissenberg et al., 1990; James and Elgin, 1986) and HDAC1/RPD3 histone deacetylase (De Rubertis et al., 1996; Mottus et al., 2000). A tremendous amount of insight into chromatin biology was achieved trough work on the PEV strain and it is still in progress as molecular function of many of the genes that modify the PEV phenotype is unknown.

Evidence for epigenetic mode of inheritance in flies was shown in a study on the Fab-7 DNA element that is recognized and modified by Polycomb Group proteins (Cavalli and Paro, 1998). When positioned next to mini-*white* allele it induces silencing, which results in modified eye color. However, the silencing can be relieved by ectopically triggered transcriptional activity at a neighboring locus during early embryonic development. This activated state was shown to persist mitotically throughout development as well as meiotically to the following generations in the absence of the initial trigger (Cavalli and Paro, 1998).

Another study used a PEV line to demonstrate heritability of heterochromatin state in flies (Seong et al., 2011). In response to heat shock or osmotic stress, dATF-2 transcription factor becomes phosphorylated and is resealed from heterochromatin. This leads to heterochromatin disruption that can be monitored by the eye color but was also demonstrated through molecular signatures such loss of heterochromatin element HP1 and H3K9me2 from the affected locus. The stress-induced loss of heterochromatin is transmitted to the next

generation. When stressed male flies were mated with non-stressed females evidence of de-silencing was observed in the eyes of the male progeny. Since the white allele lies on the X chromosome it must have been inherited from the female. This type of non-Mendelian transmission is reminiscent of 'paramutation' observed in plants and could be due to transfer of diffusible molecules such as siRNAs. The study also shows that if the stress is repeated over several generations, heterochromatin loss becomes gradually more severe and requires 3-4 generations to return to basal state in stress-free conditions (Seong et al., 2011).

Heterochromatin loss can also be triggered by dietary factors (Ost et al., 2014). Male flies that were fed with high glucose diet developed syndromes of obesity such as high tri-glyceride content in their body fat and de-regulation of metabolic genes. These symptoms were also observed in their F1 progeny. Interestingly, a glucose-poor diet in fathers also resulted in the offspring to develop obesity. Diet-induced de-silencing of heterochromatin domains defined by H3K27me3 and H3K9me3 in male sperm and stable transfer of that state through fertilization and somatic development appears to be the underlying mechanism. This is supported by requirement of heterochromatin modifiers PcG and Su(var)3-9 (responsible for deposition of H3K27me3 and H3K9me3) for the intergenerational metabolic reprogramming (IGMR). None of the phenotypes were transmitted to the subsequent F2 generation, meaning that the effect is intergenerational rather than transgenerational. Importantly, mRNA expression signatures of paternal-diet-induced obese mice defined by the overexpression of heterochromatin-embedded metabolic genes were also found in data from genetically controlled human obesity studies (Ost et al., 2014). This suggests a parallel mechanism of intergenerational gene regulation between flies and humans.

1.9.5 *C. elegans* studies

Several examples of transgenerational inheritance of a trait have been described in *C. elegans*. Studying epigenetic silencing of single copy transgenes and exo-RNAi triggered silencing (described in previous chapters) led to discovery of nuclear RNAi pathway and its role in epigenetic inheritance. Many components of that pathway, including *hrde-1, nrde-1, nrde-2* and *nrde-4* exhibit Mrt phenotype (progressive increase in sterility across generations) when grown at high temperature (Buckley et al., 2012). These defects are rescued when crossed back to wild-type background, which argues against *de novo* genetic abnormalities as an explanation.

Multi-copy transgenes, which are typically silenced in the germline (Boulin et al., 2006), can display quantitative heritable effects (Arico et al., 2011; Sha and Fire, 2005). The gamete through which the somatic *unc-119p::gfp* and *unc54p::gfp* reporters are inherited to the offspring was shown to influence the somatic expression with sperm passage resulting in up-regulation of the transgene in the inheriting progeny (Sha and Fire, 2005). Multiple passages of the transgene through the same germline required several consecutive generations to fully reset when shifted to the opposite germline. Moreover, (Rechavi et al., 2011) showed stable multigenerational silencing of virally derived *FR1gfp* transgene. The initiation of silencing required *rde-1*. Once silenced, transgene could be propagated in a silenced state for up to 4 generations in the absence of *rde-1*. Transgene silencing was accompanied by production of small RNAs (20-30 nt) antisense to the GFP and was shown to act in *trans*.

While most of the above studies utilized exogenous genetic elements to induce and/or monitor the change in gene expression, two recent studies in *C. elegans* showed that environmental perturbation can elicit a heritable

response that involves endogenous small RNAs and their cognate targets (Rechavi et al., 2014; Schott et al., 2014). Starving the worms at L1 stage for 6 days resulted in quantitative differences in transcriptome and small RNA composition of worms when they were grown to adulthood and compared to non-starved animals (Rechavi et al., 2014). Many upregulated small RNAs were transmitted for at least three consecutive generations. The targeted transcripts were enriched in genes associated with nutrition and correlate with their decreased expression. The majority of the inherited small RNAs were depleted in *rde-4* and *hrde-1* suggesting that biogenesis of these molecules is dependent on RDE-4 and their transgenerational transmission on HRDE-1 (Rechavi et al., 2014). In addition to an altered small RNA composition, the F3 progeny of starved worms also exhibited an extended lifespan (Rechavi et al., 2014).

Another study showed that rearing worms at elevated temperature resulted in changes in the transcriptome of a 4-cell embryo that were transmitted transgenerationally (Schott et al., 2014). For at least 20 genes, the expression change observed at 25°C was maintained after shifting the worms back to 20 °C for at least 1 subsequent generation. Transgenerationally affected mRNAs were enriched in PRG-1 and HRDE-1 targets. Follow-up analysis of two candidate genes revealed significant expression differences up to 2 generations after the shift. *B0286.1* transcript was decreased at 25 °C and expression increased gradually over three generations when shifted to 20 °C. Authors measured expression of one of the small RNAs antisense to that transcript and found an anticorrelated profile, with high abundance at 25 °C and gradual increase after shift to 20 °C. The second gene, *K10B3.5*, had opposite dynamics, with increased mRNA at 25 °C that drops gradually over three generations at 20°C. Here too, expression of a small antisense RNA

molecule showed an anticorrelated profile. The transgenerational memory of transcript expression was dependent on germline RNAi component *mut-2* and was inherited only through female germline (Schott et al., 2014).

In addition to studies on gene expression, various *C. elegans* phenotypes appear to depend on its ancestral environment. Apart from already described genetic and environmental perturbations affecting longevity of the future generations (Greer et al., 2011; Rechavi et al., 2014), there is evidence for transgenerational component in adaptation to odor (Remy, 2010) and stress resistance (Tauffenberger and Parker, 2014). Exposure to an odor cue in early larval development desensitizes worms to the odor in adulthood. Interestingly, this altered behavior can be transmitted for over 40 generations in the absence of the odorant indicating remarkable stability of an acquired trait (Remy, 2010). Although the mechanism of this inheritance has not been demonstrated, another study found that odor adaptation process is mediated by somatic nuclear RNAi component NRDE-3 and downstream chromatin component HPL-2 (Juang et al., 2013). High glucose content in *C. elegans* diet resulted in decreased fecundity and increased resistance against cellular stress and neurodegeneration in 2 subsequent generations (Tauffenberger and Parker, 2014). This effect was dependent on the presence of H3K4me3 components *set-2* and *wdr-5.1*.

It is still unclear how prevalent epigenetic inheritance is in *C. elegans* and to what extend does it influence its ability to adapt to changing environment. Similarly, the exact mechanism of this epigenetic inheritance remains poorly understood. In some cases chromatin modifying-enzymes were suggested to mediate the transfer of the modified phenotype across generations (Greer et al., 2014; Tauffenberger

and Parker, 2014), while other studies point to small RNAs as the inherited signal (Rechavi et al., 2011; Sapetschnig et al., 2015; Schott et al., 2014). Teasing out the relationship between the two mechanisms and their actual contribution to the process is of paramount importance and remains a subject of debate in the field (Sarkies and Miska, 2014).

1.9.6 Studies in mammals

Several studies in rodents demonstrated that exposure to stressful environment, either chemical or behavioral, can have detectable effects in subsequent generations (Szyf, 2015). Heritable epigenetic modifications at the *Agouti* locus in mice were one of the first demonstrations of a non-genetic type of inheritance in mammals (Morgan et al., 1999). An intracisternal A particle (IAP) murine retrotransposon inserted upstream of the transcription start site of the *Agouti* gene can induce its ectopic transcription leading to multiple abnormalities such as yellow fur, obesity, diabetes and increased susceptibility to tumors (Blewitt and Whitelaw, 2013). This modified phenotype exhibits high variability among pups of the same litter and can be transmitted through the maternal lineage to the offspring. Molecular analysis showed that the heritable transcriptional changes at the *Agouti* locus are associated with changes in the DNA methylation state of the gene and the neighboring retrotransposon, which escapes efficient reprogramming of the genome during early development (Blewitt et al., 2006).

Mice subjected to chronic variable stress during pregnancy sire male offspring with a stress-sensitive and dysmasculinized phenotype, which is partially transmitted to the following generation (Morgan and Bale, 2011). Analysis of the affected F2 male progeny showed decreased expression of three miRNAs that control hormonal homeostasis. Transmission of the phenotype to the F3 was not demonstrated; therefore the effect might be

due to a direct *in utero* exposure.

A certain type of paramutation, similar to what is observed in plants (Arteaga-Vazquez and Chandler, 2010), was reported in mice (Rassoulzadegan et al., 2006). Homozygous wild-type progeny of heterozygous *Kit(tm1Alf/+)* mothers showed a variable level of the phenotype characteristic of the *Kit(tm1Alf)* mutation despite inheriting two wild-type copies of the *Kit* gene. The observed phenotype was associated with decreased level of Kit mRNA and could be recapitulate if RNA isolated from the *Kit(tm1Alf/+)* mice was injected into wild-type fertilized eggs (Rassoulzadegan et al., 2006). RNA methyltransferase Dnmt2 is required maternally and paternally to mount the effect of paramutation on the locus, and no DNA methylation changes were observed, supporting the role of RNA as carrier of paramutation (Kiani et al., 2013). The paramutated locus can be propagated for at least four generations in the otherwise wild-type background (Yuan et al., 2015).

A behavioral response might also be transmitted epigenetically to subsequent generation (Dias and Ressler, 2014). Male mice were conditioned to associate a specific odor with electric shock. This generated an altered response to future exposure to the same odor. Interestingly, the F1 and F2 offspring of the F0 conditioned mice also exhibited an altered behavioral sensitivity to that odor, even though they themselves had never smelled it before the behavioral test. Both the F1 and the F2 generations showed anatomical differences in olfactory regions that are activated by the used odor. The *Olfr151* gene encoding an olfactory receptor specific to the used odor was hypomethylated in sperm from conditioned F0 males and their F1 progeny. These changes where not observed in the olfactory tissues in F1 and F2 progeny. Through *in vitro* fertilization and cross-fostering experiments, authors confirmed that

the effect was passed through gametes.

One of the key studies in support of existence of transgenerational inheritance demonstrated that exposure of pregnant rats to an endocrine disruptor resulted in decreased male fertility observed in four subsequent generations (Anway et al., 2005). Reproduction defects correlated with altered DNA methylation patterns in the germline suggesting a possible mechanism of epigenetic transmission to subsequent generations (Anway et al., 2005). However, the transgenerational effect of endocrine disruption was not reproduced when a different strain of inbred rats was used (Schneider et al., 2008). A more recent study (Iqbal et al., 2015) investigated in more detail the effects of chemical disruption of the endocrine system in mice and no evidence for transgenerational inheritance. Alterations caused by the inhibitor were detected in the germline of the G1 animals. However, progenitors of the G1 germline were already formed while the G1 animal was a fetus and present in the pregnant mother exposed to the toxins. Examination of DNA methylation and mRNA expression pattern in the G2 germline revealed no significant difference to the control animals demonstrating that germline reprogramming mechanism had reset the acquired changes.

There is also evidence for paternally heritable effect of malnutrition in rodents. Male mice fed with a low-protein diet sired offspring with an elevated level of cholesterol and altered gene expression profile for multiple genes involved in lipid and cholesterol biosynthesis (Carone et al., 2010). This was accompanied by numerous changes in DNA methylation in the hepatocytes of the affected F1 offspring. Similarly, undernourishment in pregnant mice resulted in metabolic defects detected in F2 progeny (Radford et al., 2014). In utero nutritional environment of male F1 embryos resulted in hypomethylated DNA at specific loci

detected in the germline of the F1 as they became adults. Many of the hypomethylated regions were found at the regions that resist histone-to-protamine exchange during spermatogenesis, suggesting that chromatin based inheritance mechanism might be involved in the transmission of the altered DNA methylome. Some of the hypomethylated regions were still detected in the F2 early embryos but the differences were lost entirely in differentiated tissue. Despite that, many of these regions remained differentially expressed, possibly due to defects in early development (Radford et al., 2014).

Numerous epidemiological studies in human populations show supporting evidence for intergenerational effects of environmental stress, such as increased alcohol consumption, nicotine intake, exposure to pollutants and malnutrition (Nilsson and Skinner, 2015). One example comes from the study that examined descendants of mothers pregnant during the Dutch famine at the end of the Second World War (Veenendaal et al., 2013). Individuals exposed to famine in utero had a decreased level of glucose tolerance, higher body mass index, elevated cholesterol and higher risk of developing schizophrenia later in life (Lumey et al., 2011). Whole genome-wide methylation profiling on the blood samples form the adults exposed to famine *in utero* found multiple hypomethylated regions especially at genes expressed in early development (Tobi et al., 2014). Some evidence for transgenerational effect was also reported for the Dutch famine ancestors as F2 offspring of *in utero* famished F1 mothers had increased neonatal adiposity and generally poorer health later in life (Painter et al., 2008; Veenendaal et al., 2013). It is to conclude a true transgenerational transmission in such studies as multiple confounding factor come into play. For example, children that were exposed to the Dutch famine while in utero were found to prefer fatty foods later in adulthood and had a more atherogenic lipid profile (Lussana et al., 2008),

which could in turn affect their progeny through an intergenerational mechanism.

The vast majority of phenomena described in mice models and epidemiological studies in human fall under the category of intergenerational rather than transgenerational effects (Whitelaw, 2015). There is little doubt that events happening during neonatal development are of critical importance and disruptions caused by hostile environment can ripple through to adult life and modify the phenotype. As most studies in mice and men are correlative it is impossible to exclude that some of the observed epigenetic changes correlated with altered phenotype in F1 or F2 have protective or buffering role rather than being the cause of the phenotype. Despite the isolated few examples of transgenerational transmission of environmentally induced epigenetic changes in mammals, most of the literature does not support transgenerational transmission, most likely due to efficient germline reprogramming system. Further investigations in large, controlled human cohorts and mechanistic dissection of the reported transgenerational phenomena in model organisms will be needed to gain a more profound understanding of epigenetic inheritance, which is of great importance to human health.

1.15 Summary

Transgenerational effects appear to be common in plants where stable epi-alleles can significantly affect organism's phenotype. The fact that they are usually associated with transposable elements, viruses or transgenes suggests that they might be a byproduct of germline defense system. The few examples of transgenerational inheritance in mammals demonstrate that such events are possible, however they appear to be exceptions and the governing mechanisms remain obscure and many key questions unanswered. How do loci evade epigenetic reprogramming? How is the

epigenetic information propagated across generations? Are histones involved? Can is epigenetic change sensed by somatic tissues be communicated to the gamete? What is the role of RNA and which RNA molecules can confer epigenetic inheritance?

Research in the model organism *C. elegans* has already thought us much about the mechanisms of epigenetic inheritance, yet we are still far from comprehensive understanding of this phenomenon. Very few studies so far have reported transgenerational effects triggered by an environmental cue and provided a limited mechanistic explanation for the phenomena. It is not unclear how the environmental stimulus is transmitted into a heritable response, nor whether there is any transfer of epigenetic information from the soma to the germline involved. The relative contribution of the *cis* and *trans* acting molecules in the inheritance process is also an unknown. It is possible that the basic mechanisms governing environmentally triggered epigenetic inheritance in *C. elegans* have parallels in higher organisms including humans. But even if they turn out not to, it remains a fascinating area of research with far-reaching implications for ecology and evolution.

2. Results

PART I

2.1 Heritable changes in transgene expression

2.1.1 Temperature-induced changes in the expression of multi-copy transgenes are inherited for up to 15 generations

When cultivating *C. elegans* strains carrying integrated multi-copy transgenes expressing either mCHERRY or green fluorescent protein (GFP) under the control of the *daf-21* promoter at different temperatures, we noticed that expression of the transgenes was increased at high temperature (Figure 6).

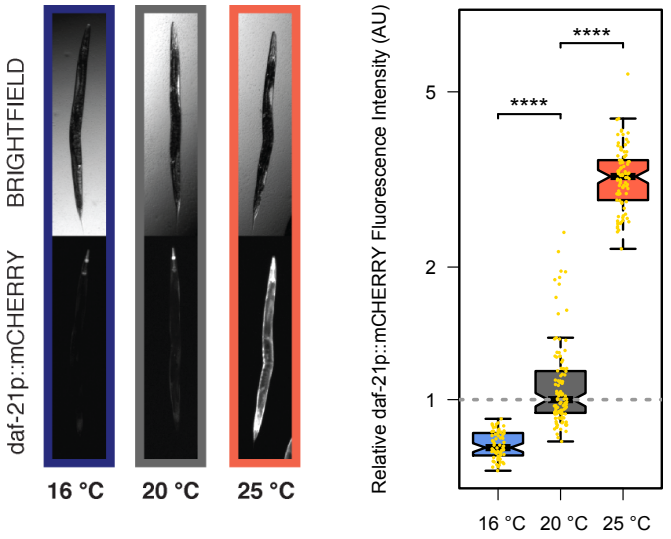

Figure 6. Expression of the *daf-21p::mCHERRY* integrated multi-copy transgene increases at high temperature. Quantification of reporter expression in adult worms grown for >20 generations at 16, 20 or 25 °C. Each yellow dot represents one worm. Worms grown at 20 °C are 1.29 times brighter than those at 16 °C and 3.21 times dimmer than those at 25 °C. P values: **** p< 0.0001 (Wilcoxon rank test). Y axis in log scale.

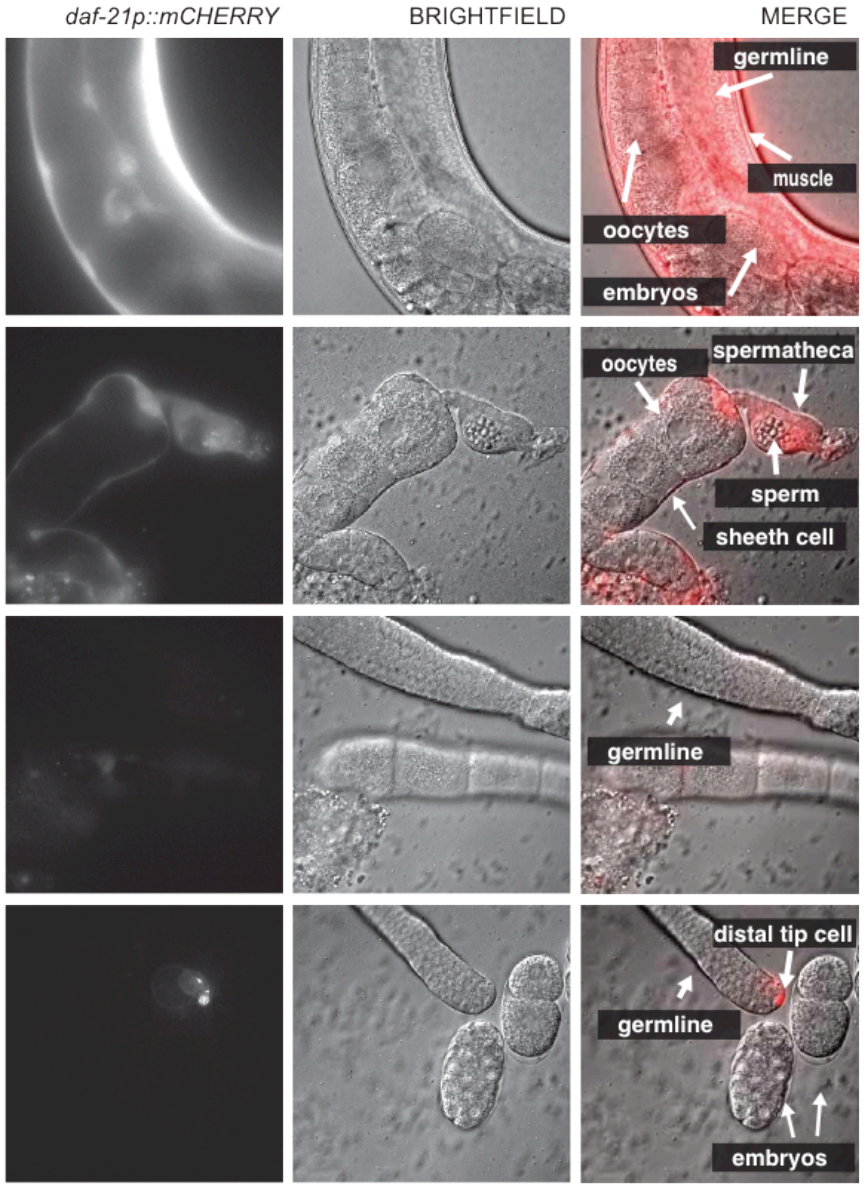

Figure 7. The *daf-21p::mCHERRY* integrated multi-copy transgene is not expressed in the germline. Florescent signal in the emission spectrum of the mCHERRY protein was detected in somatic cells including the somatic cells that support development of the gonad (spermatheca, distal tip cell, sheath cells) but not within the germline or the gametes. Worms carrying the *daf-21p::mCHERRY* transgene shifted from 20 to 25 °C for 2 generations were used for this experiment.

To investigate the dynamics of the response to temperature, we shifted animals from 16 to 25°C as embryos and quantified expression in adults in the same generation (P0) and subsequent ones (F1-F6). At 25°C the transgene exhibited a progressive increase in expression over consecutive generations before reaching a stable plateau in the third generation (Figure 8). We repeated this experiment starting from a population grown at 20 °C and observed similar results: progressive increase in transgene expression with a peak at 3rd generation followed by stabilization (Figure 9). Shifting the animals back to a lower temperature (20°C) after multiple generations at 25 °C resulted in a gradual reduction in expression, only reaching a stable plateau after 15 generations (Figure 10). The temperature-induced changes in the expression from this transgene are therefore inherited over multiple generations. Moreover, there is an asymmetry in the transgenerational dynamics of transgene expression with a fast upregulation when temperature is increased and a relatively slow recovery to baseline expression level when temperature is decreased. We found that a single generation of growth at elevated temperature (48 hours from embryo to L4/young adult at 25 °C, then shifting back to 20°C) was sufficient to induce a multigenerational change in gene expression, with a significant difference in expression detected for at least seven generations after the transient environmental perturbation (Figure 11). We observed very similar behavior with a multi-copy *daf-21p::GFP* transgene (Table 1).

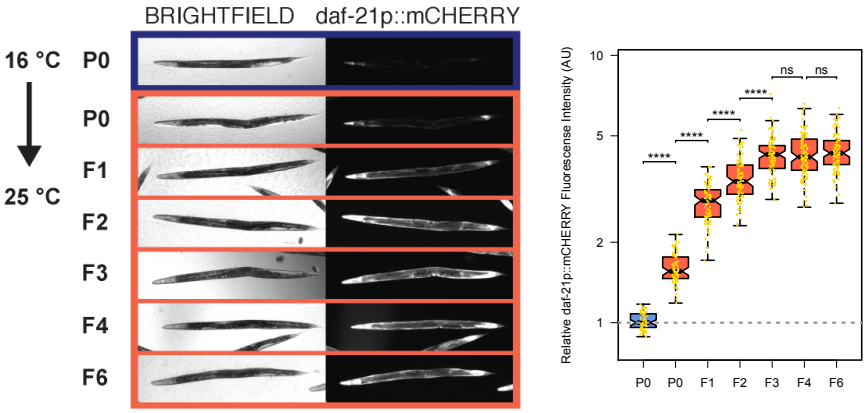

Figure 8. Increasing the temperature from 16 to 25 °C results in a progressive increase in transgene expression that plateaus at the 3rd generation. Stage-matched worms grown continuously at 16 °C were used as reference in each measurement. Expression was quantified in adults. P values: **** p< 0.0001 , ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

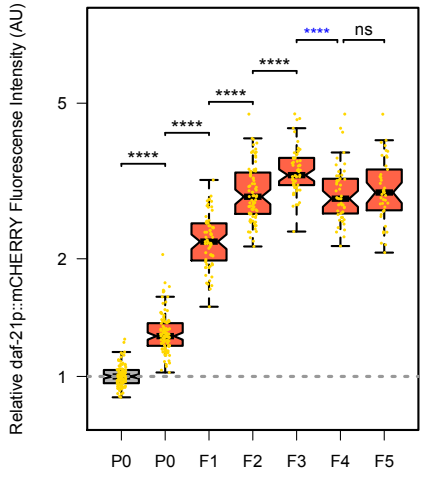

Figure 9. Increasing the temperature from 20 to 25 °C results in a progressive increase in transgene expression that plateaus at the 3rd generation. P values: **** p< 0.0001 , ns p> 0.05 (Wilcoxon rank test). Y axis in log scale. Blue asterisks indicate a significantly lowere expression in the successive generation.

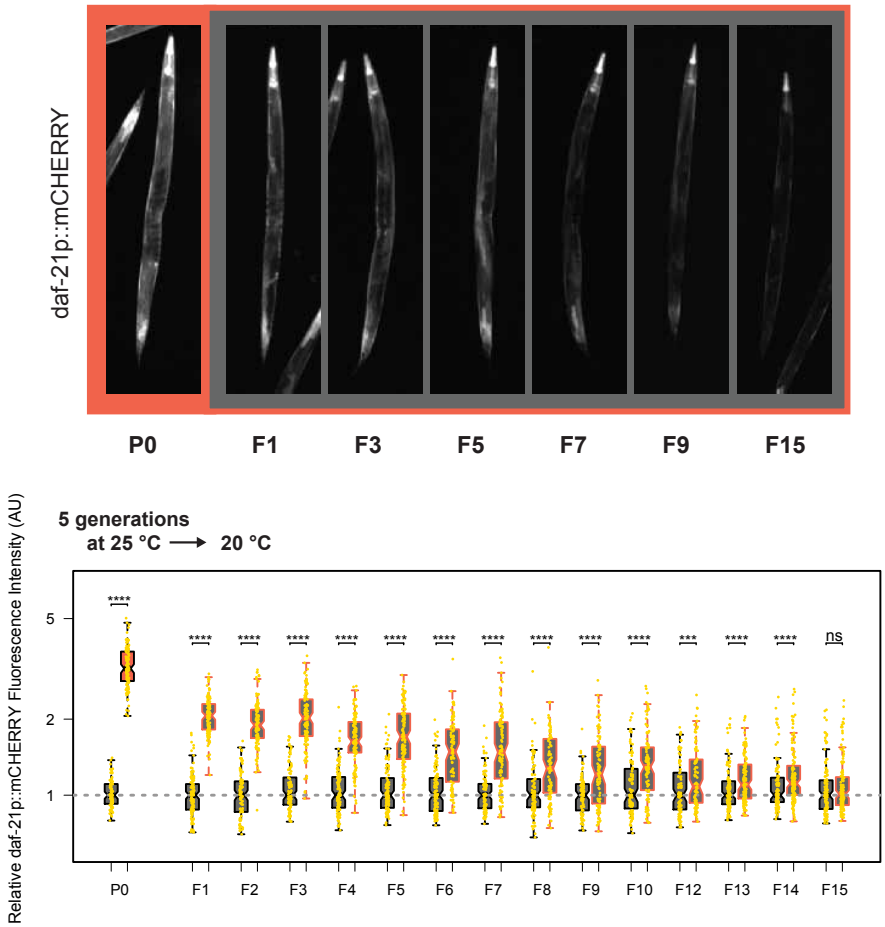

Figure 10. Expression of the *daf-21::mCHERRY* multicopy transgene reduces gradually over multiple generations after shifting worms from 25 to 20 °C. Quantification was performed in adult worms after shifting the worms from 25 to 20 °C starting with worms grown for 25 °C for 5 generations. Stage-matched worms kept constantly at 20 °C were used as reference for normalization. P values: **** $p < 0.0001$, *** $p<0.001$, ns $p > 0.05$ (Wilcoxon rank test). Y axis in log scale.

				elevated expression after shift to 20 °C		
Srain	**Transgene**	**MULTI or SINGLE Copy**	**upregulation at 25 °C**	**F1**	**F2**	**F5**
BCN1050	*daf-21::mCHERRY*	MULTI	YES	YES	YES	YES
BCN1049	*daf-21::GFP*	MULTI	YES	YES	YES	YES
BCN1082	*daf-21::GFP*	SINGLE	YES	YES	NO	NO
CL2166	*gst-4::GFP*	MULTI	YES	YES	YES	YES
CF1553	*sod-3::GFP*	MULTI	YES	YES	YES	YES
TJ375	*hsp-16.2::GFP*	MULTI	YES*	YES*	nd	nd
SJ4005	*hsp-4::GFP*	MULTI	YES	NO	nd	nd
MH1870	*sur-5::GFP*	MULTI	YES	NO	NO	nd
KM267	*hsp-16.41::hlh-1 + pRF4 (rol-6)*	MULTI	YES**	YES**	YES**	YES**

nd = not determined

* expression induced by 30min heat shock at 34 °C

** expression inferred from frequency of 'roller' phenotype

Table 1. List of transgenes tested for a trans-generational memory of expression

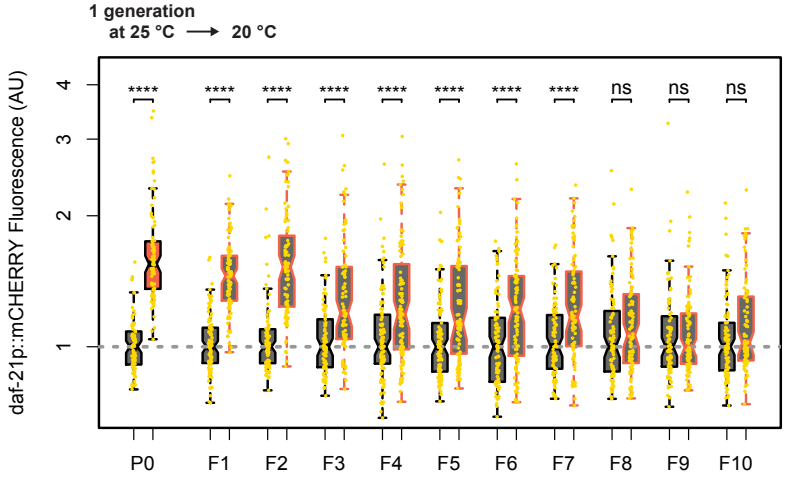

Figure 11. Growing Worms grown at 25 °C from embryos to the L4 stage is sufficient to mount a heritable change in transgene expression that last for at least 7 generations. Quantification of transgene expression in adult worms over multiple generations after shifting the worms from 25 to 20 °C starting with worms grown for 25 °C for one generation (from embryo to young adult). Stage-matched worms kept constantly at 20 °C were used as reference for normalization. P values: **** p< 0.0001 , *** p<0.001, ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

When integrated into the genome as a single copy, the *daf-21p::GFP* transgene still showed a robust increase in expression at high temperature (Figure 12). However increased expression from the single-copy transgene was not maintained in generations beyond the F1 progeny (Figure 12), indicating that the multi-generation inheritance of expression levels requires the transgene to be present in multiple copies in the genome.

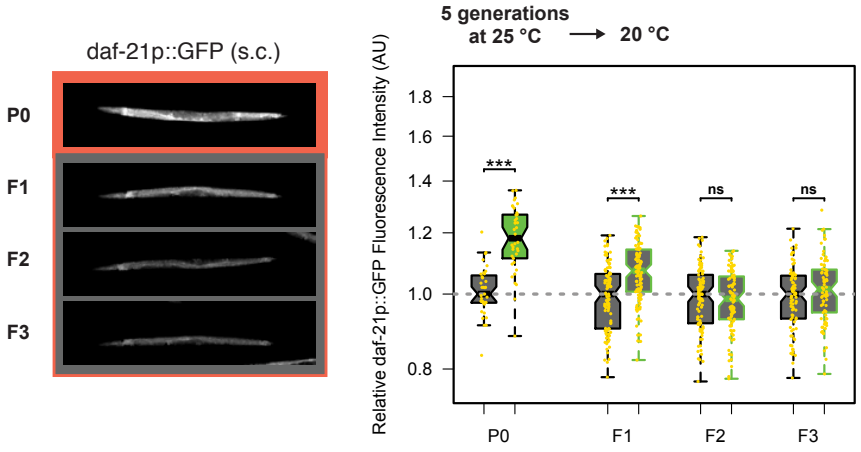

Figure 12. A single copy reporter driven by the *daf-21* promoter shows higher somatic expression at 25 °C and takes two generations to return to baseline expression after transferring to 20 °C. Stage-matched worms kept constantly at 20 °C were used as a reference for normalization. P values: *** $p<0.001$, ns $p> 0.05$ (Wilcoxon rank test). Y axis in log scale.

Testing a panel of six additional multi-copy fluorescent reporters identified two more that increased expression at high temperature: *sod-3p::GFP and gst-4p::GFP*. After transfer to 20 °C, expression of these transgenes was significantly higher in the progeny of animals previously grown at 25°C for at least five subsequent generations (Table 1). In addition we found that the KM267 strain which carries an integrated array containing a heat shock-inducible *hsp-16.41p::hlh-1* construct and a

dominant *rol-6(su1006)* allele has a higher penetrance roller phenotype at 25 °C and that this increased penetrance declines gradually over 5 generations after after returning the worms to 20 °C (Table 1).

2.1.2 Inherited differences in environmentally-triggered changes in expression manifest during embryonic development

Multi-copy transgenes are normally silenced in the germline of *C. elegans* and we observed no germline expression of the multi-copy *daf-21p::mCHERRY* and *daf-21p::GFP* transgenes at either 20 or 25°C (Figures 6 and 18). This germline silencing allowed us to test whether the heritable differences in environmentally-triggered changes in expression are apparent at the time of transgene activation during embryonic development or are re-established at a later stage of an animal's life. We transferred animals to 20°C from 16 or 25°C at the L4 larval stage and a day later, when they reached adulthood, we extracted their progeny and used time-lapse microscopy to quantify transgene expression during embryonic development (Figure 14A). Differences in expression were detected from the onset of zygotic expression (Figure 14B and D), but not earlier (Figure 14C), indicating that the heritable influences act to establish quantitatively different levels of expression from the onset of transcription.

To confirm that the transgene is not expressed in the early embryo and that transgene mRNA is not delivered maternally, we used single molecule Fluorescent *in situ* Hybridization (smFISH) (Raj et al., 2008) to detect mRNA transcripts in animals carrying the multi-copy *daf-21p::GFP* transgene array at various stages of embryonic development (Figure 15). Endogenous *daf-21* transcripts are supplied maternally and are detected at all stages of embryonic development. In contrast, mRNA transcribed from the multi-copy *daf-21p::GFP* reporter is absent in the

early embryo and becomes detectable at the 16-cell stage. At the comma stage, both the *daf-21* and *gfp* transcripts are abundant and enriched the same regions of the embryo (Figure 15).

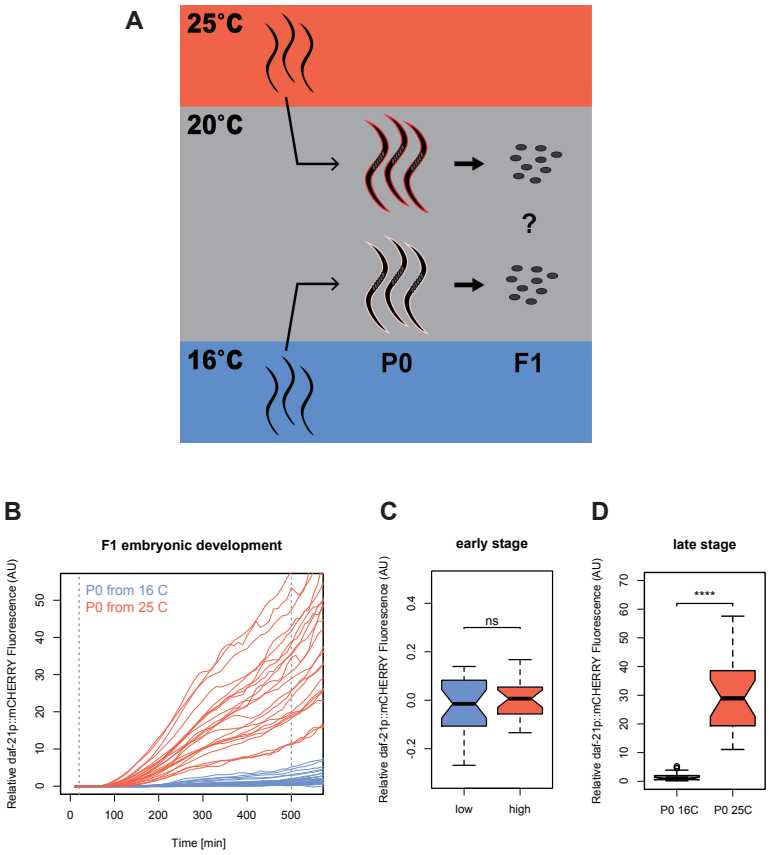

Figure 14. Epigenetic memory of environmentally-triggered change in expression manifests during embryonic development. (A) Schematic diagram of the experiment. L4 larvae from populations grown at 16 °C and 25 °C were transferred to 20 °C. The next day, after reaching adulthood (P0), embryos where extracted and the *daf-21p::mCHERRY* transgene expression quantified during embryogenesis by time-lapse microscopy. (B) Time-lapse fluorescence measurement of developing embryos derived from parents that grew either at 16 °C (blue) or 25 °C (red); one line represents one embryo. (C-D) Quantification of expression difference at the early and late time-points of development indicated by the dashed lines in (B). P values: **** $p < 0.0001$, ns $p > 0.05$ (Wilcoxon rank test).

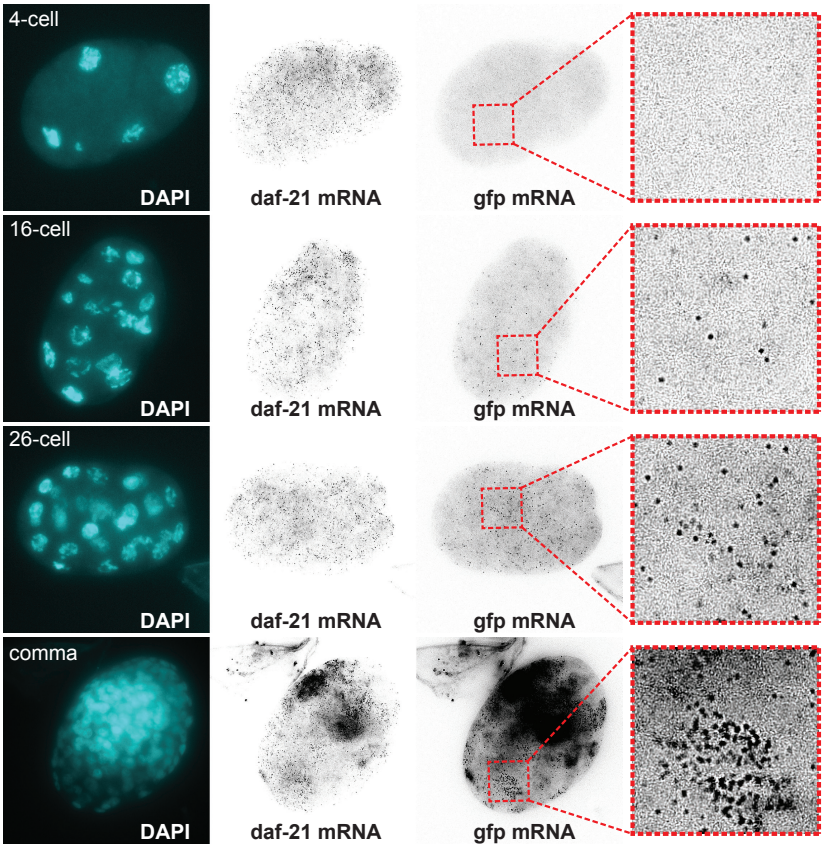

Figure 15. The *daf-21p::GFP* multicopy reporter is activated during embryonic development. Two colour single molecule FISH (smFISH) was used to simultaneously detect endogenous *daf-21* mRNA transcripts as well as *GFP* mRNA transcribed from the integrated multicopy *daf-21p::GFP* transcriptional reporter. A representative embryo from each stage of development is shown. In red squares, magnification of the indicated region.

2.1.3 Inter-individual differences in expression in a homogenous environment are also inherited

Even after long-term maintenance of animals at a constant temperature of 20 °C there is still variation in transgene expression between animals. To test whether this intra-population variation in expression is also inherited, we sorted 'high' and 'low' expressing individuals, extracted their embryos and used time-lapse microscopy to quantify expression during embryogenesis (Figure 16). Expression from the transgene in the progeny from sorted 'high' worms was on average 1.94 x higher at the 3-fold stage of development (P= 2.3e-05, Wilcoxon rank test) than in the progeny of sorted 'low' worms (Figure 16D). Thus, variation in expression between individuals in a common environment is also inherited from one generation to the next.

2.1.4 Multi-generational analysis of single copy reporter using time-lapse microscopy

Expression from a single copy *daf-21p::GFP* reporter was detected in both somatic tissues and the germline (Figure 17,18 and 19). The single-copy reporter expression appears uniform in most tissues, with a stronger signal in the intestine, while the endogenous *daf-21* transcripts exhibit strong germline enrichment (Figure 17). The multi-copy transgene is entirely silenced in the germline (Figures 17 and 18) and has a much weaker expression in the intestine compared to other somatic tissues, with the exception of the most posterior intestinal cells (Figures 17 and 31).

Protein expressed from the single-*copy* *daf-21p::GFP* transgene is detected in both early and late embryos whereas protein expressed from the multi-copy *daf-21p::mCHERRY* array is only detected in late embryos (Figure 20). GFP detected in the early embryos may represent either

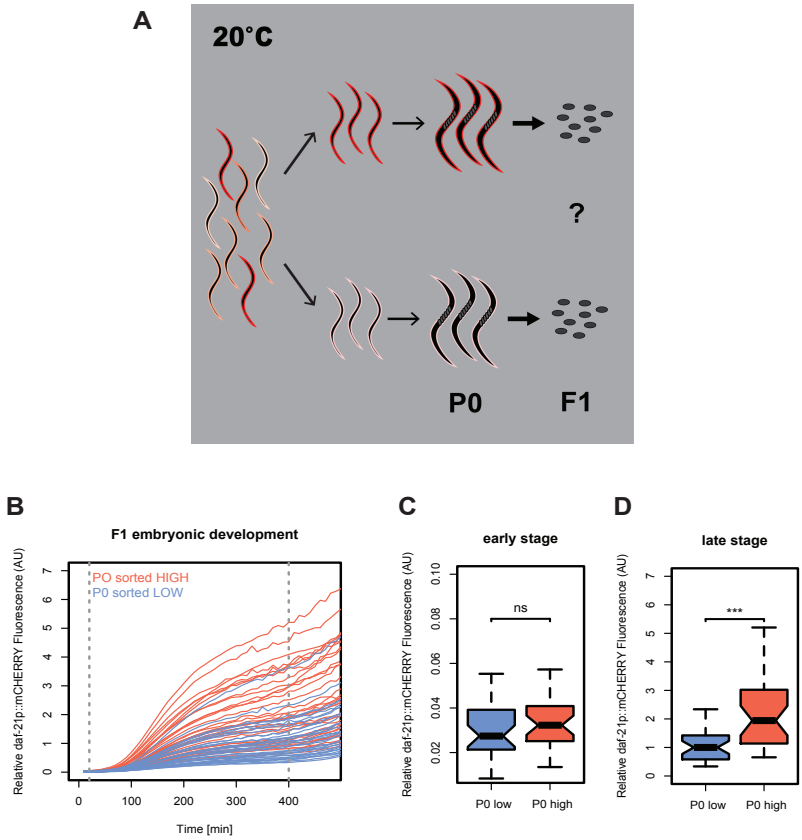

Figure 16. Worms grown continuously at 20 °C for >20 generations show variability in *daf-21p::mCHERRY* expression. (A) Schematic diagram of the experiment. "High" and "Low" *daf-21::mCHERRY* expressing larvae were picked from a stable 20 °C population and reporter expression in the progeny measured by time-lapse microscopy of embryonic development. (B) Time-lapse fluorescence measurement of developing embryos derived from sorted "High" and "Low" parents. Each line represents one embryo. (C-D) Quantification of expression at the early and late time-points of development indicated by the dashed lines in (B). P values: *** $p < 0.001$, ns $p > 0.05$ (Wilcoxon rank test).

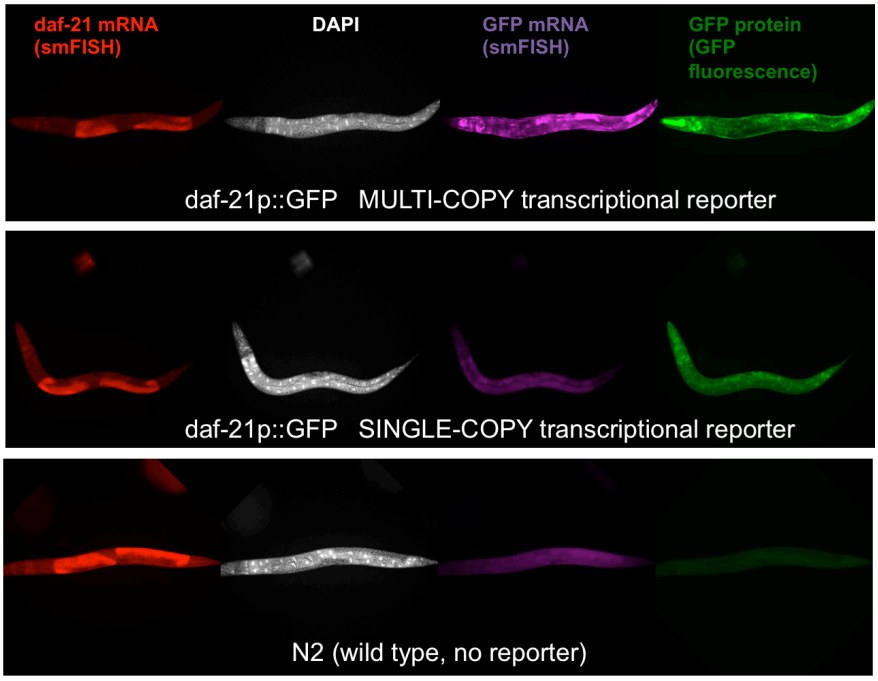

Figure 17. Comparison of multi-copy and single-copy *daf-21::GFP* transcriptional reporter activity using single molecule FISH. Endogenous *daf-21* mRNA transcripts (in red) are highly enriched in the germline. GFP mRNA transcripts (in purple) are only detected in somatic tissues in the multicopy reporter strain but also in the germline in the single copy reporter strain.

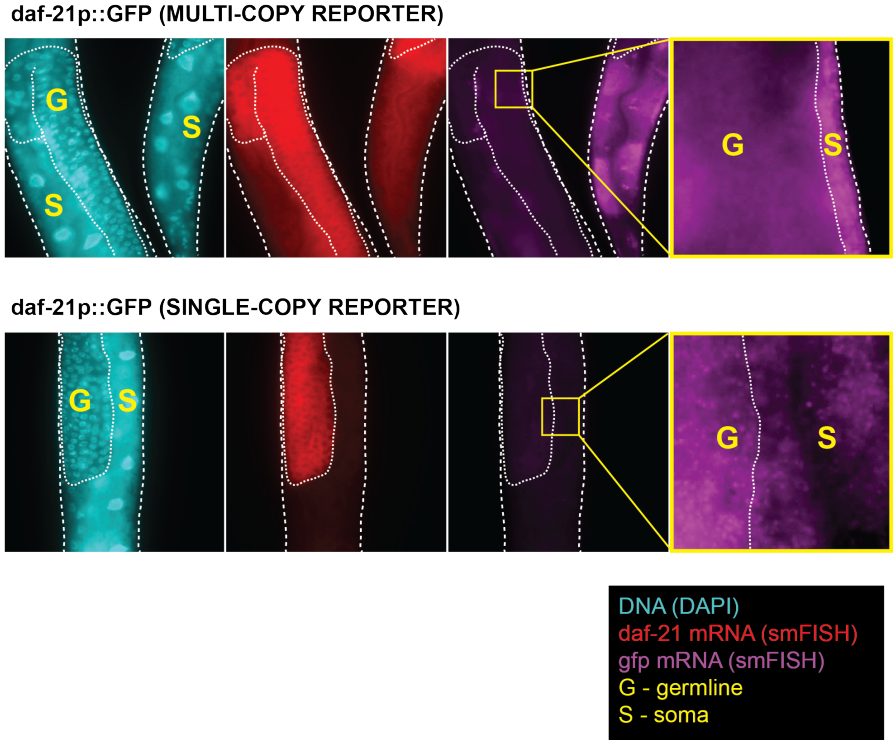

Figure 18. The *daf-21p::GFP* multi-copy reporter is expressed only in somatic tissues, whereas the single-copy reporter is active both in the germline and in the soma. Single molecule FISH staining against endogenous *daf-21* mRNA transcripts (in red) as well as GFP mRNA (in purple). In yellow squares, magnification of the indicated region.

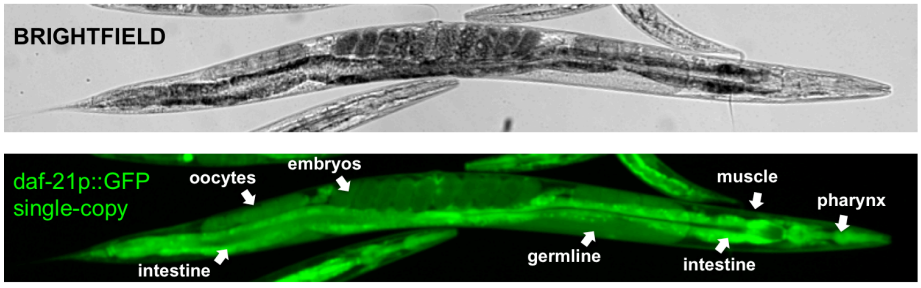

Figure 19. The single-copy *daf-21p::GFP* transcriptional reporter is active in both the germline and in somatic tissues.

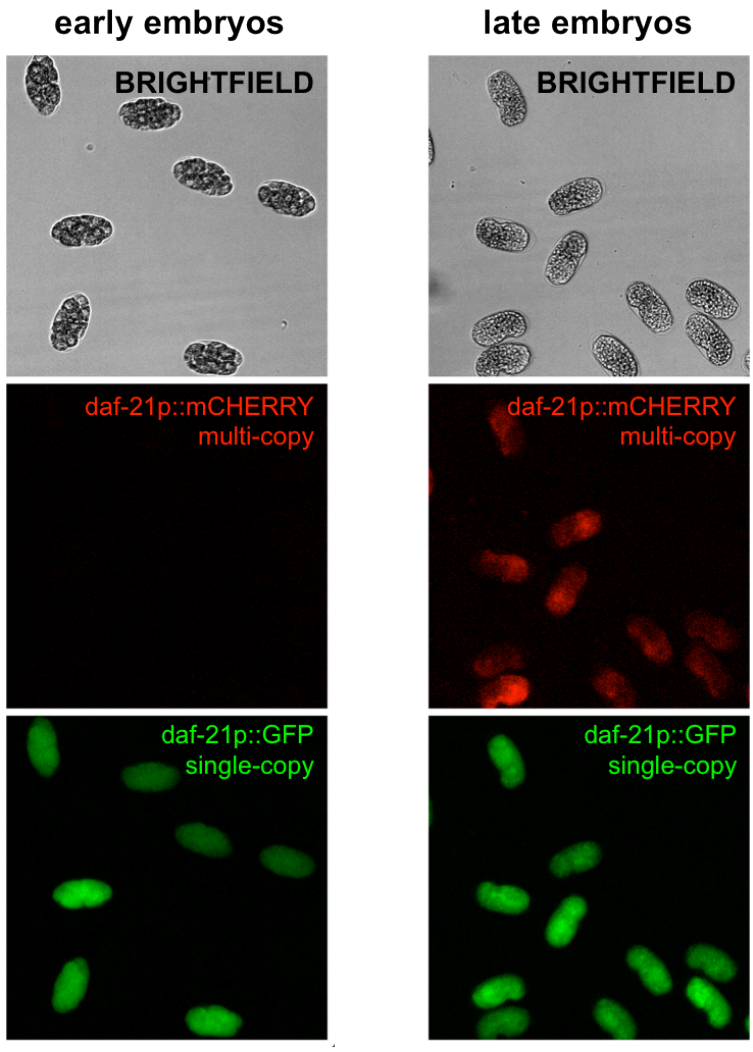

Figure 20. GFP expressed from a single copy *daf-21::GFP* reporter can be detected in both early and late embryos. In contrast, no protein expressed from the multi-copy *daf-21::mCHERRY* can be detected in early embryos.

translation of maternally deposited mRNAs or maternally-supplied GFP protein because early *C. elegans* embryos are known to be transcriptionally quiescent (Edgar et al., 1994). We carried out a multi-generational analysis of the single-copy reporter expression in embryonic development in animals with different ancestral environments. We grew worms at 16 or 25 °C for 4 weeks, and then shifted L4 animals from both populations to 20 °C. The following day, we extracted early embryos from the gravid parents and measured expression of the single-copy *daf-21p::GFP* reporter during the course of embryonic development using time-lapse microscopy (Figure 21). Embryos coming from parents (red in Figure 21) grown at 25 °C are brighter at all stages of development. Higher expression in the early embryos most likely reflects higher expression in the parental germline. Expression is also higher at the late embryonic stage. This is not just due to the higher protein concentrations in the early embryos, as the increase in expression is also higher in these embryos (Figure 21). Interestingly, in the following generation (F2) of growth at 20 °C, the descendants of worms grown at 25 °C have lower expression in the early embryos, yet the increase in expression, caused by somatic expression of the transgene, is still higher in this group ('slope' in Figure 22). In the F3 progeny, the expression in early embryos remains lower in the descendants of worms grown at high temperature. However, the increase in expression is the same in both groups (Figure 23). This trend is maintained for at least 2 subsequent generations (Figure 24). Based on this analysis we conclude that somatic induction of transgene expression is stronger in the first two generations after the shift. The fact that in the analysis of somatic expression of adult worms we detected difference in expression only in F1 worms might mean that the higher rate of somatic expression in F2 embryos (ancestral 25°C) is not maintained in larval development. Alternatively it could be due to the different

sensitivity of the two approaches. Expression in the early embryo, which most likely is a product of maternal germline activity, exhibited unexpected dynamics for which we currently have no explanation.

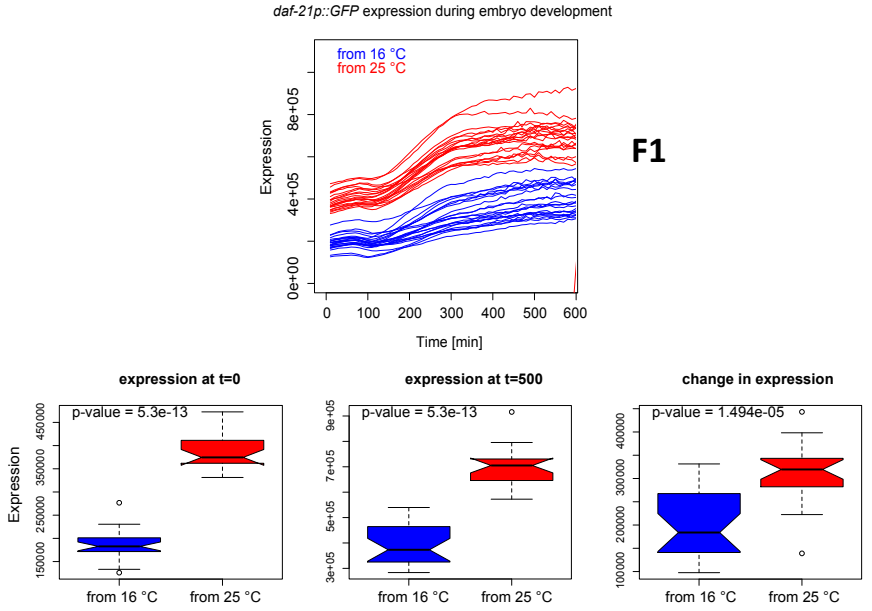

Figure 21. *daf-21p::GFP* single copy reporter expression during embryogenesis. Worms grown at 16 °C and 25 °C for > 10 generations were shifted to 20 °C as L4 larvae. A day later, embryos where extracted and GFP expression measured during the embryonic development of the F1 progeny in both groups. Each line represents one worm. The boxplots show the difference in fluorescence intensity at an early time point, late time point and the expression change between the two timepoints.

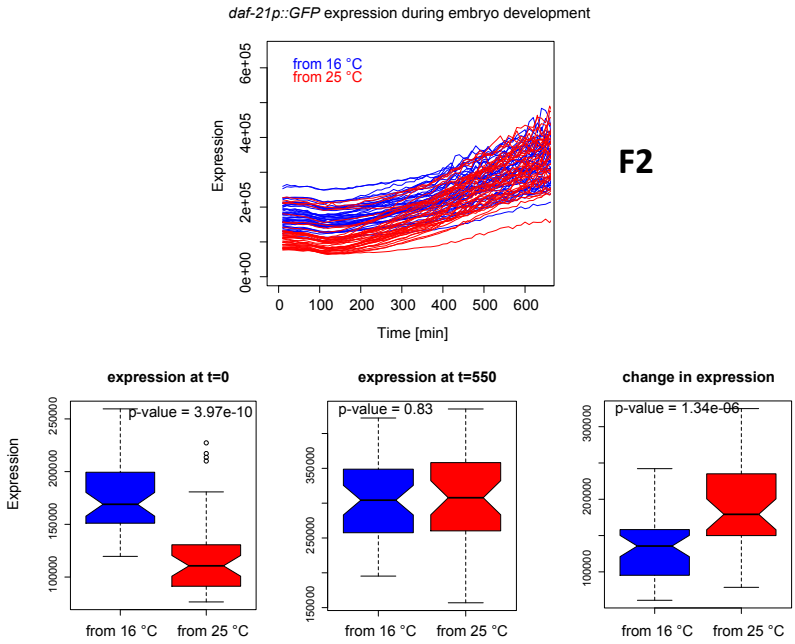

Figure 22. Measurement of *daf-21p::GFP* single copy reporter expression during embryogenesis in F2 embryos (P0 at 16 °C vs. 25 °C).

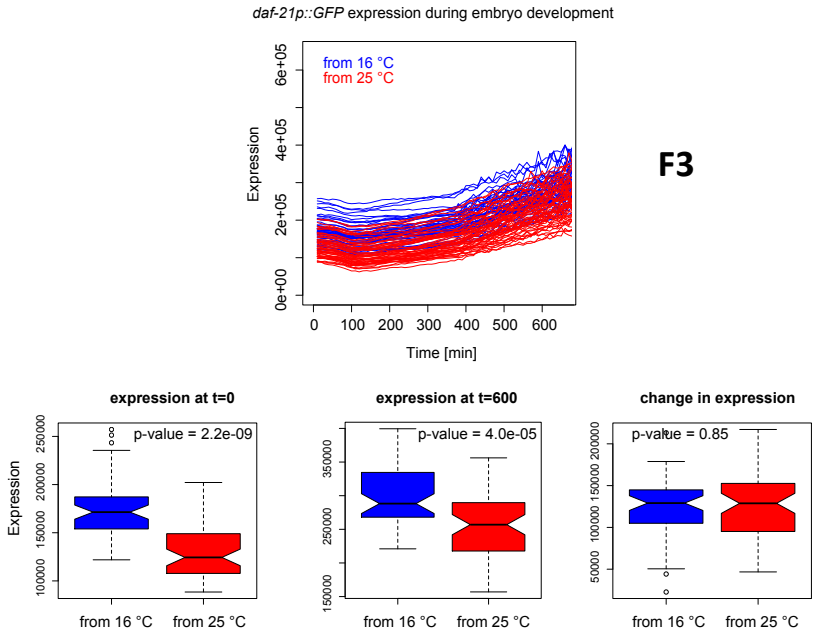

Figure 23. Measurement of *daf-21p::GFP* single copy reporter expression during embryogenesis in F3 embryos (P0 at 16 °C vs. 25 °C).

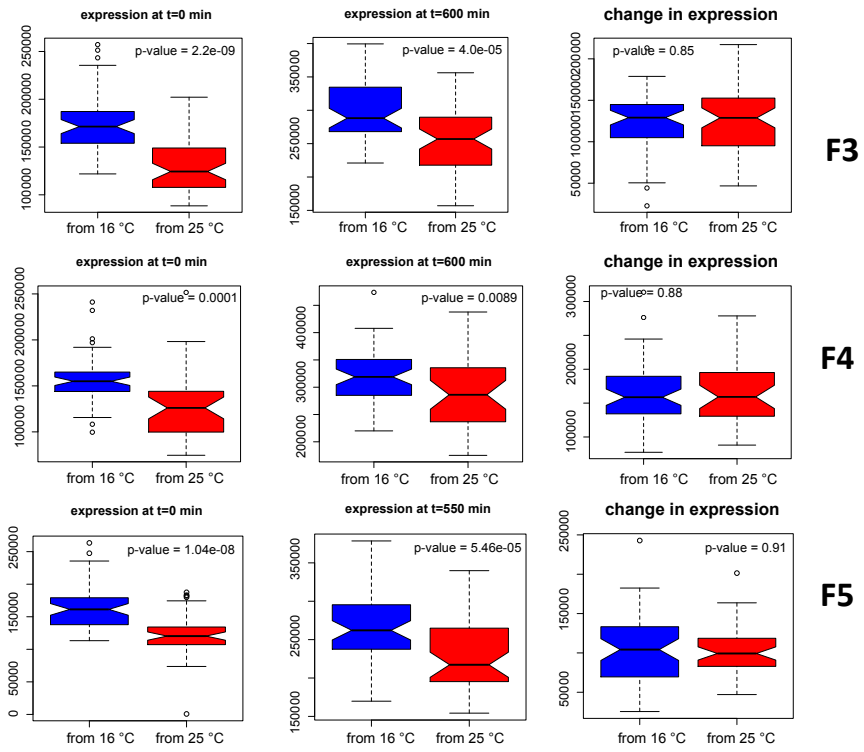

Figure 24. Measurement of *daf-21p::GFP* single copy reporter expression during embryogenesis in F3, F4 and F5 embryos (P0 at 16 °C vs. 25 °C).

2.1.5 *daf-21p::mCHERRY* expression changes evoked by temperature are transmitted through both gametes

In self-fertilizing hermaphrodites, both sperm and oocyte originate from the same individual. Therefore, the 'memory' of expression level could be inherited through either gamete or through both. To distinguish between these possibilities we performed genetic crosses between male and hermaphrodite animals where only one of the parents transmitted the *daf-21p::mCHERRY* transgene to the progeny. We generated 'high' and 'low' *daf-21p::mCHERRY* transgene populations through growth at 25 °C and 16 °C respectively. After shifting the two cohorts to 20 °C for 2

generations we crossed L4 hermaphrodites to males carrying the *daf-21p::GFP* multi-copy transgene (Figure 27A).

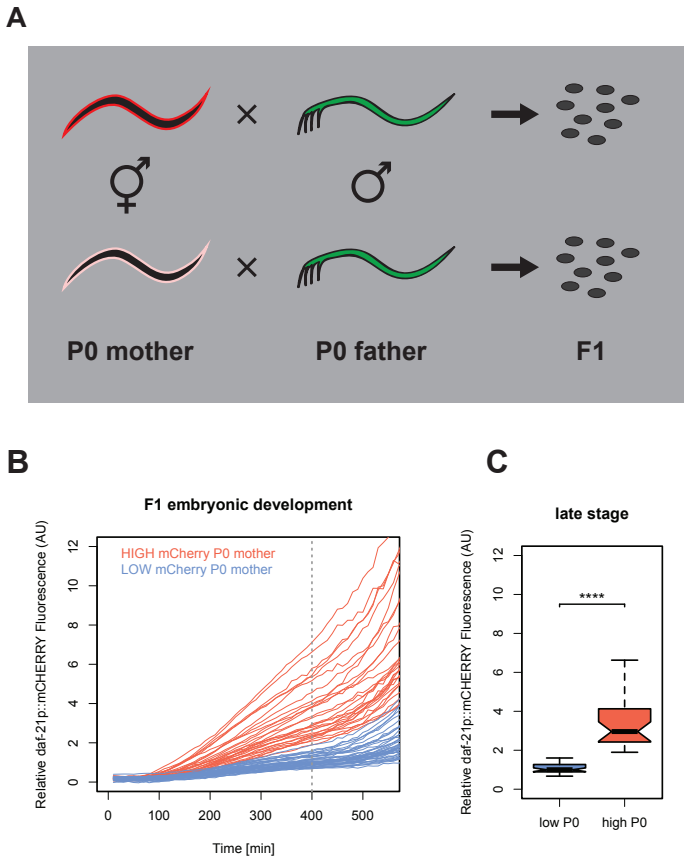

Figure 27. Transgene expression differences are transmitted to subsequent generations through oocytes. (A) *daf-21p::GFP* males were crossed to *daf-21p::mCHERRY* L4 hermaphrodites from populations grown at 16 °C and 25 °C for several generations, then transferred to 20 °C. The next day, after reaching adulthood, (P0) embryos where extracted and the oocyte-derived *daf-21p::mCHERRY* transgene expression quantified during embryonic development. Cross progeny were identified based on the presence of GFP transgene (B) Time-lapse fluorescence measurement of developing embryos derived from mothers that grew either at 16 °C (blue) or 25 °C (red); one line represents one embryo. (C) Quantification of expression at the indicated time-point (vertical line) during embryonic development. P values: **** p< 0.0001.

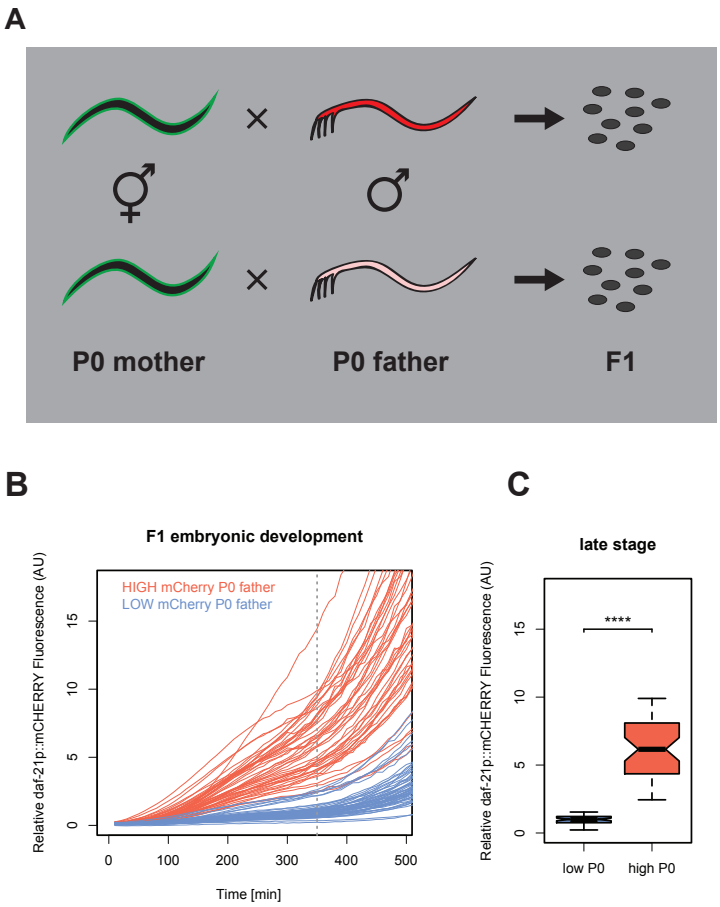

Figure 28. Expression differences are transmitted to subsequent generations through sperm. (A) *daf-21p::GFP* L4 hermaphrodites were crossed to *daf-21p::mCHERRY* males from populations bred at 16 °C and 25 °C for several generations, then transferred to 20 °C. The next day, after reaching adulthood, (P0) embryos where extracted and the sperm-derived *daf-21p::mCHERRY* transgene expression quantified during embryonic development. Cross progeny were identified based on the presence of mCHERRY transgene (B) Time-lapse fluorescence measurement of developing embryos derived from parents that grew either at 16 °C (blue) or 25 °C (red); one line represents one embryo. (C) Quantification of expression at the indicated time-point (vertical line) during embryonic development. P values: **** $p < 0.0001$.

The following day we extracted progeny of the gravid hermaphrodites and compared mCHERRY expression in the developing embryos (Figure 28B); GFP fluorescence was used to identify the cross-progeny, ensuring that *daf-21p::mCHERRY* was only transmitted via the oocytes. Measurement of *daf-21p::mCHERRY* fluorescence revealed strong separation between the two cohorts (Figure 27B and C) demonstrating that parental expression level is inherited through oocytes. In a reciprocal experiment, in which the differentially expressed *daf-21p::mCHERRY* transgene was only delivered by the males (Figure 28), we obtained similar results – 'high' males sired 'high' offspring and 'low' males sire 'low' offspring (Figure 28). Together, this demonstrates that differences in parental expression levels are inherited through both oocytes and sperm.

2.2 The mechanism

2.2.1 Inheritance of temperature-induced changes in expression requires chromatin modifying enzymes and the nuclear RNAi pathway

To identify genes required for the epigenetic inheritance of temperature-induced changes in expression, we first tested a panel of mutations in candidate genes for their effects on transgene expression (Figure 29). Mutations in the genes *rde-1, rde-4, ergo-1, nrde-3, set-2* and *rbr-2* decreased the expression of the multi-copy transgene, whereas mutations in the genes *met-1, met-2, set-25, spr-5, mut-7, hrde-1, wdr-5.1* and *nrde-2* increased the expression (Figures 29, 30 and 31). In *wdr-5.1* mutants the increase was evident only in the intestine while expression in other tissues was lower than in control animals (Figure 31). In animals carrying mutations in *spr-5, hpl-2, and met-2* and in a *met-2; set-25* double mutant we observed both a ubiquitous increase in transgene expression, and a

stronger overexpression in the intestine (Figure 30). In *met-2* mutant animals we observed a very high level of inter-individual variability (Figure 29 and 30) that disappears in *met-2; set-25* double mutants (Figure 29).

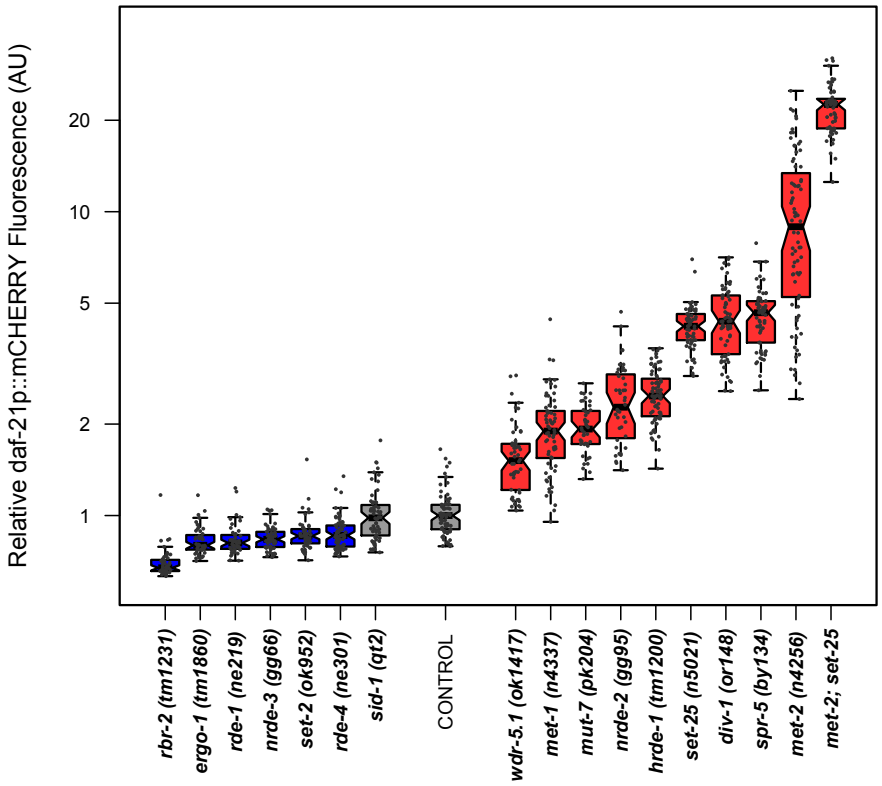

Figure 29. Multiple chromatin and small RNA pathway mutants affect expression of the *daf-21p::mCHERRY* transgene. Quantification was performed in adult worms at 20 °C. Y axis in log scale. In blue mutants with significantly lower expression, in red mutants with significantly higher expression, in gray mutant with no significant expression difference. Y axis in log scale.

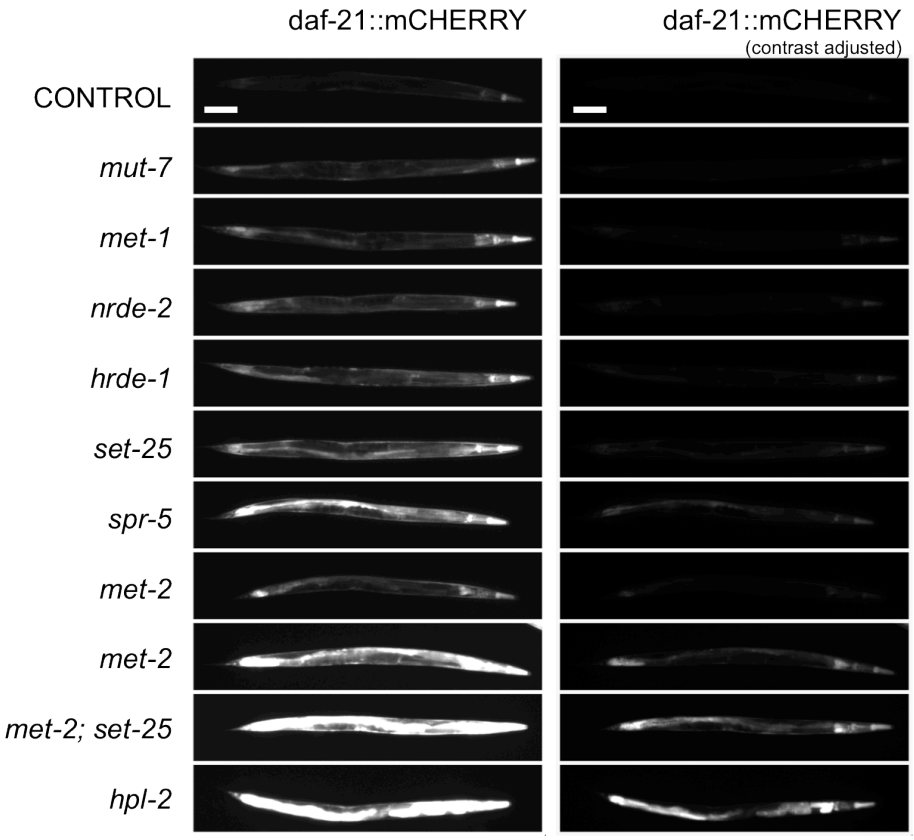

Figure 30. Mutations in multiple chromatin modifying enzymes and small RNA pathway components increase expression from the *daf-21p::mCHERRY* reporter. Scale bar = 100 μm.

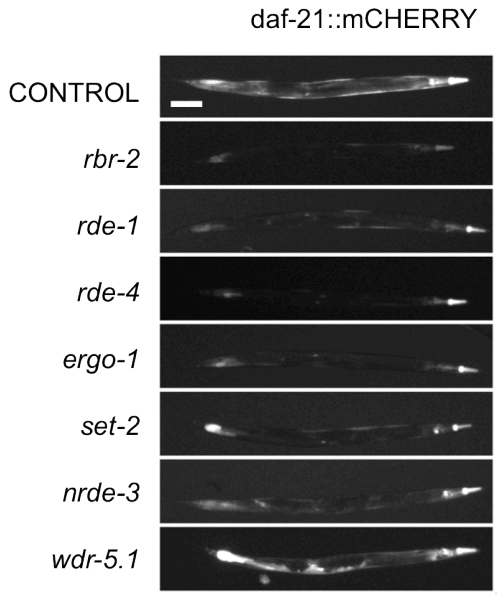

Figure 31. Mutations in multiple chromatin modifying enzymes and small RNA pathway components increase expression of *daf-21p::mCHERRY*. Scale bar = 100 μm.

We next performed experiments to test whether the genes identified as candidate *daf-21p::mCHERRY* regulators are required for the inter-generational memory of temperature-induced changes in expression. The control and mutant worms were grown at 25°C for a single generation and then shifted back to 20°C and cultivated for two more generations while monitoring expression levels via fluorescence intensity measurements (Figure 32). All of the tested mutants showed a robust response to the changing environment in the parental (P0) generation and increased expression of the transgene at 25°C (Figures 32, 33 and 34). Mutations in the genes *rde-1, rde-4, nrde-3, wdr-5.1, set-2, ergo-1* and *rbr-2*, even though they affect the baseline expression of the transgene, had little effect on the trans-generational dynamics of expression, with inter-generation inheritance similar to that in wild-type animals (Figure 34).

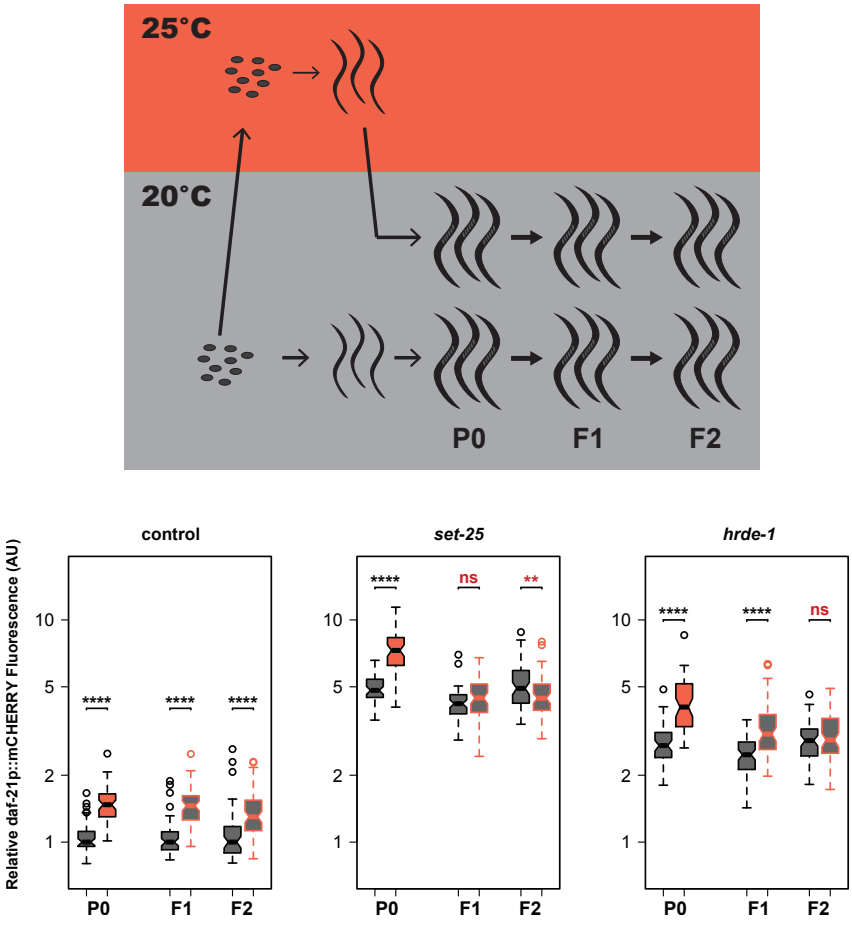

Figure 32. SET-25 and HRDE-1 are required for inheritance of temperature-induced changes in transgene expression. (A) Schematic diagram of the experiment. Early embryos, extracted from a population grown at 20 °C, were moved to 25 °C and grown for 2 days then shifted back to 20 °C and propagated for 2 more generations. Expression was quantified in adults in the 25 °C exposed parental generation (P0) as well as in two subsequent generations (F1, F2). Worms grown continuously at 20 °C were used as control. (B) Quantification of *daf-21p::mCHERRY* fluorescence intensity. Control worms at 20 ° C were used as reference for normalization in each generation. *set-25* mutant worms fail to transmit the increased expression generated by elevated temperature during development of P0 worms to the following generations. In *hrde-1* mutants, the increased expression following exposure to high temperature is retained in the F1 generation, but disappears in the F2. P values: **** $p < 0.0001$, *** $p<0.001$, ns $p > 0.05$ (Wilcoxon rank test). Y axis in log scale.

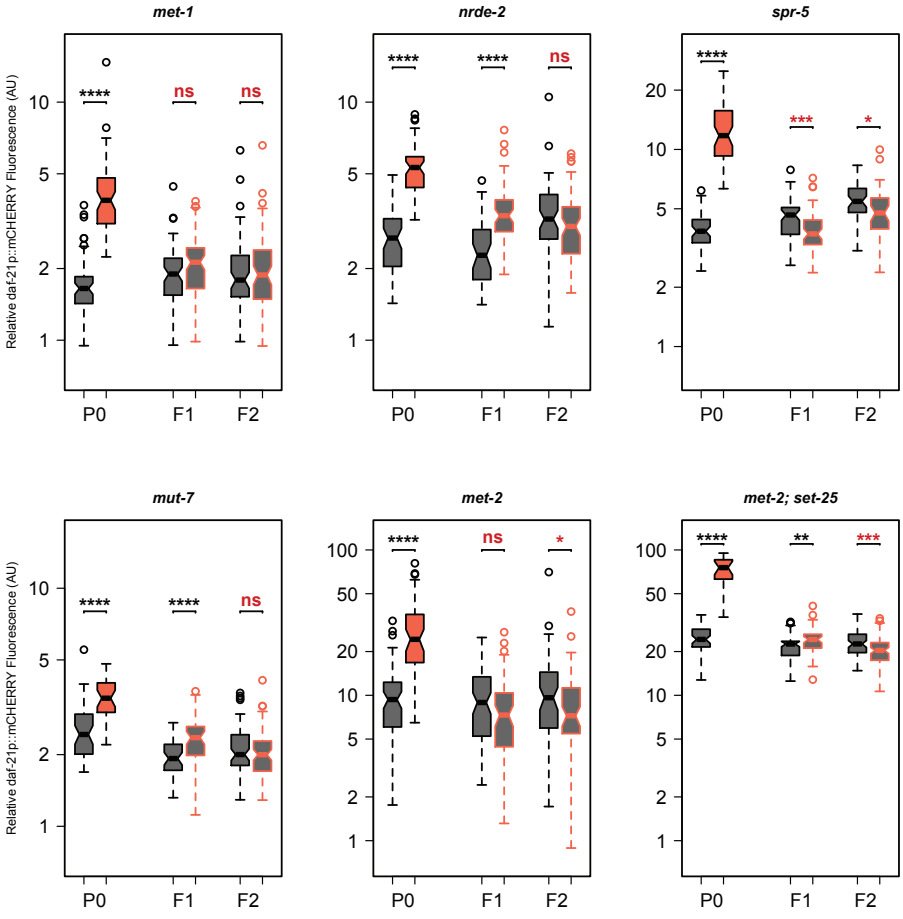

Figure 33. Multiple chromatin-modifying enzymes as well RNAi pathway components are required for inheritance of temperature-induced changes in transgene expression. Experiment done as in Figure 32. P values: **** p< 0.0001 , *** p<0.001, ** p<0.01, * p<0.05, ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

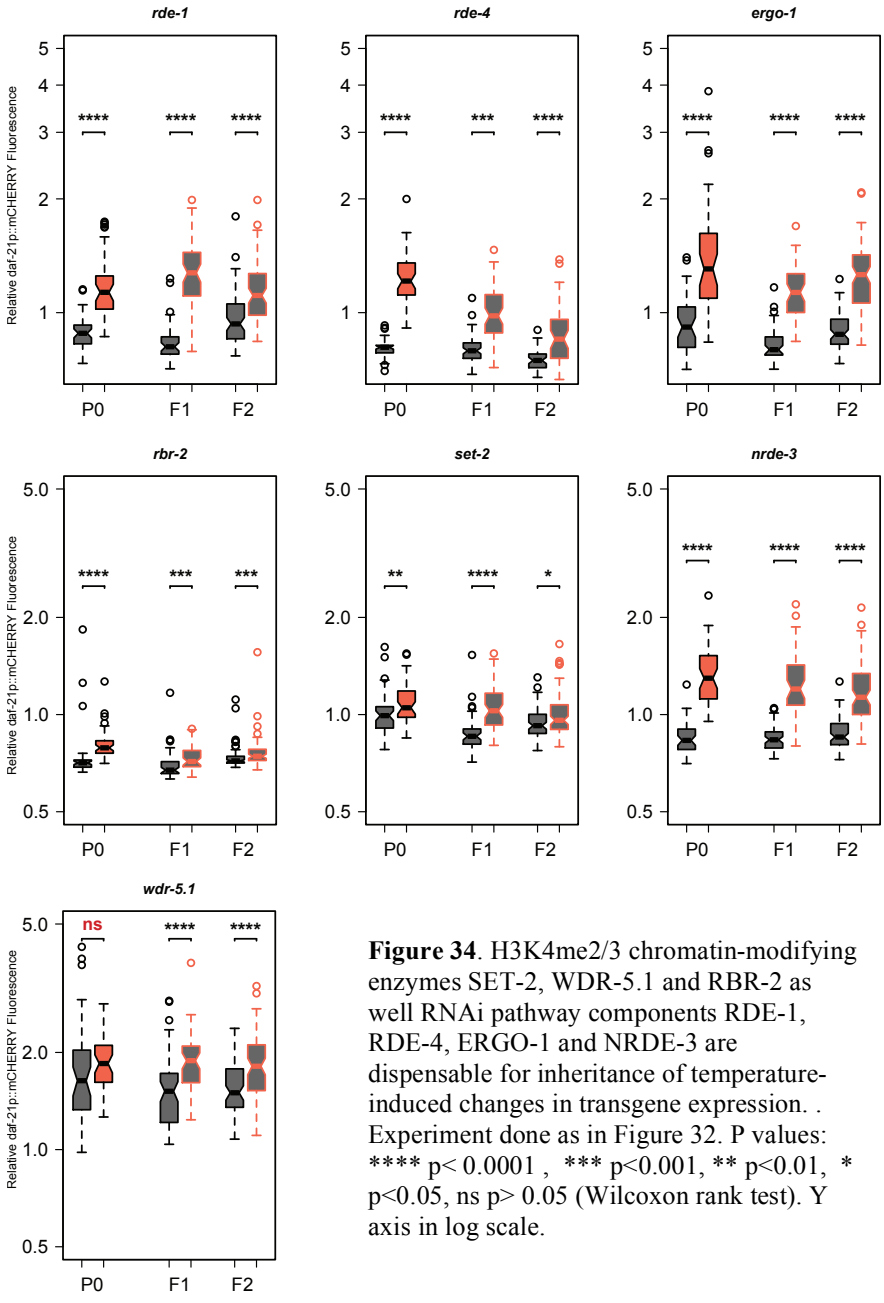

Figure 34. H3K4me2/3 chromatin-modifying enzymes SET-2, WDR-5.1 and RBR-2 as well RNAi pathway components RDE-1, RDE-4, ERGO-1 and NRDE-3 are dispensable for inheritance of temperature-induced changes in transgene expression. . Experiment done as in Figure 32. P values: **** p< 0.0001 , *** p<0.001, ** p<0.01, * p<0.05, ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

This suggests that these genes are not required for the initiation, transmission or reception of the epigenetic signal across generations. In contrast, mutations in the genes *met-1, met-2, spr-5* and *set-25*, showed reduced inheritance of the temperature-induced increase in expression of the transgene in the first (F1) generation after a temperature shift (Figure 32 and 33). These genes are therefore required for the inheritance of the environmentally triggered change in expression in the first generation. In addition, mutations in genes encoding two components of the nuclear small RNAi pathway – the Argonaute HRDE-1 and the accessory factor NDRE-2 – had little impact on the transmission of the expression state to the immediate F1 progeny after a temperature shift, but did prevent the inheritance of expression differences one generation later in the F2 animals (Figure 32 and 33). These two genes are therefore important for the long-term transmission of the epigenetic memory.

2.2.2 SET-25 and HRDE-1 mediate inheritance of inter-individual expression differences developed in a homogenous environment

We next asked if the genes required for efficient transmission of environmentally triggered transgene up-regulation are also essential for inheritance of the pre-existing expression variance that is observed in populations maintained at constant temperatures. For each mutant, we sorted animals with high and low transgene expression from a synchronized population and followed the expression in their progeny for two generations. Strikingly, only the *set-25* mutant failed entirely to pass the expression differences to the subsequent generations (Figure 35 and 36). In the *hrde-1 (-)* mutant the differences established by sorting where partially maintained in the subsequent generations, but to a lesser extent than for the wild-type (Figure 35). Combined with the previous results, this indicates that inter-generational memory of newly triggered changes in expression depends on the genes *set-25, hrde-1, met-1* and *met-2*,

whereas inter-generational memory of pre-established expression variation only requires *set-25* and *hrde-1*.

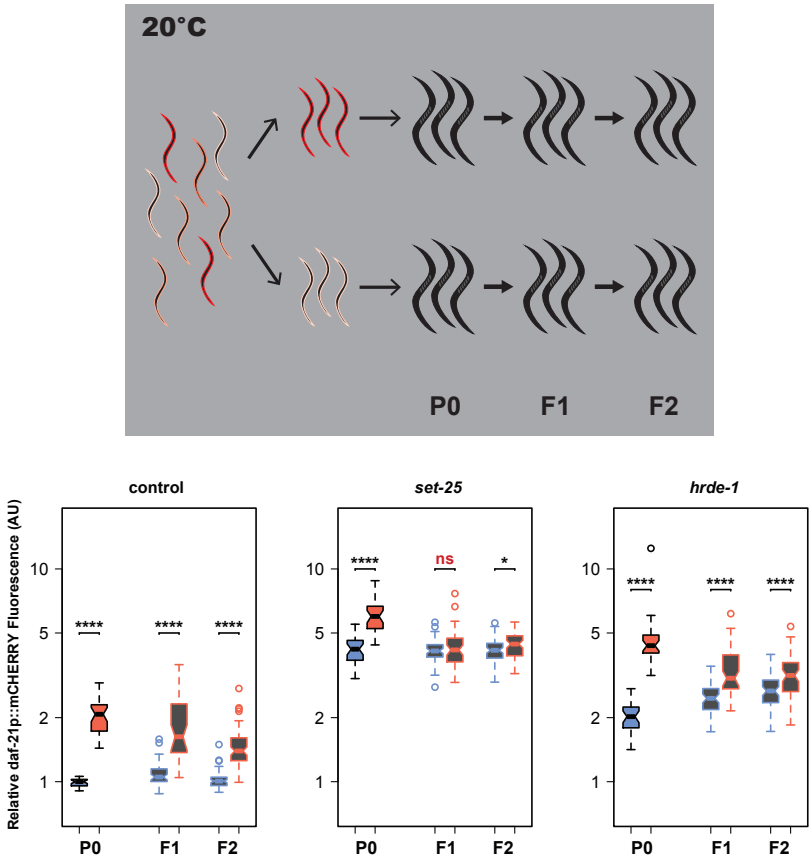

Figure 35. SET-25 is required for the inheritance of inter-individual differences in the expression of the *daf-21::mCHERRY* reporter. (A) Schematic diagram of the experiment. Using worms grown for >20 generations at 20 °C, two cohorts of 'high' and 'low' *daf-21p::mCHERRY* expressing individuals were established by picking the bright and dim worms under a fluorescent microscope. Expression was measured when those worms reached adulthood (P0) and in adults in the subsequent two generations. (B) Quantification of *daf-21p::mCHERRY* fluorescence intensity in each generation of the sorted worms. *set-25* mutant worms fail to transmit differences generated in the parental generation by sorting. In *hrde-1* mutants, expression differences are retained in the F1 and F2 generations but are smaller than in wild-type animals (see also Figure 65). P values: **** p< 0.0001 , *** p<0.001, ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

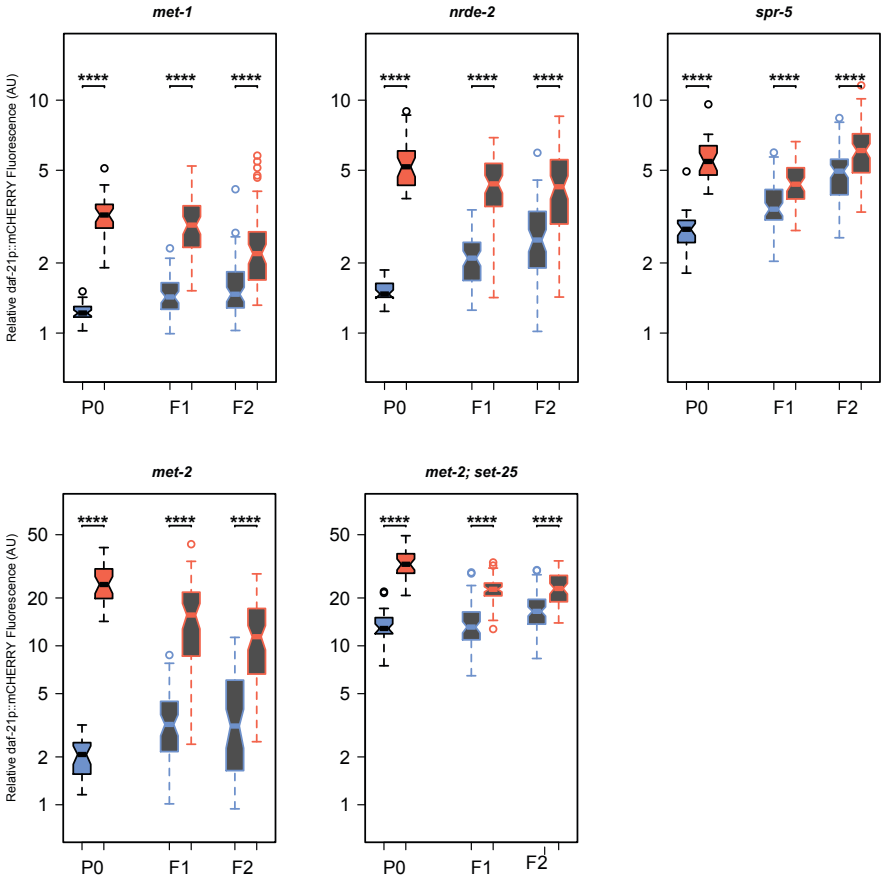

Figure 36. *met-1, met-2, nrde-2, spr-5* mutants and *met-2, set-25* double mutants transmit inter-individual differences in the expression of the *daf-21p::mCHERRY* reporter to subsequent generations. Experiment as in Figure 35. P values: **** p< 0.0001 , ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

2.2.3 SET-25 is required to both transmit and receive the environmentally triggered epigenetic memory

Two non-mutually exclusive sites of action are possible for a gene implicated in the epigenetic inheritance of an environmentally-induced change in expression – either the gene is required in the parental generation to initiate the 'memory' of the environmentally-triggered change in expression or it is required in the offspring to receive and/or propagate the signal across the cellular divisions of the developing organism.

To test whether a gene activity is required in the early embryos to propagate the epigenetic signal, we crossed mutant worms that did not carry a multi-copy transgene to males expressing either high or low levels of *daf-21p::mCHERRY*. Whereas embryos derived from mothers carrying mutations in the genes *met-1, met-2, spr-5, nrde-2* or *hrde-1* still established differences in transgene expression between transgenes inherited from 'high' and 'low' fathers, those derived from *set-25* mutant mothers did not (Figures 37 and 28). Thus, a maternal supply of MET-1, MET-2, SPR-5, NRDE-2 and HRDE-1 activity is not required for early embryos to receive and/or use the epigenetic information from the sperm. In contrast, SET-25 activity is essential in the early embryo. Using this experimental setup we were also able to test for a requirement for the genes *mes-2* and *mes-4*. *mes-2* and *mes-4* are required maternally for deposition of H3K27me3 and H3K36me3 respectively and their inactivation results in maternal-effect sterility, precluding analysis of their role in the previous assays. Although transgene expression was increased in the progeny of *daf-21p::mCHERRY* males when they were crossed to *mes-2* or *mes-4* mutant hermaphrodites, the differences in expression between transgenes derived from 'high' and 'low' male parents was

maintained. Therefore *mes-2* and *mes-4* are unlikely to play a role in epigenetic inheritance of transgene expression level described here.

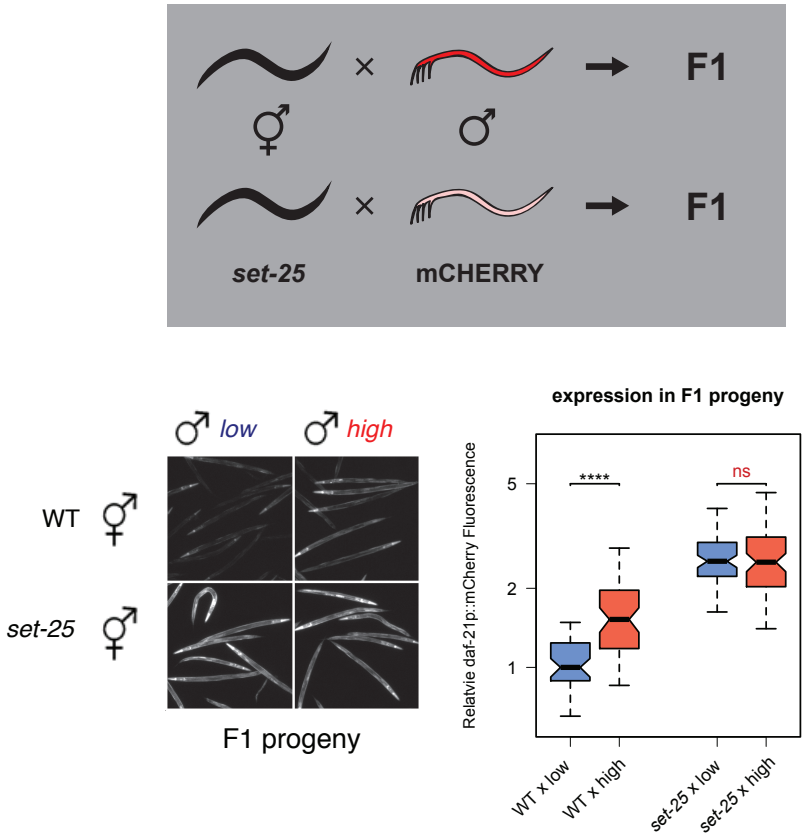

Figure 37. SET-25 is required maternally for the transmission of a temperature-induced change in *daf-21p::mCHERRY* expression to subsequent generations. (A) Schematic diagram of the experiment. *set-25* mutant hermaphrodite mothers were crossed to males expressing either high or low mCHERRY due to ancestral growth at either 16 or 25 °C. Fluorescence intensity was measured in F1 adults. Control F1 samples were made by crossing worms from the same two populations of 'high' and 'low' *daf-21p::mCHERRY* males to wild-type hermaphrodite mothers. P values: **** $p < 0.0001$, ns $p > 0.05$ (Wilcoxon rank test). Y-axis in log scale.

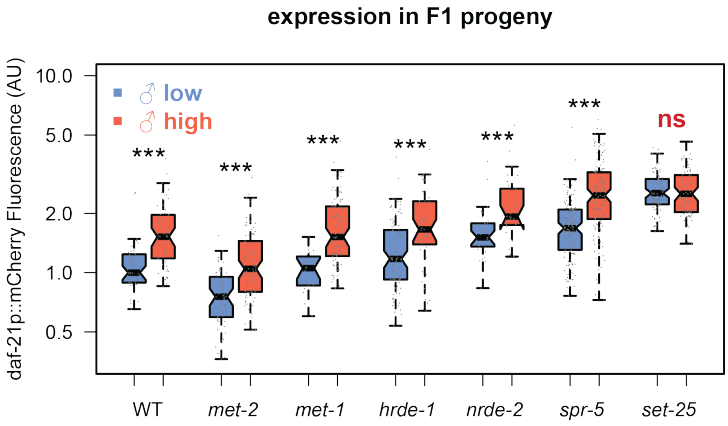

Figure 38. MET-1, MET-2, HRDE-1, NRDE-2 and SPR-5 are not required maternally for the transmission of a temperature-induced change in *daf-21p::mCHERRY* expression to subsequent generations. Experimental design as in Figure 37. The WT and *set-25* data are same as in Figure 37 and plotted for comparison. P values: *** p< 0.001, ns p> 0.05 (Wilcoxon rank test). Y axis in log scale.

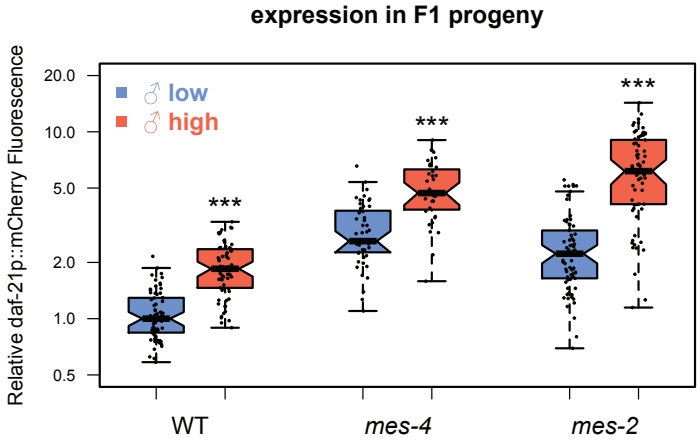

Figure 39. MES-2 and MES-4 are not required maternally for the transmission of a temperature-induced change in *daf-21p::mCHERRY* expression to subsequent generations. Phenotypically genotyped *mes-2* and *mes-4* homozygous progeny of *mes-2*/+ and *mes-4*/+ parents were used for the crosses. Phenotypic identification of the genotypes was possible due to linkage of the mutant *mes-2* and *mes-4* alleles to recessive alleles that cause Uncoordinated and Dumpy phenotypes respectively. P values: *** p< 0.001 (Wilcoxon rank test). Y axis in log scale.

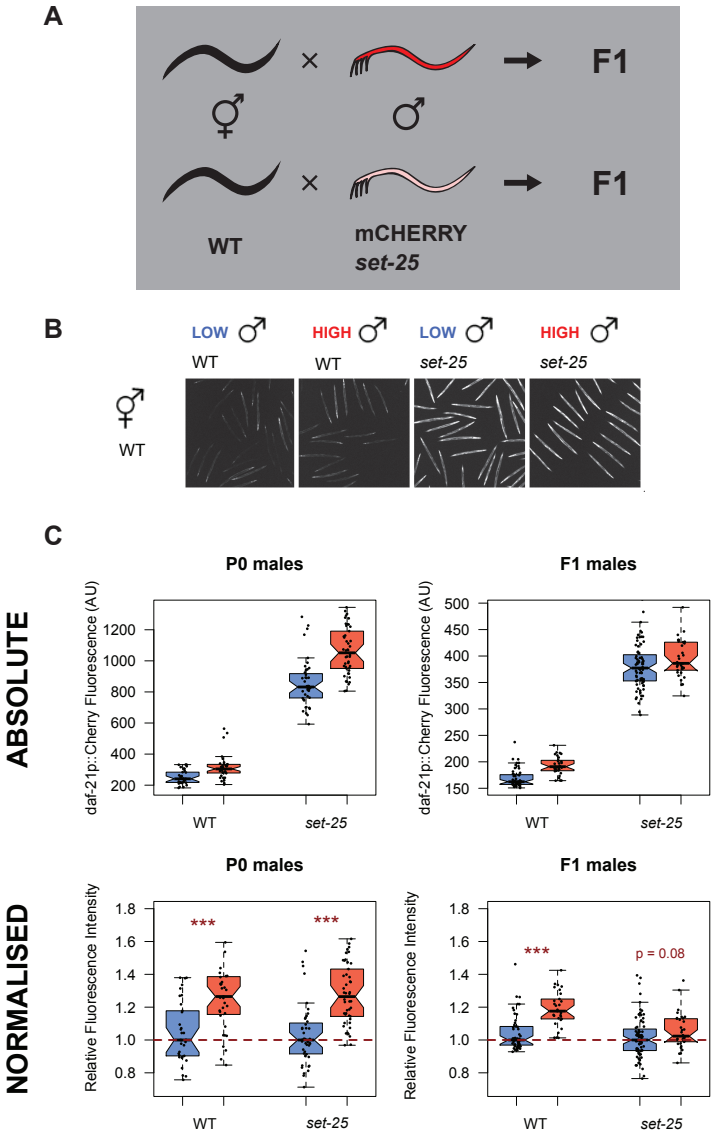

Figure 40. SET-25 is required parentally for inheritance of an environmentally-triggered expression change. (A) Schematic diagram of the experiment. 'High' cohorts was generated by growing either WT or *set-25* males at 25 °C for 48 hours, then shifted back to 20 C before mating. "Low" males were kept at 20 °C. Expression was measured in F1 males. (B) Example images of F1 males. (C) Growth of parental *daf-21::mCHERRY* male worms at 25 °C resulted in 1.18 fold increase in the F1 offspring (P=4.2e-8, Wilcox rank test), while *set-25 (-)* mutant males carrying the transgene show reduced temperature-induced differences in expression to the following generation at a significant level (1.02 fold change, P=0.08, Wilcox rank test).

To establish whether SET-25 is also required in parents to transmit the epigenetic memory, we used mutant *set-25* males carrying the transgene and generated 'high' and 'low' populations by growing the worms at 25 °C and 20 °C for one generation. We crossed these worms to wild-type hermaphrodites and quantified the expression in the F1 males (Figure 40A). While control males generated in the same manner transmitted the 'high' expression efficiently to the next generation, the *set-25* mutant males did not (Figure 40C). Thus, SET-25 activity is required both in parents to establish the epigenetic memory and in their progeny embryos to maintain it.

2.2.4 H3K9me3 is reduced on the transgene in early embryos with an ancestral high temperature history

set-25 encodes a putative H3K9me3 methyl-transferase (Andersen and Horvitz, 2007). No H3K9me3 is detected in *set-25* mutants indicating that it is required for all H3K9me3 deposition in *C. elegans* (Towbin et al., 2012). H3K9me3 is associated with silenced heterochromatin and is often enriched on repetitive multi copy transgenes in *C. elegans* (Bessler et al., 2010; Meister et al., 2011). Since SET-25 is required for the transmission of expression state across generations (Figure 32 and 35), we asked if the H3K9me3 levels on the transgene array were altered by the ancestral environment. We took worms grown at 16 and 25 °C and shifted them as L4s to 20 °C. Four days later, the second generation of embryos were harvested, fixed and co-stained with an antibody that specifically recognizes the H3K9me3 epitope and a DNA FISH probe that is complementary to the sequence of the *daf-21p::mCHERRY* transgene. The progeny of the low-expressing parents, whose ancestors were bred at 16 °C, have an increased level on H3K9me3 mark on the transgene compared to the progeny of high-expressing worms, with ancestral history of 25 °C (Figure 41). Importantly the differences are apparent in the very early

embryos, before the onset of transgene activation (Figure 41 and Figure 15).

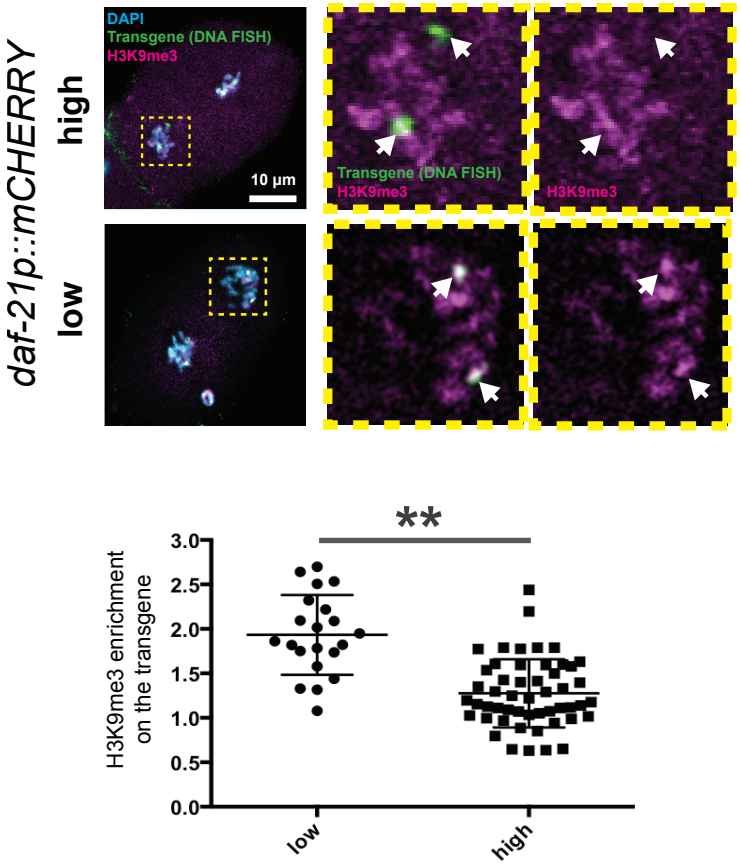

Figure 41. H3K9me3 mark is depleted from the transgene locus in the early embryos extracted from daf-21p::mCHERRY 'high' parents. Early embryos extracted from either 'high' or 'low' *daf-21p::mCHERRY* worms were fixed and stained with DAPI (blue), anit-H3K9me3 (pink) and a DNA FISH probe complementary to mCHERRY (green). H3K9me3 at the transgene was quantified by dividing the H3K9me3 signal intensity within the transgene foci stained by FISH by the signal of the entire nucleus defined by DAPI staining. Only two- and four-cell stage embryos were used for this analysis. White arrows indicate the transgene locus identified through DNA FISH staining.

Immunofluorescence staining, imaging and quantification were carried out by a colleague, Cristina Hidalgo.

2.2.5 Small RNAs restore the level of transgene repression after environmental perturbation

Since small RNAs have been implicated in transgenerational response to environment (Rechavi et al., 2014) and are proposed to act upstream of SET-25 in gene silencing (Ashe et al., 2012b) we sequenced the small RNA content in animals derived from different ancestral environments. We generated large populations of 'high' and 'low' worms by rearing them at 25 and 16 °C respectively, then shifted the populations as L4 larvae to 20 °C and propagated each population for two more generations before harvesting RNA from gravid adult animals (Figure 42A). The RNA samples were used to generate small RNA libraries followed by sequencing with Illumina's HiSeq 2000 in three biological replicates for both 'high' and 'low' populations. We found abundant small RNA molecules antisense to the transgene, with about 50 % of these reads targeting mCHERRY coding sequence (Figure 42B and Table 2). About half of the sRNAs (small RNAs) targeting the mCHERRY sequence as well as the entire transgenic construct belonged to the 22G class of small RNAs (Table 2). Comparing the two experimental conditions revealed that the 'high' population, which inherited an elevated transgene expression from environmentally perturbed ancestors, also had higher levels of 22G RNAs (in proportion of the total 22G RNA content) antisense to mCHERRY (Figure 42D). This was surprising because positively acting 22G RNAs bound to the CSR-1 argonaute only target genes expressed in the germline (Claycomb et al., 2009) and our multi-copy transgene is silenced in the germline (Figure 7). We found no difference in the relative abundance of the other 2 endogenous small RNA classes, the 21U RNAs and the 26G RNAs (Figure 42D). In fact only several 26G and a few dozen 21U reads mapped to the mCHERRY sequence, suggesting that these two classes are not relevant for transgenerational control of *daf-21p::mCHERRY* transgene expression.

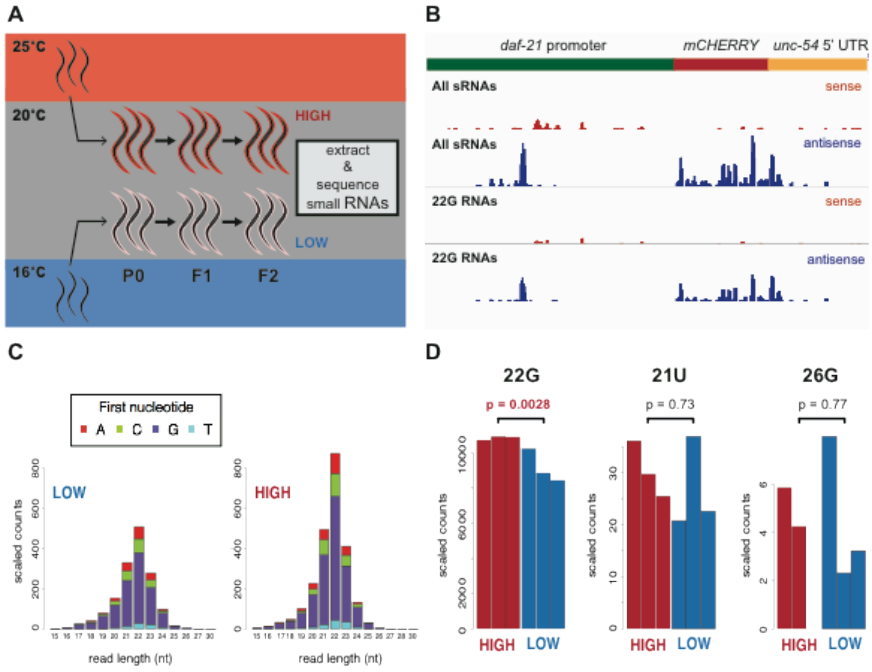

Figure 42. 22G small RNAs antisense to *mCHERRY* are more abundant in *daf-21p::mCHERRY* 'high' worms. (A) Worms kept at 16 and 25 °C were shifted to 20 °C, and propagated for two more generations in three replicates for each condition. Synchronized gravid F2 worms were used for total RNA extraction followed by library preparation and sequencing. (B) Profile of unique small RNA reads mapped to the *daf-21p::mCHERRY* construct in one of the 'high' samples. Multiple small RNA molecules mapped antisense to mCHERRY coding sequences were found, the majority of which belonged to the 22G RNA class. Profiles were very similar for all 6 samples but differed in the quantity of mapped reads between 'high' and 'low' cohorts (see panel C and D). (C) Distribution of small RNA molecules antisense to mCHERRY coding sequence according to relative abundance, size and the first nucleotide. Each profile corresponds to one of the experimental replicates. (D) Comparison of different classes of RNAs defined by length and the first nucleotide between the two conditions. Worms with higher *daf-21p::mCHERRY* expression contain more 22G RNAs targeting the mCHERRY coding sequence. Data for each replicate is plotted. Counts were scaled using the sequencing depth estimated by microRNA expression. P-values calculated using Wald test.
Library preparation and sequencing was performed by the CRG Genomics Facility. Small RNA sequence analysis was carried out by a collaborator, Eduard Casas from Tanya Vavouri's Laboratory at IMPPC, Badalona, Spain.

	high 1	high 2	high 3	low 1	low 2	low 3
Number Total Reads	10838054	13198335	15292980	11811422	15992687	13579245
22G Reads	2313037	2809256	3010761	1678310	3304500	2366283
Mapped Reads	33941100	40815005	49155884	45314572	49185456	46439356
22G Mapped Reads	4523459	5376621	5814368	3322445	6249540	4467723
MIRNA-estimated seq.depth	1.024	1.176	1.215	0.627	1.299	0.929
Reads antisense of transgene	38675	46332	48604	23370	45548	31392
22G Reads antisense of transgene	20152	24174	24955	12073	23112	15938
Reads antisense of mCHERRY	21992	25589	27073	12572	23329	15591
22G Reads antisense of mCHERRY	10981	12854	13249	6425	11503	7842
Scaled 22G Reads antisense of mCHERRY	10724	10930	10903	10245	8855	8441
22G / total sRNA	0.213	0.213	0.197	0.142	0.207	0.174
mapped 22G / mapped total sRNA	0.133	0.132	0.118	0.073	0.127	0.096
22G transgene / total transgene sRNA	0.521	0.522	0.513	0.517	0.507	0.508
22G mCHERRY / total mCHERRY sRNA	0.499	0.502	0.489	0.511	0.493	0.503

Table 2. Antisense small RNAs (sRNA) that target the transgene are enriched in 22G RNAs. Number of reads for each of the replicated samples in each category is given. Between 9.6 % and 13.3 % of the mapped genomic small RNAs belong to the 22G class. Between 49.3 % and 51.1 % of sRNAs antisense to mCHERRY sequence belong to the 22G RNA class.

To test whether the small RNAs enriched in the 'high' worms are repressive, we crossed either 'high' or 'low' heterozygous *daf-21p::mCHERRY* feminized worms to males homozygous for *daf-21p::mCHERRY* (Figure 44). We then measured expression from the transgene in the F1 progeny of the two crosses and *a posteriori* determined the genotype of each F1 adult worm by observing the segregation of the transgene in their F2 progeny. While only half of the progeny of each cross will be homozygous for the transgene, inheriting it from both parents, all of the progeny will inherit the maternally supplied small RNAs (Figure 45). Comparing the level of transgene expression between heterozygous F1 animals derived from 'high' and 'low' mothers thereofore allowed us to test whether the maternally supplied small RNAs have any effect on the paternally derived transgene. Consistent with the small RNAs acting to repress expression from the transgene, we found that the heterozygous progeny of 'high' mothers had lower expression of the paternally inherited transgene than the heterozygous progeny of 'low' mothers (Figure 45). The homozygous progeny inherited a transgene from both parents, therefore fluorescence intensity is due to activity of

both alleles. We found that the progeny of 'high' mothers have higher transgene expression than those of the 'low' mothers (Figure 45). This was not surprising, since our previous experiments demonstrated that shifting worms from high to low temperature requires multiple generations to reset the expression level.

It is possible that the repressive effect observed in the heterozygous progeny is mediated by differential loading of an upstream transcription factor or another *trans*-acting factor, rather than the small RNAs. To discriminate between these scenarios, we crossed heterozygous *daf-21p::mCHERRY* 'high' and 'low' hermaphrodites to worms carrying a GFP reporter driven by an identical *daf-21* promoter region and 5' untranslated region (Figure 46). We measured and compared the fluorescence in the F1 progeny of the two crosses using time-lapse microscopy and found the same expression of the paternally derived *daf-21p::GFP* transgene, regardless of having inherited the *daf-21p::mCHERRY* transgene or not (Figure 46). This shows that the repressive element is sequence specific. Taken together, our results demonstrate a *cis*-acting signal that correlates with the expression state of the parent and a similarly correlated *trans*-acting sequence-specific silencing signal.

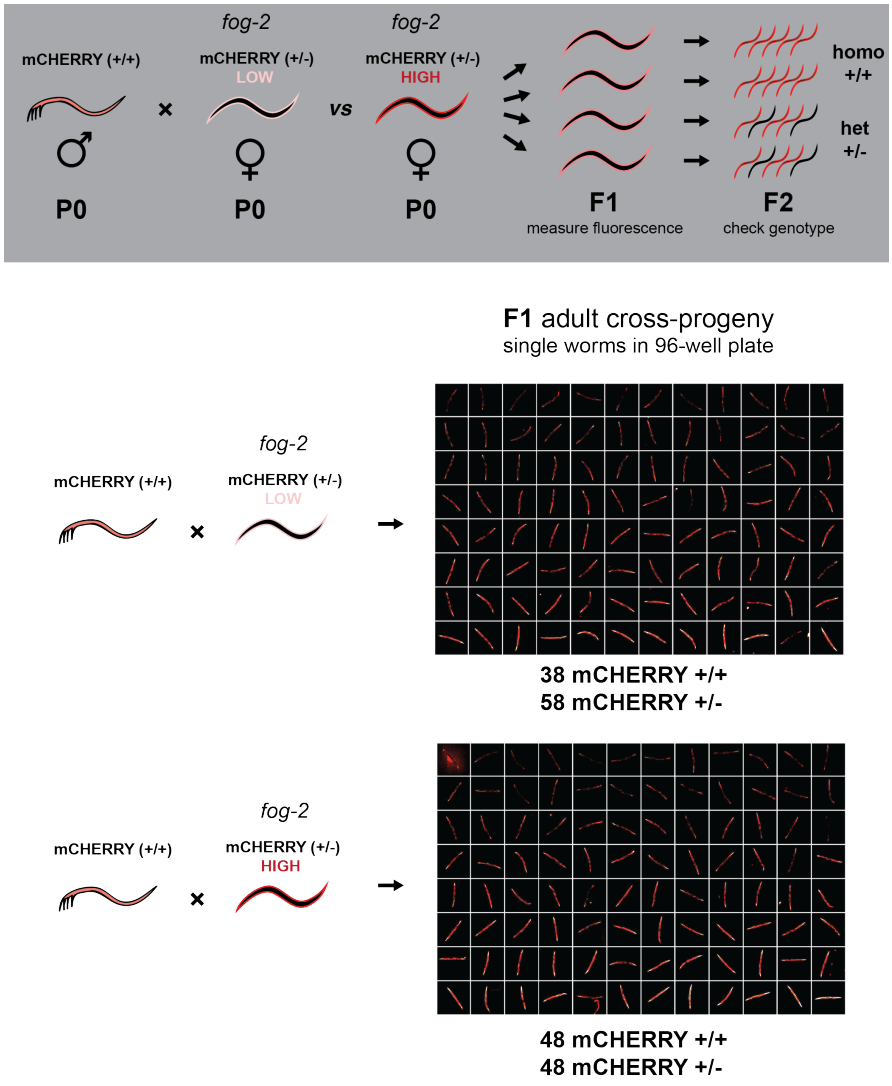

Figure 44. Diagram of the experiment designed to measure the effect of maternally inherited *trans*-acting molecules on the paternally inherited transgene. Males carrying the *daf-21p::mCHERRY* transgene were crossed to heterozygous *fog-2* feminized hermaphrodites conditioned ancestrally with temperature to express either high or low levels of *daf-21p::mCHERRY*. Between 105-120 F1 hermaphrodite L4 progeny were picked to single wells three days later from each of the two crosses. The worms were allowed to develop into adults and lay F2 embryos for one day. The F1 adults were then removed and imaged in single wells. The genotype of each F1 worm was determined *a posteriori* as heterozygous or homozygous for the reporter based on the *daf-21p::mCHERRY* fluorescence in the F2 populations.

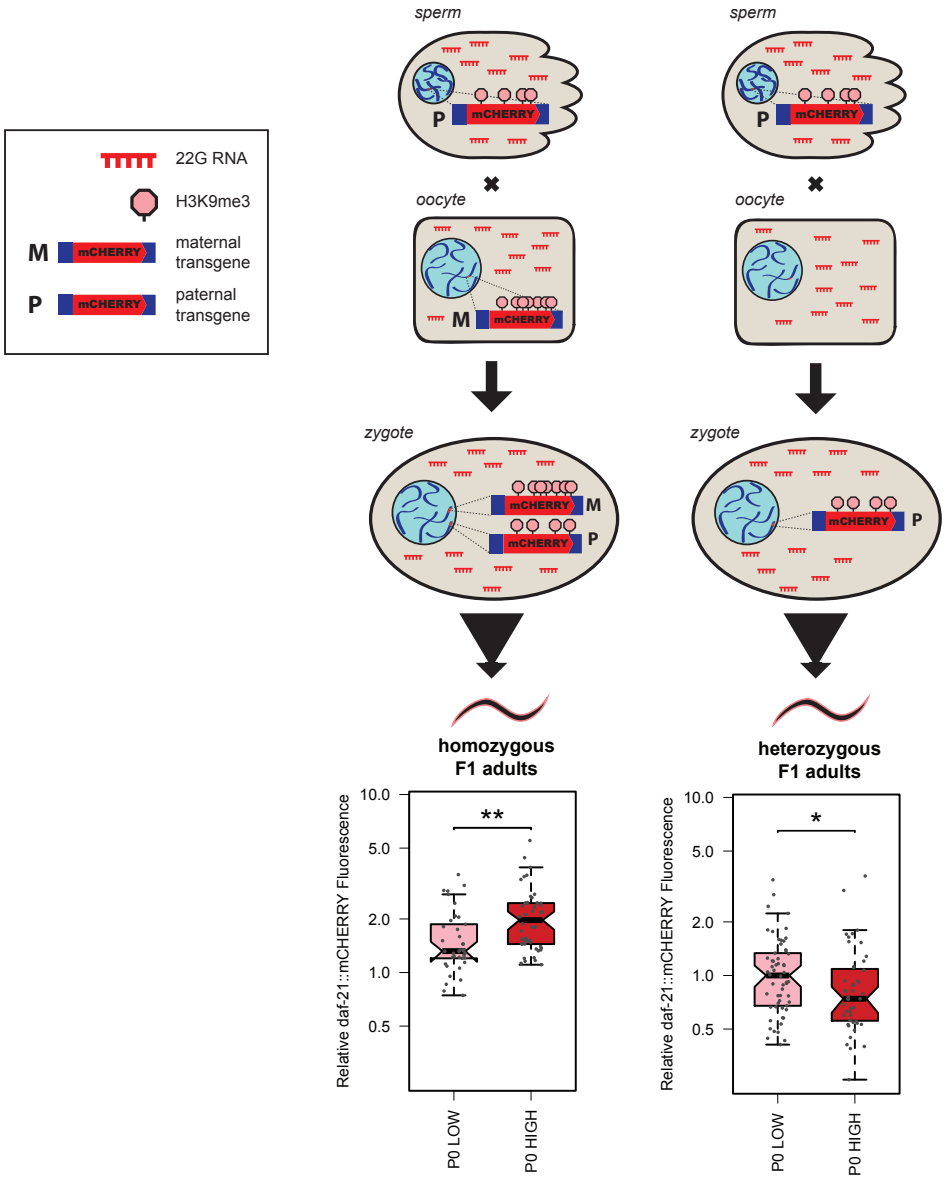

Figure 45. Evidence for a heritable, silencing *trans*-acting signal that correlates with transgene expression in parental generation. The heterozygous F1 worms, which inherited the transgene only from the father, have a 1.35 times lower expression (P=0.029, Wilcox rank test) if the mother had 'high' expression compared to if the mother had 'low' expression. Homozygous F1 cross-progeny of the 'high' mother are 1.49 times brighter (P=9.3e-4, Wilcox rank test) than those of the 'low' mother. Y axis in log scale.

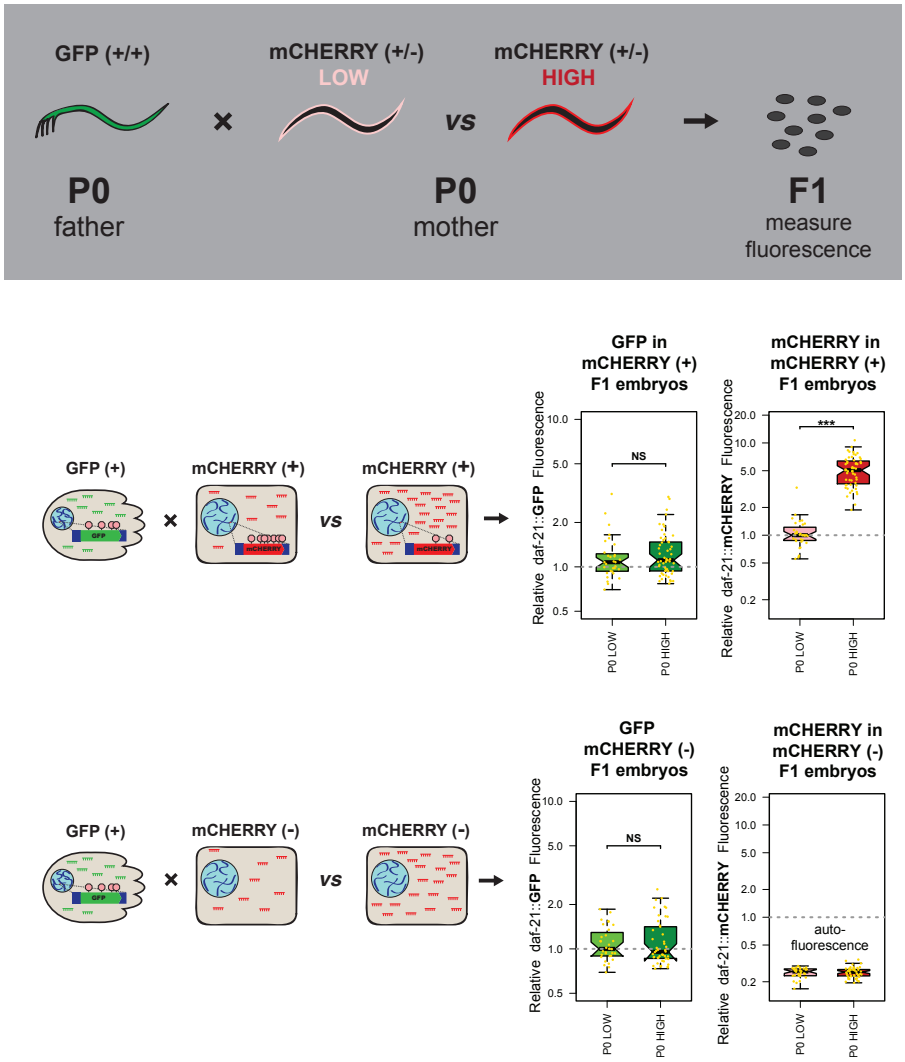

Figure 46. Heritable trans-acting signal is sequence-specific. Male worms carrying *daf-21p::GFP* transgene were crossed to high or low heterozygous *daf-21p:mCHERRY* hermaphrodites. One day later, embryos were extracted from each cohort and expression of both GFP and mCHERRY measured using time-lapse microscopy. Cross-progeny were identified based on the presence of GFP. Expression of GFP and mCHERRY in the late stage of embryogenesis (about 3-fold stage) was compared between offspring sired by high and low mothers. No significant effect on the paternal *daf-21::GFP* transgene was detected. P values: **** $p < 0.0001$, ns $p > 0.05$ (Wilcoxon rank test). Y axis in log scale.

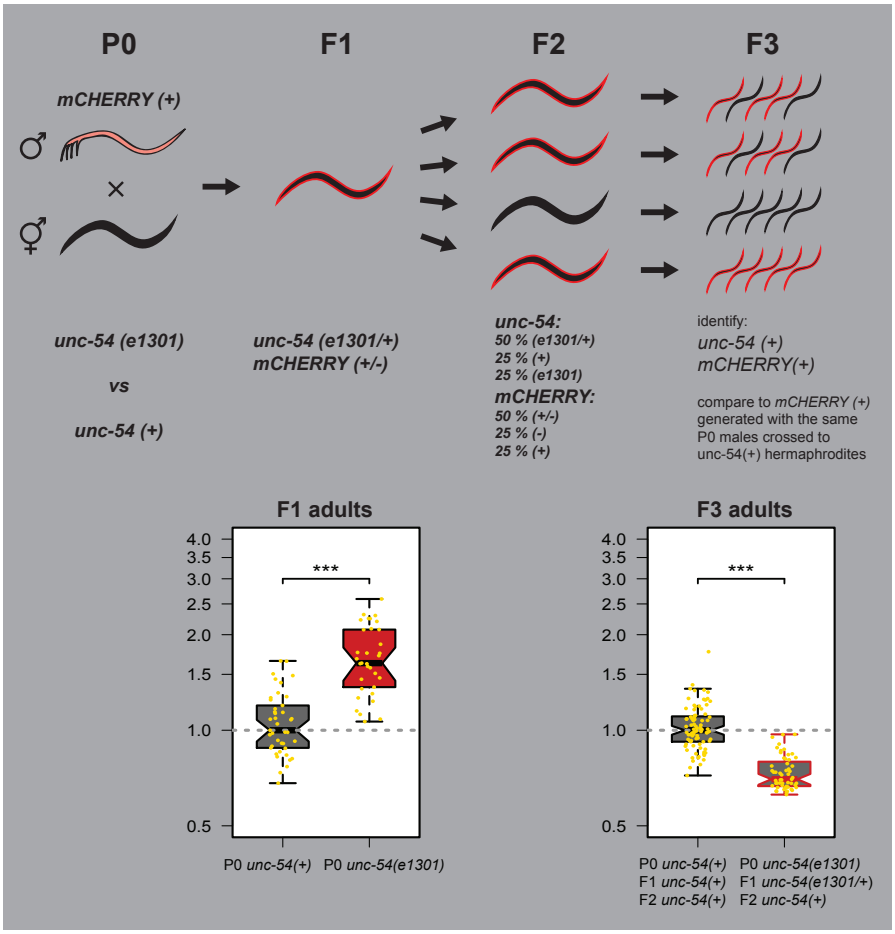

Figure 47. Transient transgene up-regulation in the soma results in decreased expression in the future generations. Males carrying the *daf-21p::mCHERRY* transgene were crossed to *unc-54(e1301)* mutant worms. A 1.63 fold up-regulation was observed in the heterozygous F1 progeny (P=3.2e-11, Wilcoxon rank test) resulting form muscle-specific up-regulation (see Figure 48). The *unc-54(e1301)* mutation was removed by letting the F1 worms self and picking multiple F2 worms to single wells. The F2 worms were allowed to self and produce F3 progeny. Based on the presence of uncoordinated behavior and the distribution of transgene expression, worms homozygous for *daf-21p::mCHERRY* and not carrying the *unc-54* mutation were identified and their fluorescence measured. *unc-54(+)* F3 progeny of the *unc-54*(e1301/+) F1 worms were 1.43 x less bright (P=2.9e-19, Wilcoxon rank test) than the *unc-54(+)* progeny of the *unc-54(+)* ancestors. To generate controls for each measurement, P0 *daf-21p::mCHERRY* males were crossed in parallel to wild-type *(unc-54(+))* worms and propagated across the generations in the same manner as the *unc-54(e1301)* mutant worms. Y-axis in log scale.

2.2.6 Increased transgene expression in somatic tissues results in decreased expression in subsequent generations

Expression from the multi-copy *daf-21p::mCHERRY* transgene array is only detected in somatic tissues. However, the epigenetic modification evoked by increased temperature must be transmitted through the germline to the next generation. We therefore tested whether up-regulation of the transgene only in somatic cells was sufficient to mount a heritable change in expression. To trigger increased *daf-21p::mCHERRY* expression in somatic cells we crossed males carrying the reporter to a hermaphrodites carrying a dominant mutation in the muscle myosin heavy chain gene *unc-54* that results in protein misfolding and upregulation of the *daf-21* promoter (van Oosten-Hawle et al., 2013). Muscle-specific up-regulation of the transgene was evident in the heterozygous *unc-54(e1301/+)* F1 progeny of the cross (Figure 47), with transgene expression in other tissues indistinguishable from that in control animals (Figure 48). We isolated individual F2 progeny of the self-fertilizing F1 hermaphrodites and allowed them to grow and produce progeny. We then identified the populations where the *e1301* allele had been lost and *daf-21p::mCHERRY* transgene fixed (Figure 47 and 48) based on the locomotion phenotype and mCHERRY fluorescence. We found that the wild-type offspring of the *e1301/+* worms had decreased levels of transgene expression compared to control animals generated in an analogous procedure by mating the same cohort of P0 males with wild-type P0 mothers (Figure 47 and 48). This shows that up-regulation of the transgene in the soma alone does not lead to an increased expression in subsequent generations. On the contrary, the experiment shows that up-regulation of the transgene in muscle cells generates a repressive signal that is transmitted through the germline to subsequent generations.

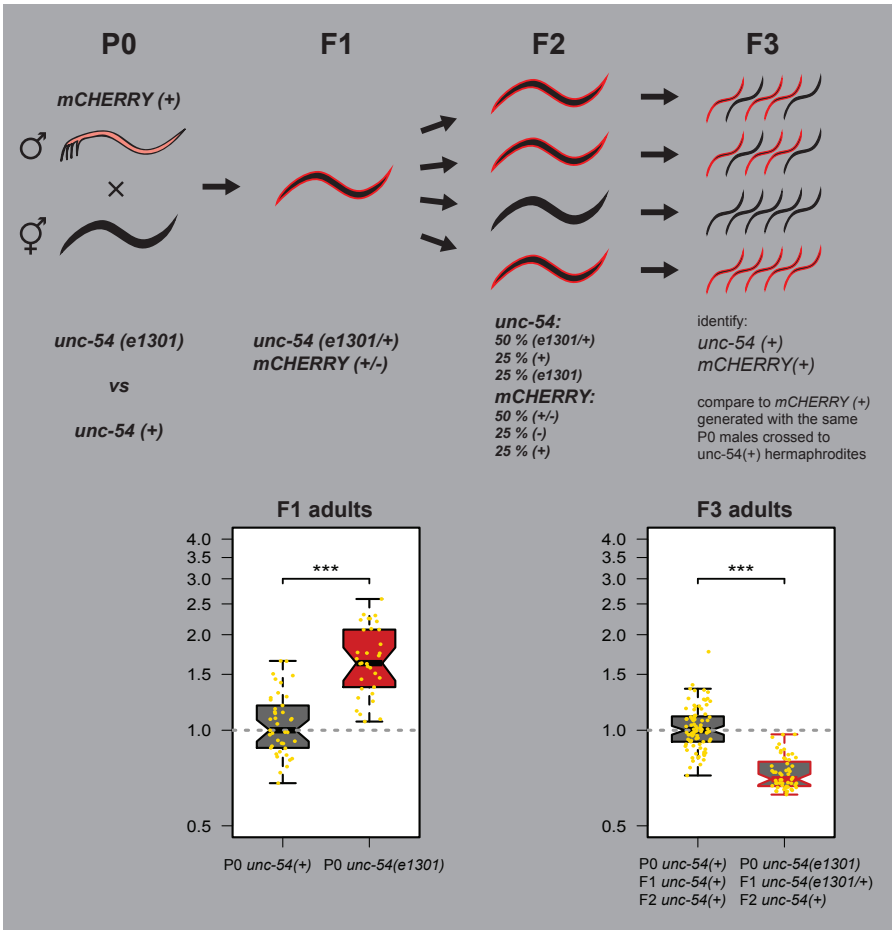

Figure 47. Transient transgene up-regulation in the soma results in decreased expression in the future generations. Males carrying the *daf-21p::mCHERRY* transgene were crossed to *unc-54(e1301)* mutant worms. A 1.63 fold up-regulation was observed in the heterozygous F1 progeny (P=3.2e-11, Wilcoxon rank test) resulting form muscle-specific up-regulation (see Figure 48). The *unc-54(e1301)* mutation was removed by letting the F1 worms self and picking multiple F2 worms to single wells. The F2 worms were allowed to self and produce F3 progeny. Based on the presence of uncoordinated behavior and the distribution of transgene expression, worms homozygous for *daf-21p::mCHERRY* and not carrying the *unc-54* mutation were identified and their fluorescence measured. *unc-54(+)* F3 progeny of the *unc-54*(e1301/+) F1 worms were 1.43 x less bright (P=2.9e-19, Wilcoxon rank test) than the *unc-54(+)* progeny of the *unc-54(+)* ancestors. To generate controls for each measurement, P0 *daf-21p::mCHERRY* males were crossed in parallel to wild-type *(unc-54(+))* worms and propagated across the generations in the same manner as the *unc-54(e1301)* mutant worms. Y-axis in log scale.

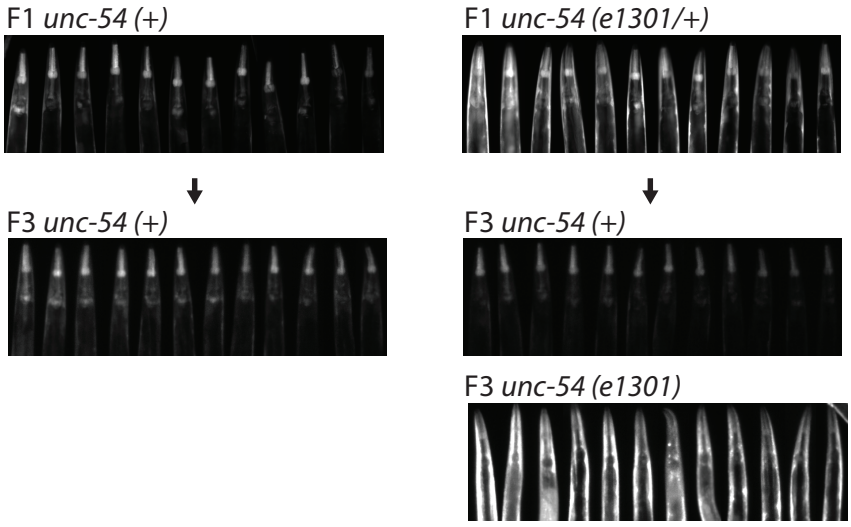

Figure 48. Example images of worms from experiment outlined in Figure 47. In F1 *unc-54 (e1301/+)* worms up-regulation is evident only in body wall muscles. The F3 *unc-54 (+)* descendants of the *unc-54 (e1301/+)* grandparents experience decreased expression in all tissue. Mutant F3 *unc-54 (e1301)* homozygotes show a pronounced increase in expression only in muscle tissues and appear to have reduced expression in the pharynx compared to wild type.

2.2.7 Transport of dsRNA from soma to germline enforces transgene repression affecting expression in the following generations

In *C. elegans* dsRNA can spread between tissues and mediate systemic silencing (Winston et al., 2002). Import of the dsRNA into cells require a dsRNA channel SID-1 (Shih and Hunter, 2011; Winston et al., 2002). Recently, it was shown that somatically expressed dsRNA molecules can trigger an HRDE-1-mediated silencing of homologous sequences in the *C. elegans* germline and that initiation, (but not maintenance) of this repression requires SID-1 (Devanapally et al., 2015). We found that inactivation of *sid-1* had no effect on the steady state expression of the multi-copy transgene at 20 °C (Figure 29). In addition, shifting *sid-1* mutant animals to 25 °C for 48 hours during development from the embryo to L4/young adult stage resulted in increased expression, similarly to that observed in control animals (Figure 49 and Figure 11). However, upon returning these animals to 20 °C and quantifying transgene expression in subsequent generations we found that the multi-generational recovery of expression level to baseline levels was delayed (Figure 49). That is, *sid-1* mutants showed a prolonged trans-generational memory of the transient environmental exposure.

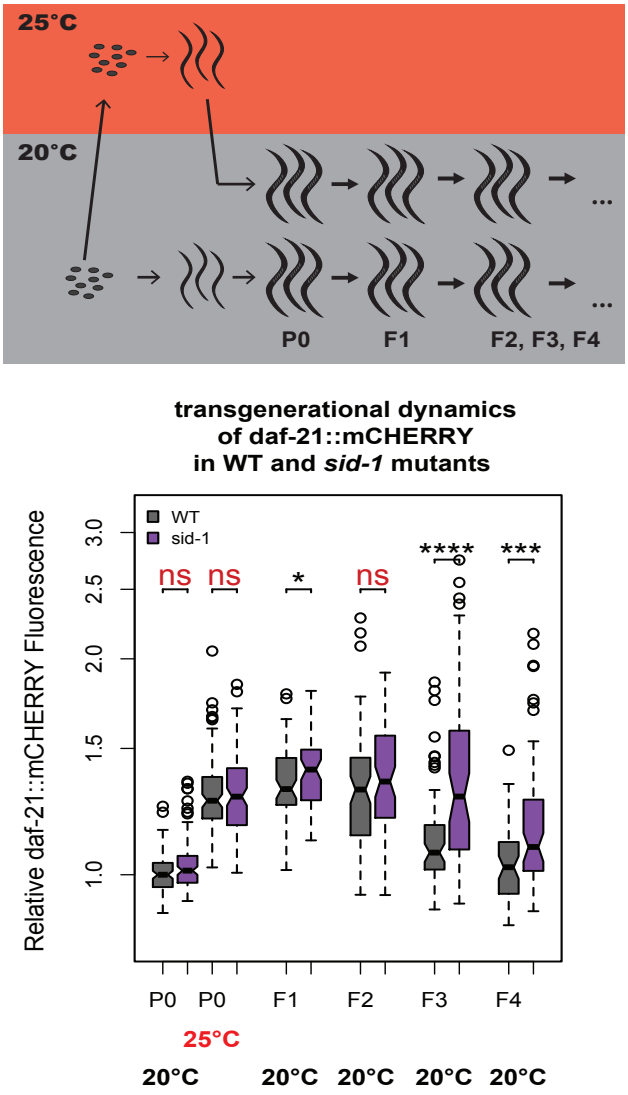

Figure 49. SID-1 is required for robust recovery of *daf-21p::mCHERRY* repression after environmental perturbation. Worms carrying *daf-21p::mCHERRY* transgene in WT or *sid-1(qt2)* background were grown at 25 °C from embryo to L4 stage, then shifted back to 20 °C. Expression was monitored in four subsequent generations. Wild type worms kept at 20 °C were used as reference for normalization in each generation. No significant difference was observed between WT and *sid-1* mutant worms kept constantly at 20 °C. Y-axis in log scale.

2.2.7 An incoherent feed forward loop involving chromatin and small RNAs governs transgenerational dynamics of transgene expression

We show here that transgenerational dynamics of transgene expression in response to temperature change exhibits hysteresis. When the worms are shifted from 20 to 25 °C the increase in expression is relatively fast reaching its maximum at the third generation (Figure 9). Upon return to 20 °C, expression drops more gradually and needs 15 generations to reach a steady state (Figure 10). Therefore the expression state in any individual animal depends not only on the current temperature, but also on the expression state of the preceding generation.

Which molecules confer this memory? Temperature shift experiments identified several candidate genes required for the inheritance process: *set-25, hrde-1, met-1, met-2, nrde-2, mut-7* (Figures 32 and 33). However, expression of the transgene is constitutively high in all of these mutants at 20 °C (Figures 29 and 30) with similar or higher fluorescence intensity as the wild-type strain at 25 °C (compare Figure 6 and 29). This confounds our ability to test the effect of these mutations on inheritance using a temperature shift assay. Therefore we performed a second assay based on sorting (Figure 35 and 36) and found that only *set-25* mutants failed entirely to transmit the differential expression state from one generation to the next (Figure 35). We show that SET-25 is required maternally to transmit the expression state of the paternal allele indicating that it acts in the embryo to receive and re-establish the epigenetic memory (Figure 37), and also that it is needed in the environmentally perturbed fathers to establish the epigenetic memory that is passed to their progeny (Figure 40). SET-25 mediates deposition of H3K9me3 (Towbin et al., 2012) and we find that repression of the transgene in the soma correlates well with amount of H3K9me3 on the transgene in the early

embryo before activation of zygotic transcription (Figure 41). Together this points to H3K9me3 as the transgenerational carrier of the epigenetic state. We also show that the high somatic expression state is associated with a higher amount of small RNAs targeting the transgene and genetic experiments indicate that they are inherited and repressive (Figures 45 and 46). Therefore we postulate that heritable small RNAs that act in *trans* are responsible for the progressive restoration of transgene repression over multiple generations. Consistent with the role of 22G RNAs in mediating transgenerational silencing, we find that the transgene is upregulated in *hrde-1* and *nrde-2* mutants (Figures 29 and 30).

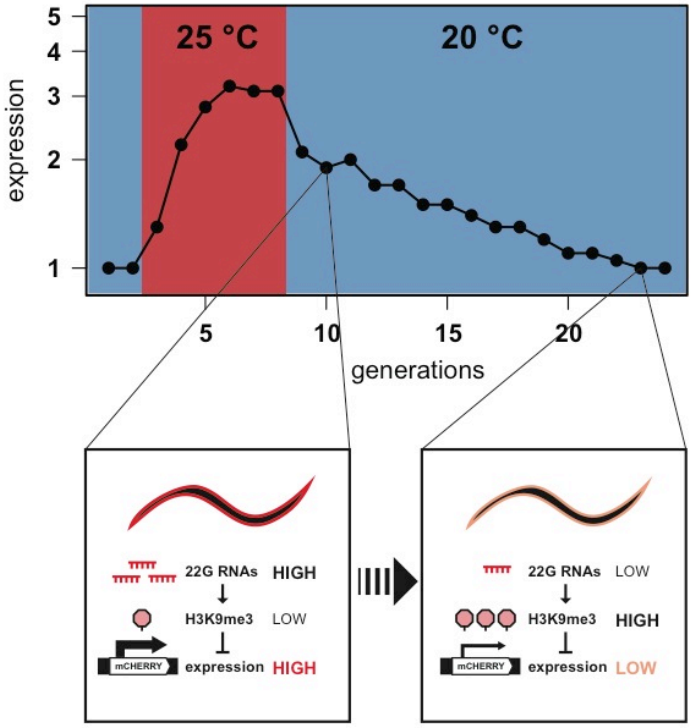

Figure 50. Summary model for transgenerational dynamics of *daf-21p::mCHERRY* transgene expression.

Moreover, increasing transgene activity specifically in a somatic tissue appears to have a repressive effect on subsequent generations (Figure 47). We propose that somatic expression of the transgene is accompanied by production of mobile dsRNA that migrate to germline through SID-1 channel providing substrate for the nuclear RNAi pathway that controls the level of transgene repression. In agreement with this hypothesis, *sid-1* mutant worms exhibit a delayed recovery after environmental perturbation. Therefore an in creased expression of a repetitive transgene in somatic cells generates a negative feedback on the expression in subsequent generation through interaction with the germline creating an incoherent feed-forward motif (Figure 51).

The defining characteristic of an incoherent feed forward motif is that the same input generates two conflicting signals, one activating and one inhibitory, that converge on the same output (Kashtan et al., 2004). If the negative/inhibitory experiences a delay in arrival at the output such a motif is going to generate a biphasic behavior characterized by a sharp increase followed by a gradual decay of the output signal (Kim et al., 2008). When the transgene is depleted from H3K9me3 as a result of high temperature, somatic expression increases, which simultaneously boosts the production of the repressive dsRNA. This promotes acquisition of the H3K9me3 on the transgene via the nuclear RNAi pathway, leading to gradual restoration of the repressed state (Figure 51).

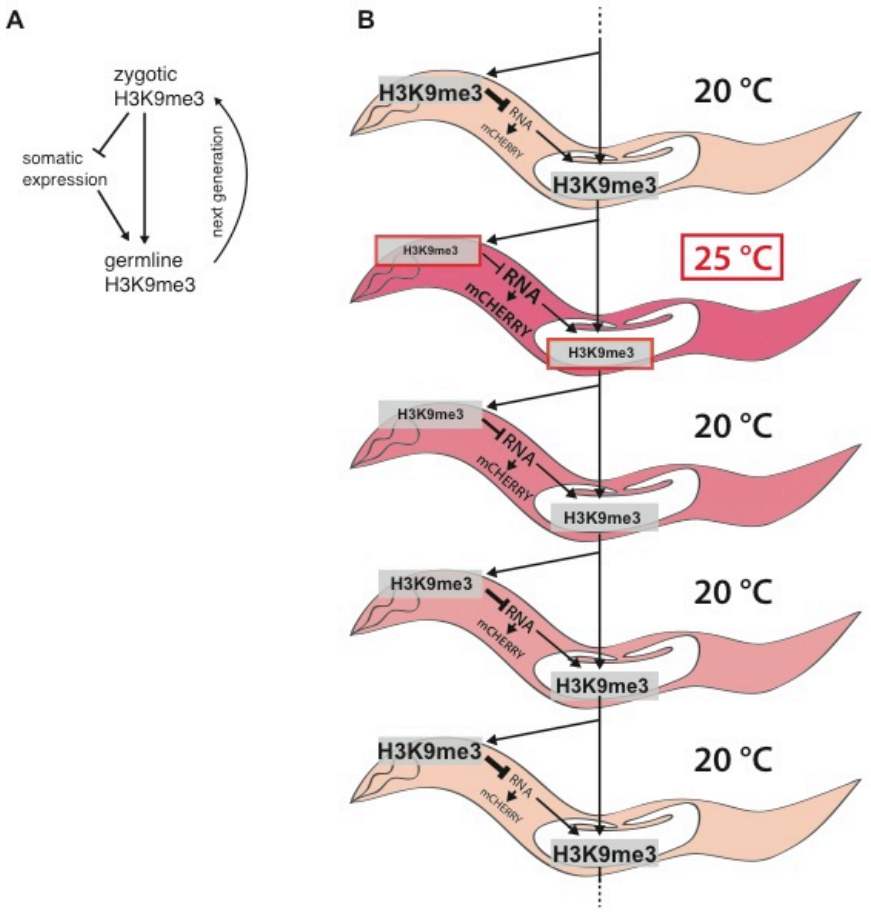

Figure 51. An incoherent feed-forward loop between the soma and germline participates in the recovery of transgene repression (A) The level of H3K9me3 on the transgene array determines its expression in the soma and is inherited across generations. High H3K9me3 decreases somatic expression of the transgene. Somatic expression promotes deposition of additional H3K9me3 in the germline affecting expression in the following generation and contributing to the re-establishment of repression over multiple generations (B).

PART II

3.1 Replication stress interferes with epigenetic inheritance

3.1.1 Identification of genes that regulate expression of a multi-copy transgene

In a large scale RNAi screen we identified a large number of genes that when depleted, altered the expression of a multi-copy *hsp-16.2p::GFP* transgene in embryos in response to a heat shock (Figure 51). The screen was carried out in a 96-well format with liquid feeding based on (Lehner et al., 2006) and was a secondary screen aimed to discard the false positives from a list of putative regulators of cellular reprogramming generated in a different research project. (In that project we identified 292 primary hits through screening through a sub-library, which contained all genes whose knockdown resulted in any type of phenotype reported in (Kamath et al., 2003) and (Simmer et al, 2003)). Expression of GFP in embryos trapped in killed gravid adults was determined after 1h heat shock at 34 °C and 2 h incubation at 20 °C through visual inspection. We identified 35 genes whose inactivation resulted in increased GFP expression (Table 3) and 87 genes that showed a decreased expression after heat shock (Table 4). The genes that reduced expression of *hsp-16.2p::GFP* (Table 4) included many essential genes required for general transcription such as *tbp-1, ama-1*, and *cpsf-2* and likely affect global transcription. Other hits could potentially be positive regulators of transgene expression that participate in the loss of multi-copy transgene repression during embryonic development. Our list of putative 'transgene activators' includes multiple subunits of the nuclear pore complex and 2 subunits of SWI-SNF chromatin remodeling (Table 4). The screen also identified the master regulator of the heat shock response, HSF-1.

Therefore the list might include other specific regulators of the heat shock response that act similar to HSF-1. On the other hand, genes whose knockdown results in a stronger activation of *hsp-16.2p::GFP* after heat shock could either be negative regulators of the heat shock response or affect expression of multi-copy transgenes, possibly through interfering with epigenetically inherited memory of transgene repression described in the previous chapter.

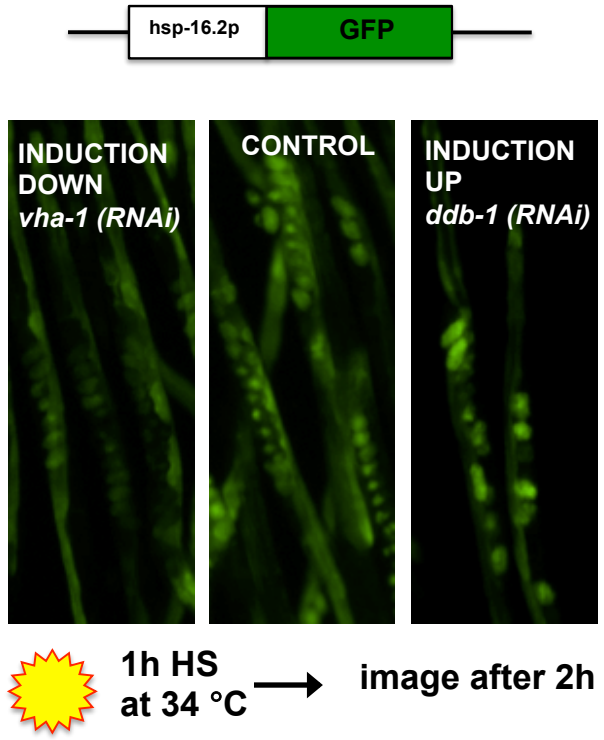

Figure 51. A large scale screen identifies genes that affect heat-induced expression of the *hsp-16.2p::GFP* multicopy reporter in the embryos. Example images obtained in the screen. Embryos are trapped in gravid worms killed by a brief exposure to bleach. One hour heat shock at 34 °C induced expression of *hsp-16.2p::GFP* and its strength, relative to control animals (fed with control RNAi) was used to discover regulators of transgene expression.

Category	Gene	Description
Cell Cycle	fzy-1	Anaphase promoting complex, Cdc20, Cdh1, and Ama1 subunits
Cell Cycle	gld-2	Catalytic subunit of a cytoplasmic poly(A) polymerase (PAP)
Cell Cycle	egg-5	A phosphatase required for egg to embryo transition
Chaperone	hsp-1	Molecular chaperones HSP70/HSC70, HSP70 superfamily
Chaperone	hsp-2	Pseudogene homologous to hsp70A
Chromatin	dpy-30	Part of MLL/COMPASS and dosage compensation complex
Chromatin	his-2	H3 histone
Chromatin	his-3	H2A histone
Chromatin	his-42	H3 histone
Chromatin	let-711	Part of CCR4/NOT complex
Chromatin	rba-1	Nucleosome remodeling factor, subunit CAF1/NURF55/MSI1
Chromatin	rbbp-5	Part of MLL/COMPASS complex
Chromatin	swd-2.2	Part of MLL/COMPASS complex
Degradation	pbs-6	20S proteasome, regulatory subunit beta type PSMB1/PRE7
Degradation	pbs-7	20S proteasome, regulatory subunit beta type PSMB4/PRE4
Degradation	rpn-3	26S proteasome regulatory complex, subunit RPN3/PSMD3
Degradation	rpt-6	26S proteasome regulatory complex, ATPase RPT6
Degradation	uba-1	Ubiquitin-activating enzyme
Degradation	ubq-2	Ubiquitin/60s ribosomal protein L40 fusion
DNA Replication	ddb-1	Damage-specific DNA binding complex, subunit DDB1
DNA Replication	div-1	DNA polymerase alpha-primase complex, polymerase-associated subunit B
DNA Replication	F33H2.5	DNA polymerase epsilon, catalytic subunit A
DNA Replication	lrr-1	Leucine Rich Repeat-containing protein required for proper DNA replication
DNA Replication	pole-2	DNA polymerase epsilon, subunit B
DNA Replication	rev-1	Translesion DNA polymerase - REV1 deoxycytidyl transferase
DNA Replication	rfc-3	Replication factor C, subunit RFC3
DNA Replication	rpa-2	Single-stranded DNA-binding replication protein A (RPA), 30 kD subunit
Metabolism	C27D9.1	Orthologous to mutliple human fucosyltransferase genes
Metabolism	mel-32	Glycine/serine hydroxymethyltransferase
Nuclear transport	npl-4.1	Nuclear pore complex, rNpl4 component (sc Npl4)
Nuclear transport	npl-4.2	Nuclear pore complex, rNpl4 component (sc Npl4)
Structural	vab-10	Spectraplakin, required for nuclear and cell migration
Unknown	R53.9	Non-coding RNA
Unknown	F27B3.6	Unknown
Unknown	tag-297	Unknown

Table 3. Genes identified in the screen whose inhibition results in enhanced expression of the multicopy *hsp-16.2p::GFP* reporter in embryos upon heat shock treatment.

Category	Gene	Description
ATPase	unc-32	Vacuolar proton-translocating ATPase (V-ATPase), subunit unc-32
ATPase	vha-1	Vacuolar H+-ATPase V0 sector, subunits c/c'
ATPase	vha-10	Vacuolar H+-ATPase V1 sector, subunit G
ATPase	vha-12	Vacuolar H+-ATPase V1 sector, subunit B
Cell Cycle	car-1	Uncharacterized mRNA-associated protein RAP55
Cell Cycle	cks-1	Cyclin-dependent protein kinase CDC28, regulatory subunit CKS1, and related proteins
Cell Cycle	cyl-1	Ortholog of human CCNL2 (cyclin L2) and CCNL1 (cyclin L1)
Cell Cycle	czw-1	Centromere/kinetochore protein zw10 involved in mitotic chromosome segregation
Cell Cycle	smc-4	Structural maintenance of chromosome protein 4 (chromosome condensation complex Condensin, subunit C)
Cell Cycle	uri-1	Unconventional prefoldin RPB5 (RNA polymerase subunit 5)
Chromatin	his-64	Histone H4
Chromatin	hmg-1.2	HMG box-containing protein
Chromatin	let-526	SWI-SNF chromatin remodeling complex, let-526 subunit
Chromatin	snfc-5	SWI-SNF chromatin remodeling complex, Snf5 subunit
Degradation	cpl-1	Cysteine proteinase Cathepsin L
Degradation	F09D1.1	Spindle pole body protein - Sad1p
Degradation	gei-17	SUMO E3 protein ligase
Degradation	smo-1	C. elegans ortholog of SUMO
Degradation	Y46G5A.4	Ortholog of human SNRNP200 (small nuclear ribonucleoprotein (U5))
Metabolism	F27D4.1	Electron transfer flavoprotein, alpha subunit
Metabolism	gob-1	Trehalose-6-phosphatase
Metabolism	K08E3.5	Ortholog of human UGP2 (UDP-glucose pyrophosphorylase 2)
Metabolism	nrf-6	Predicted acyltransferase
Nuclear Transport	F23F1.5	m3G-cap-specific nuclear import receptor (Snurportin1)
Nuclear Transport	imb-2	Nuclear transport receptor Karyopherin-beta2/Transportin (importin beta superfamily)
Nuclear Transport	npp-1	Nuclear pore complex, p54 component (sc Nup57)
Nuclear Transport	npp-2	Nuclear pore complex component (sc Nup85)
Nuclear Transport	npp-21	Ortholog of human TPR (translocated promoter region, nuclear basket protein)
Nuclear Transport	npp-3	Homologous to mouse nuclear pore complex protein Nup205
Nuclear Transport	npp-6	Homologous to mouse nuclear pore complex protein Nup160
Nuclear Transport	tsr-1	Nuclear transport regulator
Nuclear Transport	xpo-2	predicted to function in nuclear transport of proteins required for mitotic progression
RNA Binding	cfim-2	Putative cleavage and polyadenylation specificity factor
RNA Binding	dpy-22	Thyroid hormone receptor-associated protein complex, subunit TRAP230
RNA Binding	smgl-2	DEAH helicase orthologous to the Drosophila CG12211 protein.
RNA Synthesis	mdt-26	Ortholog of human TCEANC2 (transcription elongation factor A (SII) N-terminal and central domain containing 2)
RNA Synthesis	ama-1	RNA polymerase II, large subunit
RNA Synthesis	C07A9.2	G10 protein/predicted nuclear transcription regulator
RNA Synthesis	C55A6.9	Putative RNA polymerase II regulator
RNA Synthesis	cpsf-2	mRNA cleavage and polyadenylation factor II complex, subunit CFT2 (CPSF subunit)
RNA Synthesis	csr-1	Argonaute protein required for chromosome segregation
RNA Synthesis	ddx-23	U5 snRNP-like RNA helicase subunit
RNA Synthesis	M03C11.7	Putative u4/u6 small nuclear ribonucleoprotein
RNA Synthesis	mex-5	CCCH-type Zn-finger protein, required for assymetry of early embryonic divisiona
RNA Synthesis	taf-10	Transcription initiation factor TFIID, subunit TAF10 (also component of histone acetyltransferase SAGA)
RNA Synthesis	taf-5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor
RNA Synthesis	tbp-1	TATA-box binding protein (TBP), component of TFIID and TFIIIB
RNA Synthesis	Y59A8B.6	HAT repeat protein
Signalling	C32E8.5	Transcriptional regulator SNIP1, contains FHA domain
Signalling	cdk-9	Cyclin T-dependent kinase CDK9
Signalling	inx-14	Innexin-type channels
Signalling	kin-10	Casein kinase II, beta subunit
Signalling	let-92	Serine/threonine protein phosphatase 2A, catalytic subunit
Signalling	lit-1	Serine threonine protein kinase, required for assymetric divisions in embryo
Signalling	rme-2	Low-density lipoprotein receptors containing Ca2+-binding EGF-like domains
Small Molecule Transp	cua-1	Copper-transporting E1-E2 ATPase
Small Molecule Transp	nkb-1	Na+/K+ ATPase, Beta subunit
Splicing	emb-4	Nuclear protein orthologous to human AQR/IBP160, an intron-binding spliceosomal 'Aquarius' protein
Splicing	F19F10.9	U4/U6.U5 snRNP associated protein

Table 4. Genes identified in the screen whose inhibition results in reduced expression of the multicopy *hsp-16.2p::GFP* reporter in embryos upon heat shock treatment.

Splicing	prp-17	mRNA splicing factor
Splicing	sap-49	Splicing factor 3b, subunit 4
Splicing	T10C6.5	Ortholog of human CWC15 (CWC15 spliceosome-associated protein)
Splicing	T10F2.4	mRNA splicing factor
Structural	gei-4	intermediate filament interacting protein
Structural	nmy-1	Myosin class II heavy chain
Structural	pat-3	Integrin beta subunit (N-terminal portion of extracellular region)
Structural	zyg-9	Microtubule-associated protein
Traffic	chc-1	Vesicle coat protein clathrin, heavy chain
Traffic	rab-10	GTP-binding protein SEC4, small G protein superfamily, and related Ras family GTP-binding proteins
Traffic	sar-1	Vesicle coat complex COPII, GTPase subunit SAR1
Traffic	vps-35	Related to yeast Vacuolar Protein Sorting factor
Transcription Factor	C08B11.3	Predicted transcriptional regulator, contains ARID domain
Transcription Factor	dve-1	DVE (Defective proVEntriculus in Drosophila) homolog
Transcription Factor	hsf-1	Heat shock transcription factor
Unknown	atx-2	Ortholog of human ataxin-2
Unknown	C01A2.5	Uncharacterized conserved protein
Unknown	C23G10.8	Unknown
Unknown	C28H8.11	Unknown
Unknown	C46G7.1	Ortholog of human RNASEK (ribonuclease, RNase K)
Unknown	dic-1	Human DICE1 (Deleted In Cancer) homolog; localizes to mitochondria
Unknown	F20D12.2	Ortholog of human MCM3AP (minichromosome maintenance complex component 3 associated protein).
Unknown	F44E5.1	Unknown
Unknown	gad-1	WD repeat-containing protein, required for gastrulation
Unknown	Y116A8C.466	Non-coding RNA
Unknown	Y71H10B.1	Ortholog of human NT5DC4 (5'-nucleotidase domain containing 4)
Unknown	ZK1128.3	Unknown
Unknown	ZK688.9	Ortholog of Saccharomyces cerevisiae TIP41

Table 4. continued

3.1.2 Aberrant DNA replication results in increased expression of multi-copy transgenes

We noticed that 8 out of 35 genes that increase *hsp-16.2p::GFP* transgene expression upon knockdown encode factors involved in DNA replication including several subunits of DNA polymerase complex, a DNA damage binding protein, replication factor C and a primase (Table 3). For further analysis we selected *div-1* ,which encodes a homolog of the B subunit of the DNA polymerase alpha-primase complex. RNAi mediated inhibition of *div-1* did not result in high embryonic lethality but resulted in a strong and penetrant increase in transgene expression (Figure 52). To test for a general effect on transgene expression, we fed worms carrying a *daf-21p::mCHERRY* transgene with RNAi expressing bacteria targeting either a non-expressed pseudogene (*ctrl*) or *div-1*. We observed a potent increase of *daf-21p::mCHERRY* transgene expression in progeny of worms fed with *div-1 (RNAi)* bacteria (Figure 52).

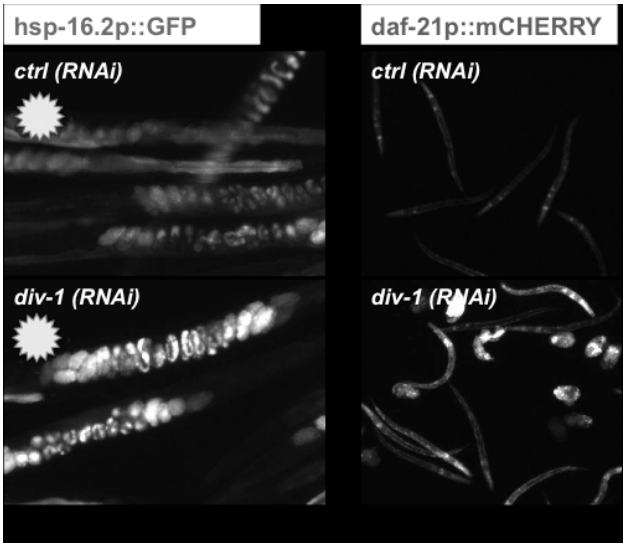

Figure 52. Depletion of *div-1* by RNAi feeding results in reduced expression of the multi-copy reporters *hsp-16.2p::GFP* and *daf-21p::mCHERRY* in F1 progeny. Expression of *hsp-16.2p::mCHERRY* was triggered in embryos trapped in killed gravid worms by a 1h heat shock at 34 °C and assayed 2 hours later. For the *daf-21p::mCHERRY* strain, embryos were extracted form gravid adults fed with RNAi bacteria, left to hatch overnight in M9, then imaged. Star indicates heat shock.

Upregulation of daf-21p::mCHERRY in response to div-1 (RNAi) was first observed by a colleague, Kadri Reis.

To test whether the effect is due to the multi-copy character of both transgenes, we took advantage of *hsp-16.2p::GFP* transgene integrated into the genome in a single copy (Mendenhall et al., 2012). We crossed this transgene into the strain containing the *daf-21p::mCHERRY* multi-copy construct that was used as a control for the *div-1 (RNAi)* effect. We then subjected the worms to RNAi feeding from L1 to adulthood, extracted synchronous populations of embryos, subjected them to a heat shock and imaged 2 hours later. Unlike the multi-copy transgene, *hsp-16.2p::GFP* integrated in the genome in a single copy was not affected by *div-1 (RNAi)* (Figure 53). In fact, a slight decrease in expression was

observed. At the same time, *daf-21p::mCHERRY* was strongly up-regulated in the *div-1 (RNAi)* condition (Figure 53).

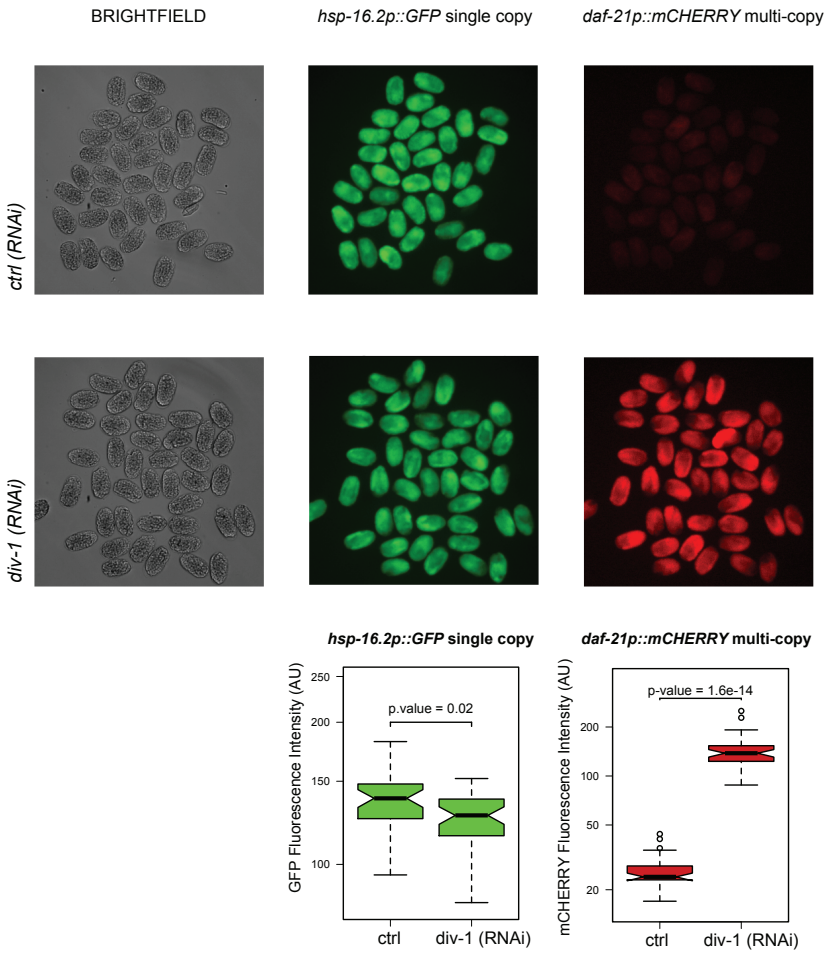

Figure 53. Single copy *hsp-16.2p::GFP* reporter is not up-regulated in response to depletion of *div-1* gene product by RNAi. Worms carrying both *daf-21p::mCHERRY* multi-copy and *hsp-16.2p::GFP* single copy transgene were used. Synchronized embryos extracted from adult worms fed with RNAi expressing bacteria were used and heat shocked for 30 min. at 34 °C. Images where taken two hours after the heat shock. Y-axis in log scale.

We then used a single copy *daf-21p::GFP* reporter and measured expression after treatment of the parents with *div-1 (RNAi)* through feeding. Expression in the embryos was assayed using time-lapse microscopy, which also allowed us to confirm the effectiveness of RNAi treatment as evidenced by a delay in embryonic development, which is a known consequence of inhibiting *div-1* activity (Encalada et al., 2000). Measurement of expression revealed a slightly less than a two-fold increase in *div-1 (RNAi)* embryos (Figure 54).

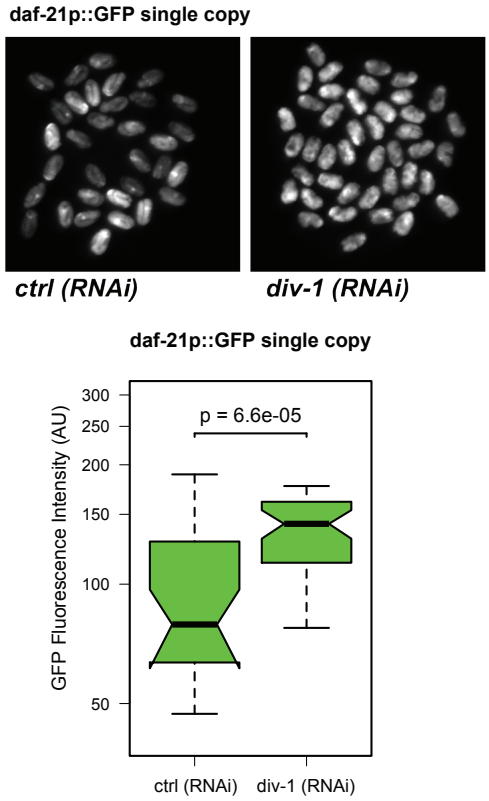

Figure 54. Depletion of *div-1* by RNAi results in elevated expression of *daf-21p::GFP* single copy reporter. Comparison of reporter expression in synchronized late-stage embryos extracted from adult worms fed with RNAi expressing bacteria. Y-axis in log scale.

3.1.3 Transgene upregulation is caused by inefficient DNA replication during embryonic development

We crossed the *daf-21p::mCHERRY* transgene into a *div-1 (or148)* mutant background and observed a robust increase in expression in all tissues (Figure 55). Moreover we detected a high incidence of sterility in *div-1 (or148)* mutant worms cultivated at 20 °C (Figure 56). This is possibly due to defects in spermatogenesis, since the sterile adults don't show any abnormalities in female germline morphology and have numerous oocytes (Figure 56). The *or148* mutation was previously shown to cause a delay in embryonic development (Encalada et al., 2000), most likely due to stalling of DNA replication forks. We observed embryonic delay and a potent increase in *daf-21p::mCHERRY* expression in the embryos carrying the *div-1 (or148)* allele (Figure 57). To determine whether the up-regulation of the transgene is a result of replication defects happening in the germline or in the early embryo we used males as donors of the transgene and hermaphrodite *div-1 (or148)* mutant worms. We crossed the transgene-bearing males to wild type or *div-1 (or148)* mutant hermaphrodites and measured expression in the progeny (Figure 58). If passage through a *div-1 (or148)* mutant germline is required for the upregulation observed in mutants and in the RNAi experiments, then the male derived transgene should not be affected. Alternatively, the transgene could be upregulated during embryonic development. In our experiment a strong up-regulation of the transgene was evident in the F1 progeny suggesting that stalled replication in early embryonic development is responsible for transgene upregulation (Figure 58).

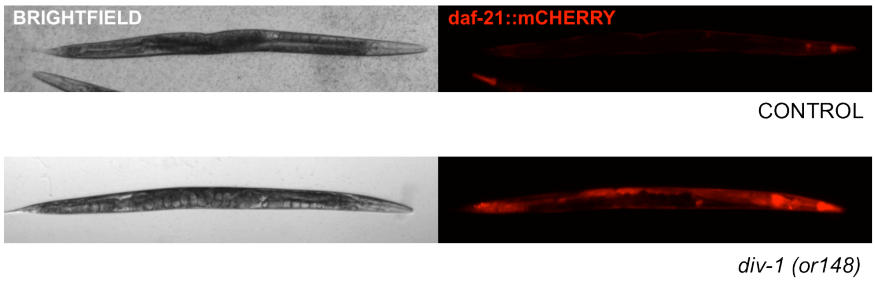

Figure 55. Expression of *daf-21p::mCHERRY* in mutant *div-1 (or148)* adult worms.

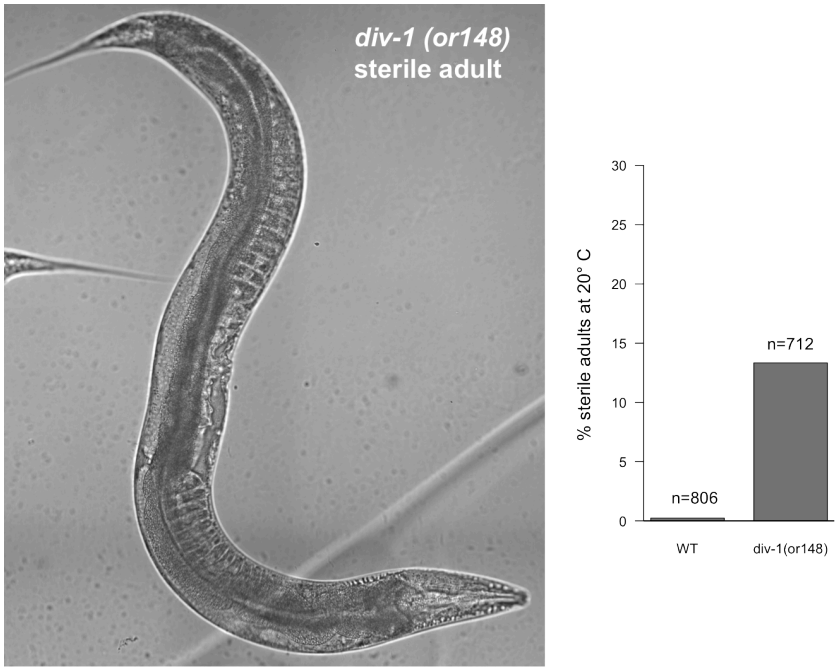

Figure 56. Worms carrying the *div-1 (or148)* are partially sterile. Sterility was scored by visual inspection of synchronized animals in the second day of adulthood by the presence or absence of embryos inside the worm.

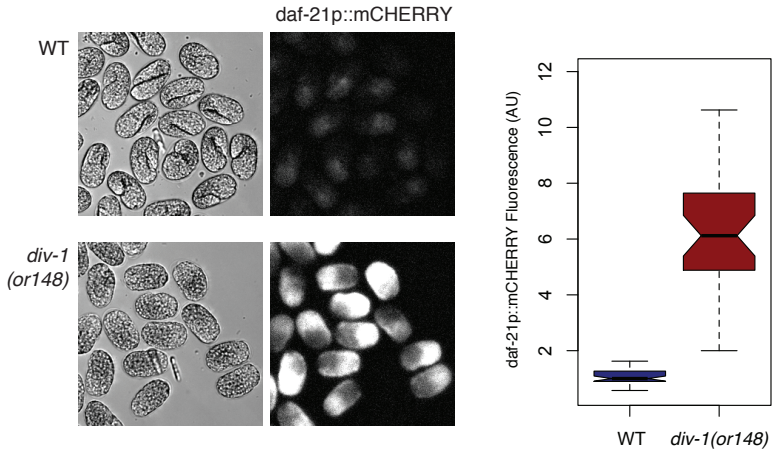

Figure 57. Embryonic development is delayed in *div-1 (or148)* embryos and *daf-21p::mCHERRY* expression is elevated. Expression of *daf-21p::mCHERRY* in mutant *div-1 (or148)* embryos synchronized at the 4-cell stage and allowed to develop for 6.5 hours at 20 °C. The delay in development in *div-1(or148)* is estimated to be approximately 1 hour based on embryo morphology.

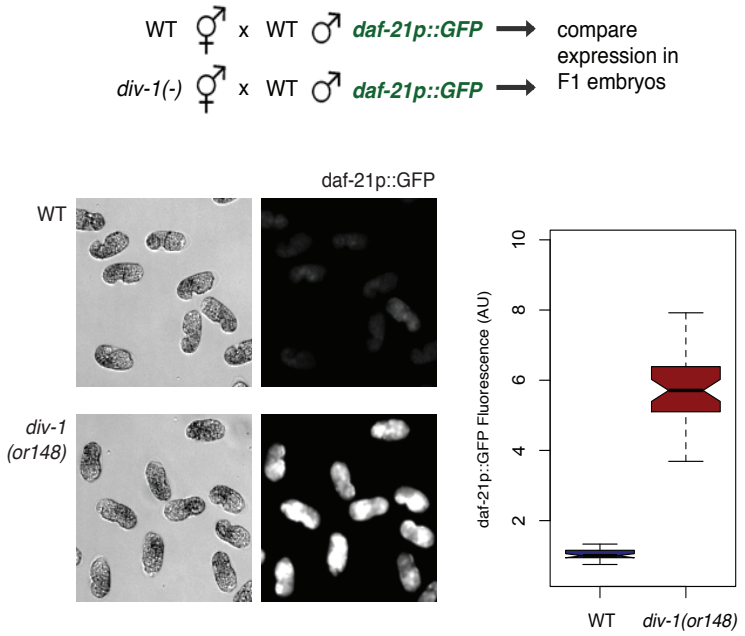

Figure 58. Maternal *div-1(or148)* mutation triggers upregulation of paternally inherited *daf-21p::GFP* multi-copy transgene during embryonic development. Male worms carrying d*af-21p::GFP* multi copy transgene were crossed either to WT or *div-1* mutant hermaphrodites. Paternally inherited *daf-21p::GFP* transgene is upregulated in the F1 progeny of *div-1* mutant mothers.

3.1.4 Transgene upregulation evoked by *div-1 (RNAi)* proceeds normally in multiple chromatin mutant backgrounds

In an attempt to identify the mechanism responsible for the effect the stalled replication has on transgene expression we subjected various mutant strains to *div-1 (RNAi)* and measured the effect on *daf-21p::mCHERRY* expression (Figures 59 – 63). We found a robust increase in transgene expression after *div-1 (RNAi)* treatment in *set-25, set-2, nrde-1, nrde-2* and *eri-6* mutant worms (Figure 59). Some quantitative differences where observed (*eri-6* mutants respond stronger than WT, while *nrde-1/2* mutant worms respond less than WT), which are most likely due to the altered sensitivity to exogenous RNAi of the mutant strains (Fischer et al., 2008; Zhuang et al., 2013a). Expression of *daf-21p::mCHERRY* in *met-1* and *met-2* mutant backgrounds increased in response to *div-1 (RNAi)* suggesting that these two genes are not required for the observed effect. Lack of response to *div-1 (RNAi)* in *drh-3, rde-1* (Figure 59) and *mut-7* (Figure 60) was expected due to inability of these mutants to trigger RNAi (Gu et al., 2009; Ketting et al., 1999; Tabara et al., 1999). To test whether these genes are involved in replication stress-triggered up-regulation, one would need to introduce the *div-1* mutation into those mutant strains and measure the effect on *daf-21p::mCHERRY* expression. We also tested if the absence of HRDE-1 impairs *div-1 (RNAi)* mediated transgene upregulation (Figure 61). In this experiment we also tested if environmental pre-conditioning (ancestral growth at 16 and 25 °C for several generations) has any obvious effect on *daf-21p::mCHERRY* expression after *div-1 (RNAi)*. We found that *hrde-1* mutant worms upregulate the transgene in response to *div-1 (RNAi)*, similarly to environmentally pre-conditioned 'high' control animals (Figure 61). Therefore HRDE-1 is dispensable for the increase in transgene expression triggered by replication stalling.

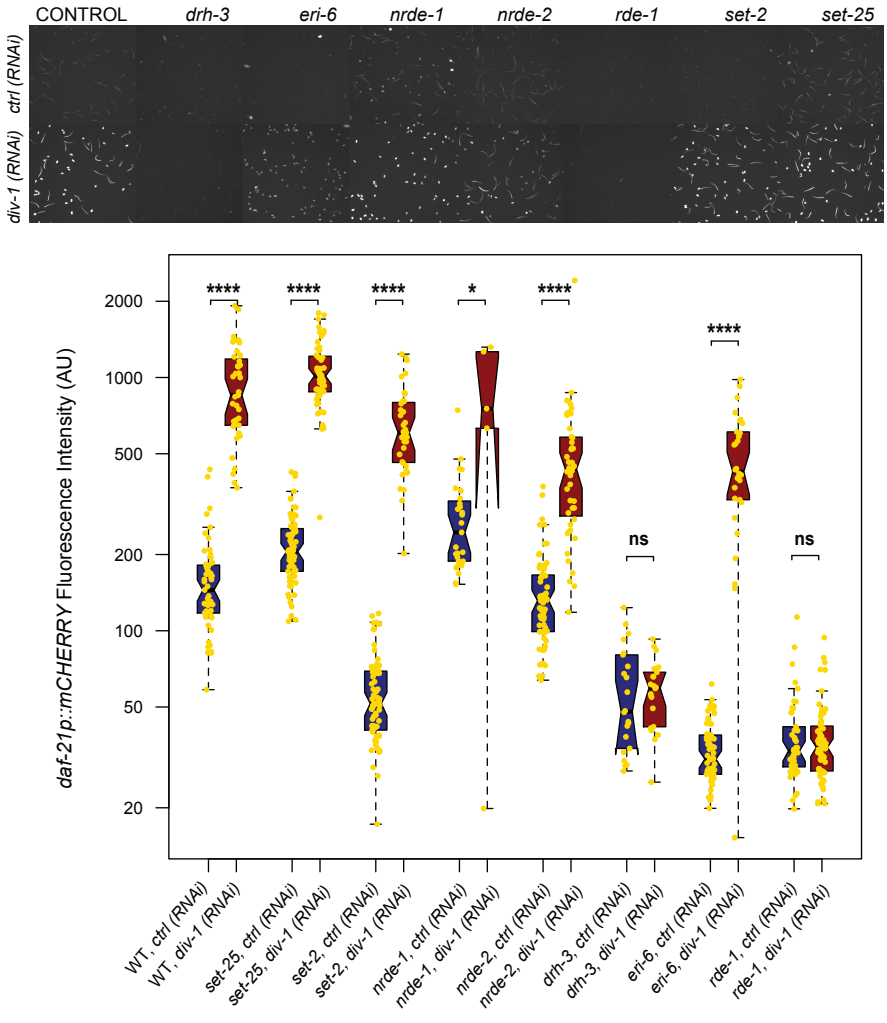

Figure 59. Depletion of DIV-1 by RNAi results in increased expression of *daf-21p::mCHERRY* in F1 progeny of *set-25, set-2, nrde-1, nrde-2* and *eri-6* mutant worms. Expression was measured in L1 worms. *drh-3* and *rde-1* strains show no effect due to RNAi resistance. P values: **** $p < 0.0001$, * $p < 0.05$, ns $p > 0.05$ (Wilcoxon rank test). Y axis in log scale.

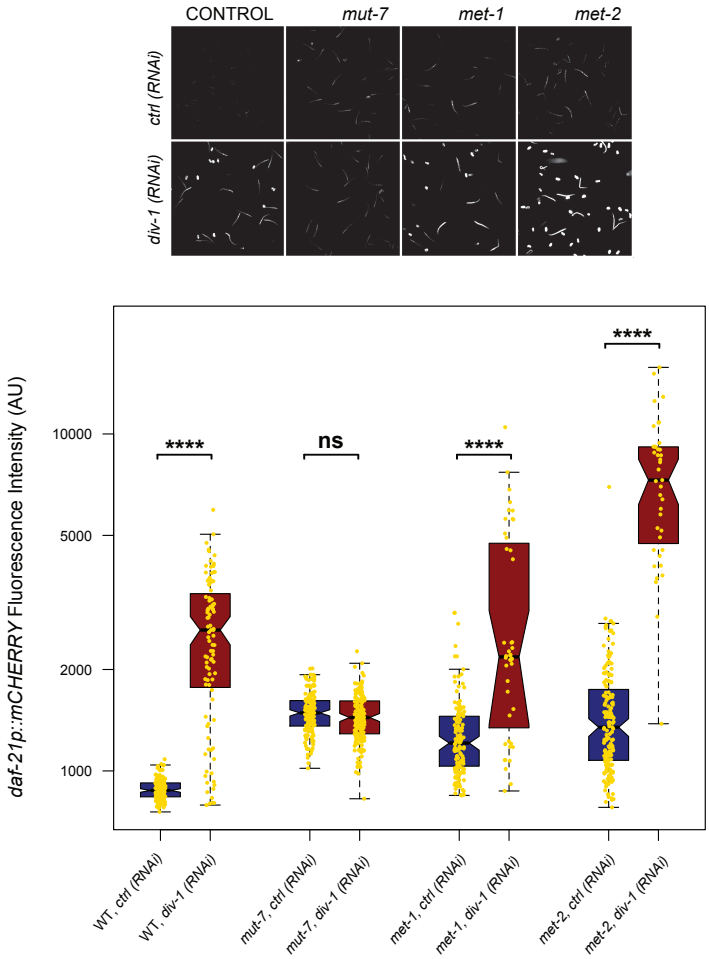

Figure 60. Depletion of DIV-1 by RNAi results in increased expression of *daf-21p::mCHERRY* in *met-1* and *met-2* mutant backgrounds. *mut-7* strain shows no effect due to RNAi resistance. Expression was measured in L1 worms. P values: **** $p < 0.0001$, ns $p > 0.05$ (Wilcoxon rank test). Y-axis in log scale.

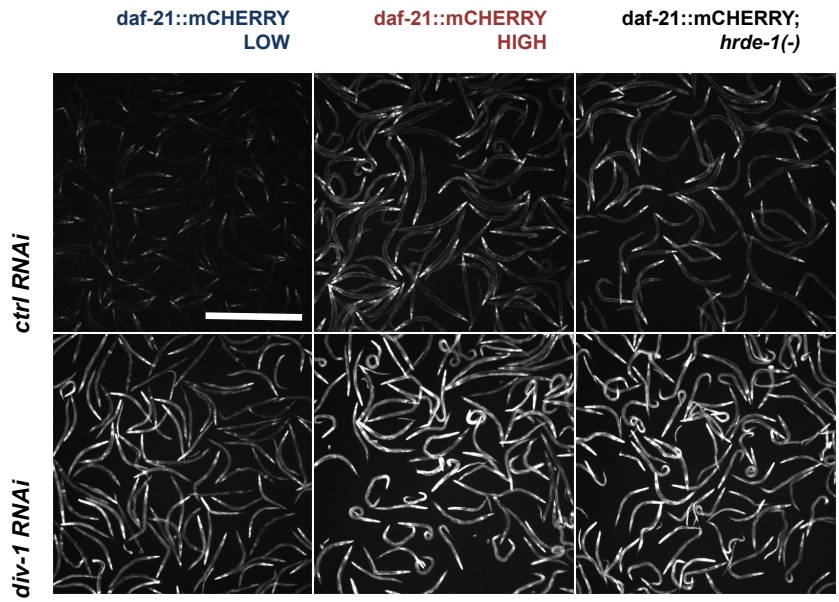

Figure 61. Depletion of DIV-1 by RNAi in parents results in increased *daf-21p::mCHERRY* expression in the F1 progeny of in *hrde-1* mutants. For comparison, *div-1 (RNAi)* mediated upregulation in worms preconditioned through ancestral growth at 25 °C (HIGH) and 16 °C (LOW) is shown. Expression is visualised at young adult stage. Scale bar = 1 mm.

3.1.5 Loss of heterochromatin marks in the embryo reduces the effect of *div-1* depletion on transgene expression

We hypothesized that replication stalling caused by knockdown of *div-1* by RNAi might interfere with mitotic inheritance of heterochromatin marks during embryonic development. In *C. elegans* heterochromatin marks H3K9me and H3K27me3 are not essential for embryonic development. If the primary cause of *div-1 (RNAi)* mediated transgene upregulation is indeed interference with mitotic inheritance of heterochromatin marks, then embryos that lack those marks should experience little or no upregulation in response to *div-1* knockdown. Previous studies demonstrated that two histone methyl transferases MET-2 and SET-25 participate in formation of methylated H3K9 on chromatin and their simultaneous depletion results in loss of all detectable H3K9me1/2/3 marks (Towbin et al., 2012). We crossed *daf-21p::mCHERRY* transgene into a *met-2; set-25* double mutant background and observed a strong de-repression of transgene expression in most somatic tissues, especially intestine (Figures 29 and 30). We then subjected the worms to *div-1 (RNAi)* through feeding and measured expression in the progeny (Figures 62 and 63). The increase in transgene expression was not abolished in *met-2, set-25* animals. In fact, it was as strong as in single *set-25 (-)* mutant animals and in wild type worms (Figure 63).

Next, we tested if the absence of another major heterochromatin mark H3K27me3 mark abolishes the effect of *div-1 (RNAi)* on transgene expression. This mark is lost in the absence of any of the core PRC2 elements *mes-2, mes-3* or *mes-6* (Bender et al., 2004). We crossed the *daf-21p::mCHERRY* transgene into a balanced strain SS186, which can be maintained as *mes-2/+* heterozygotes. Homozygous *mes-2* mutant progeny can be identified by an uncoordinated (Unc) phenotype. They are

fertile but produce 100% sterile offspring. We subjected homozygous *mes-2* worms to *div-1 (RNAi)* and quantified transgene expression in their offspring. We detected a strong upregulation of transgene expression (Figure 62). This means that loss of either H3K9me1/2/3 or H3K27me3 has little or no effect on transgene upregulation induced by replication stress.

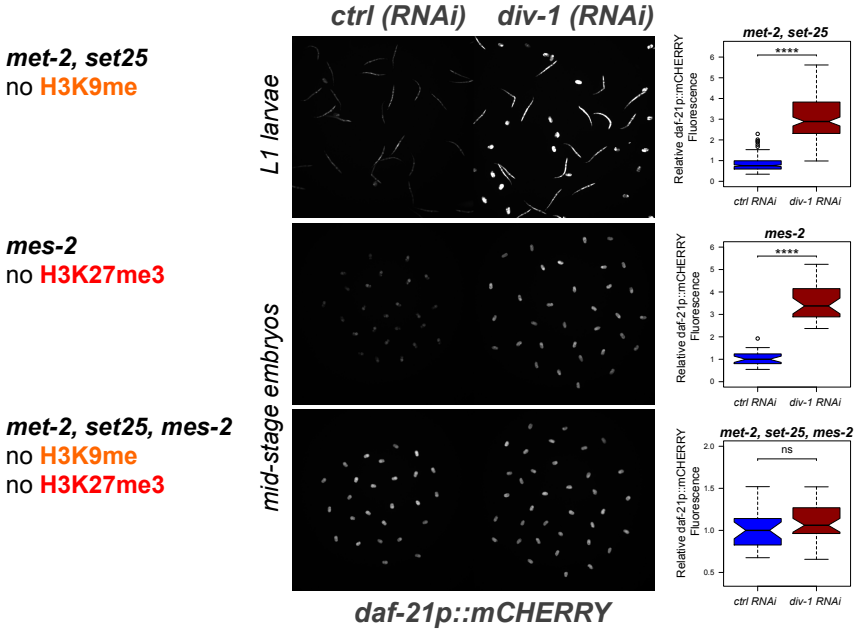

Figure 62. In the absence of H3K9me1/2/3 and H3K27me3, *div-1 (RNAi)* depletion has little effect on *daf-21p::mCHERRY* transgene expression measured in embryonic development. P values: *** p< 0.001, ns p> 0.05 (Wilcoxon rank test).

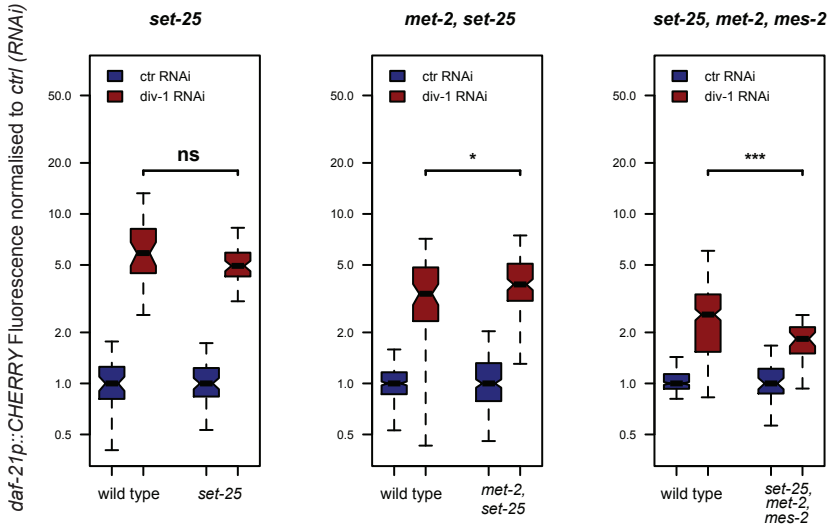

Figure 63. Quantification of *daf-21p::mCHERRY* in hatched L1s after *div-1 (RNAi)* in heterochromatin mutants. In *set-25; met-2; mes-2;* triple mutant background *div-1 (RNAi)* depletion has a significantly diminished effect on daf-21::mCHERRY transgene expression. Values were normalized with median expression of *ctrl (RNAi)* treatment for each genetic background to compare the relative effect of *div-1 (RNAi)* knockdown between the genotypes. P values: *** $p < 0.001$, * $p < 0.05$, ns $p > 0.05$ (Wilcoxon rank test). Y-axis in log scale.

Rather than interfering with faithful mitotic inheritance of specific histone marks during embryonic development, aberrant DNA replication could affect multiple repressive chromatin pathways. We measured the effect of *div-1 (RNAi)* on animals carrying *daf-21p::mCHERRY* transgene in *mes-2, met-2, set-25* triple mutant background. The experiment was performed as above with the *mes-2* mutant, uncoordinated homozygous *mes-2, met-2, set-25* gravid hermaphrodites fed with RNAi bacteria from L1 to adulthood were dissected and expression in the F1 progeny measured using time-lapse microscopy. As seen in Figure 62, *div-1 (RNAi)* has no obvious effect on transgene expression in *mes-2, met-2, set-25* mutant background. Unfortunately the experiment was confounded by the fact

that both groups of embryos failed to hatch and arrest at around the bean stage of embryonic development. We noticed that the triple heterochromatin mutant embryos are hypersensitive to temperature and often failed to hatch during time-lapse microscopy measurements. This phenotype was more frequent after *div-1 (RNAi)* feeding. We therefore repeated the experiment and instead of subjecting the embryos to time-lapse microscopy, we kept the embryos in M9 overnight and measured *daf-21p::mCHERRY* expression in hatched L1s the following day. In these conditions, we detected a significant upregulation of the transgene in the *mes-2, met-2, set-25* mutant background but of a significantly lower magnitude than in control animals (Figure 63). This suggests that in the absence of both repressive chromatin modification pathways (H3K27me3/PRC2 and H3K9me3), replication stress has a reduced effect on transgene expression.

3.1.6 Replication stress interferes with the multigenerational inheritance of transgene expression

We took *div-1 (or148)* mutant worms and investigated the dynamics of *daf-21p::mCHERRY* expression after temperature perturbation (Figure 64). The experiment was carried out in parallel to the one shown in Figure 32 and the control worms were used to normalize the data. Mutant *div-1* animals responded to increased temperature by a robust *daf-21p::mCHERRY* upregulation (Figure 64, left panel, P0). However, this strong upregulation was not efficiently transmitted to the following generations with a small, albeit significant, difference between the two cohorts in the F1 generation and no difference at all in F2 animals (Figure 64, left panel). We also carried out the 'sorting' experiment explained in Figure 35 in a *div-1* mutant background (Figure 64), in parallel to the control *daf-21p::mCHERRY* worms (shown in Figure 35). In *div-1* mutants the difference between the two populations was still present in the

F1 and F2 generations (Figure 64). To visualize the rate at which the expression difference between the sorted populations changes over the generations we calculated the logarithm of the ratio between the expression medians of the two cohorts for each mutant at every generation (Figure 65). This shows that in the *div-1* mutant background, the differences in transgene expression are less stably maintained over the generations, similarly to what is observed in *hrde-1* and *spr-5* mutants (Figure 65).

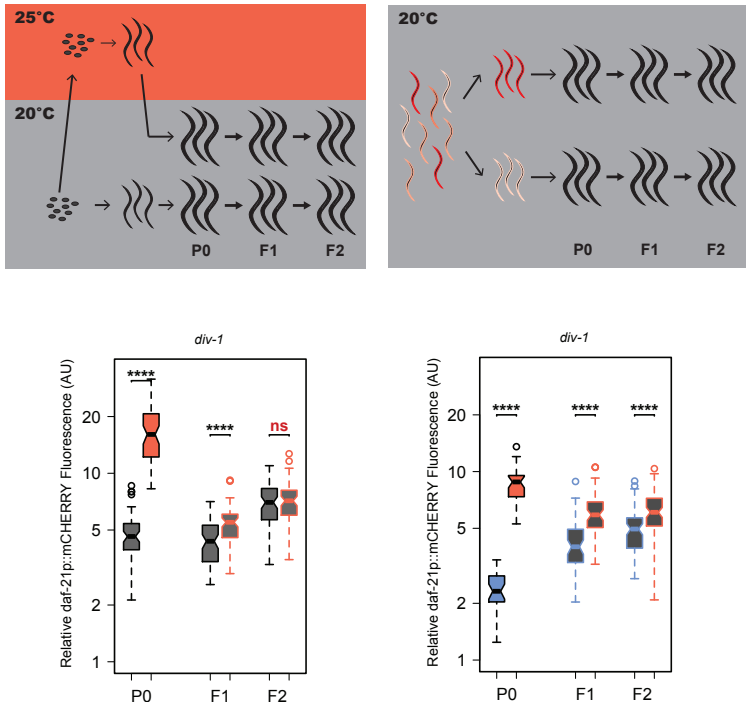

Figure 64. *div-1* mutants fail to maintain environmentally triggered differences in *daf-21p::mCHERRY* expression for more than one generation. Difference in expression in populations separated by manual sorting is maintained in F1 and F2 generations. Control worms with *daf-21p::mCHERRY* in wild-type genetic background propagated in parallel at 20 ° C were used as reference for normalization in each generation. P values: **** $p < 0.0001$, ns $p > 0.05$ (Wilcoxon rank test). Y-axis in log scale.

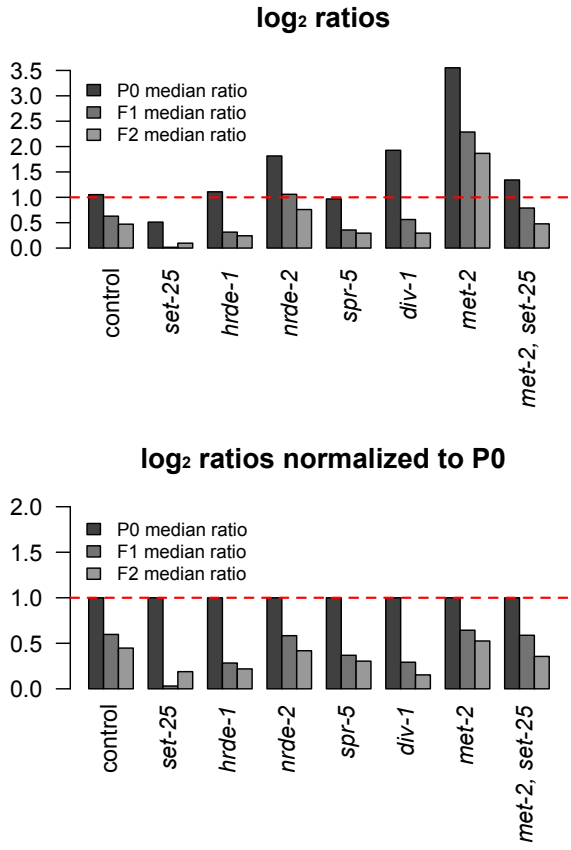

Figure 65. Transgenerational decay of *daf-21p::mCHERRY* expression difference between 'high' and 'low' populations established by sorting in P0 in different mutant backgrounds expressed as log2 ratios of median expression values. Data from Figures 35, 36 and the right panel of Figure 64.

3.1.7 The change in expression triggered by replication stress is partially heritable

To test if the increase in *daf-21p:mCHERRY* expression triggered by replication stress is heritable we crossed males carrying the transgene to either wild type or *div-1 (or148)* hermaphrodites (Figure 66). Male worms used for this experiment were preconditioned to express relatively low levels of *daf-21p::mCHERRY*, through ancestral growth at 16 °C (Figure 66, left panel). We measured the expression in F1 male cross progeny of the two cohorts when they reached adulthood. As shown before (Figure 58) presence of the maternally supplied mutant DIV-1 protein in the early embryo resulted in up-regulation of the paternally derived transgene. We then crossed the two populations of males (wild type and *div-1/+*) to wild-type hermaphrodites and measured transgene expression in the resulting F2 male offspring. Both populations experienced a wild type early embryonic environment. Strong transgene over-expression, triggered in the F1 embryos by maternal mutant DIV-1, was largely lost in the subsequent generation, but remained higher than in the control animals (Figure 66, left panel). This demonstrates that defective replication during early embryonic development can lead to changes in expression that are, to some extent, inherited in the next generation.

In parallel to the above experiment, we carried out an analogous crossing scheme but using P0 males environmentally preconditioned to express high levels of *daf-21p::mCHERRY* through ancestral growth at 25 °C (Figure 66, right panel). We detected a strong up-regulation in F1 male progeny sired by *div-1* mothers. However, F2 progeny of those worms, sired by wild-type mothers, was not significantly brighter than progeny of control F1 males (Figure 66, right panel). Therefore, if the transgene expression is increased by the means of an environmental perturbation, the heritable effect on expression caused by replication stress in masked.

This epistatic interaction between the environmentally and genetically triggered responses suggests that they converge on a common pathway.

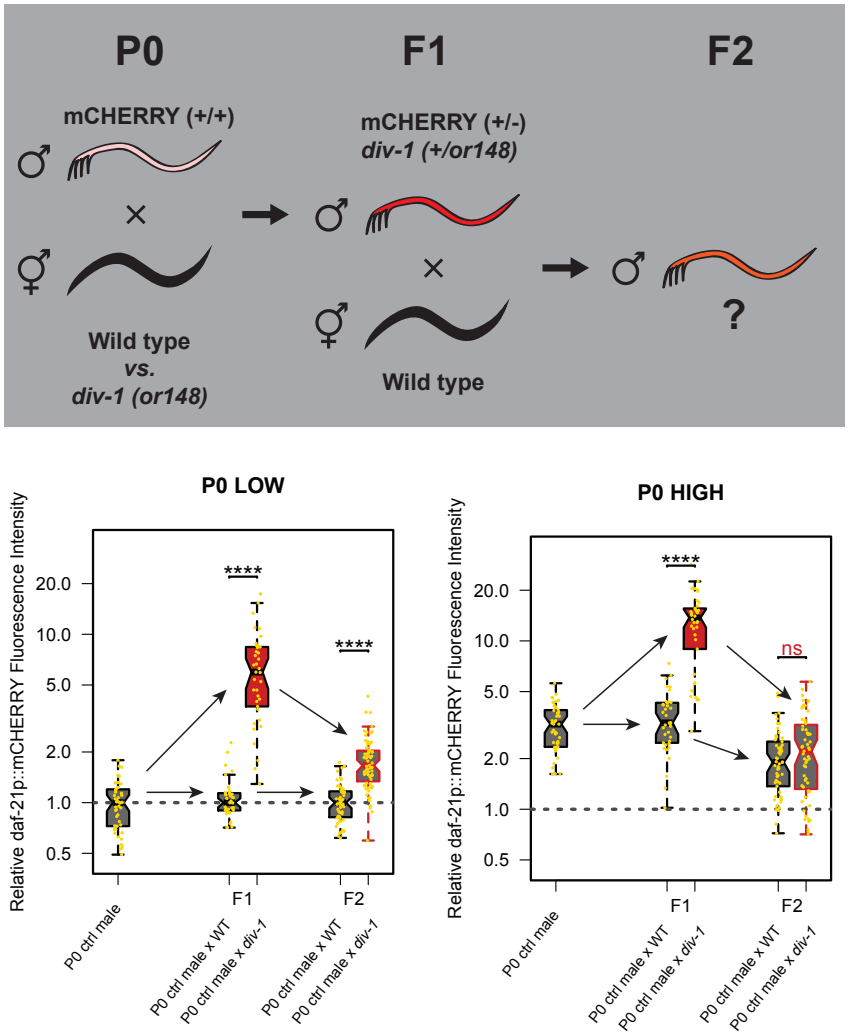

Figure 66. Upregulation of *daf-21p::mCHERRY* caused by passage through *div-1* mutant background is heritable. The effect is masked if the transgene had been previously upregulated by temperature (P0 HIGH). P values: **** $p < 0.0001$, ns $p > 0.05$ (Wilcoxon rank test). Y-axis in log scale.

3.1.8 Knockdown of CSR-1 pathway components prevents increased transgene expression triggered by replication stress

To gain more insight into the mechanism behind transgene de-repression triggered by replication stress we performed a screen to identify genes required for the increased transgene expression when *div-1* is inhibited. One of the defects caused by the *div-1 (or148)* allele is inefficient sequestration of P-granules to the germline lineage (Encalada et al., 2000). We hypothesized that transgene upregulation is related to this event, therefore the set of tested genes contained multiple P-granule components and regulators of P-granule sorting (Updike and Strome, 2010; Updike and Strome, 2009). We fed *div-1 (or148), daf-21p::mCHERRY* worms with RNAi-expressing bacteria and visually assayed the effect on expression in F1 progeny. Interestingly, we found that knockdown of several components of the CSR-1 pathway: *csr-1*, *drh-3, ego-1* and *ekl-1,* resulted in a substantial decrease in transgene expression in the progeny of RNAi treated animals (Figure 67).

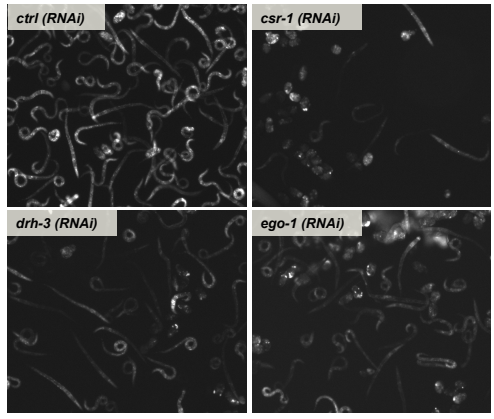

Figure 67. Inhibition of components of the CSR-1 pathway rescues upregulation of *daf-21p::mCHERRY* caused by *div-1* mutation. L1 progeny of *daf-21p::mCHERRY, div-1(or148)* worms fed with RNAi targeting 3 different components of CSR-1 pathway and a control RNAi strain.

Using time-lapse microscopy on staged embryos, we confirmed the reduction of *daf-21p::mCHERRY* expression after *ego-1* RNAi and also found it to be similar in *div-1 (or148)* and WT genetic backgrounds (Figure 68).

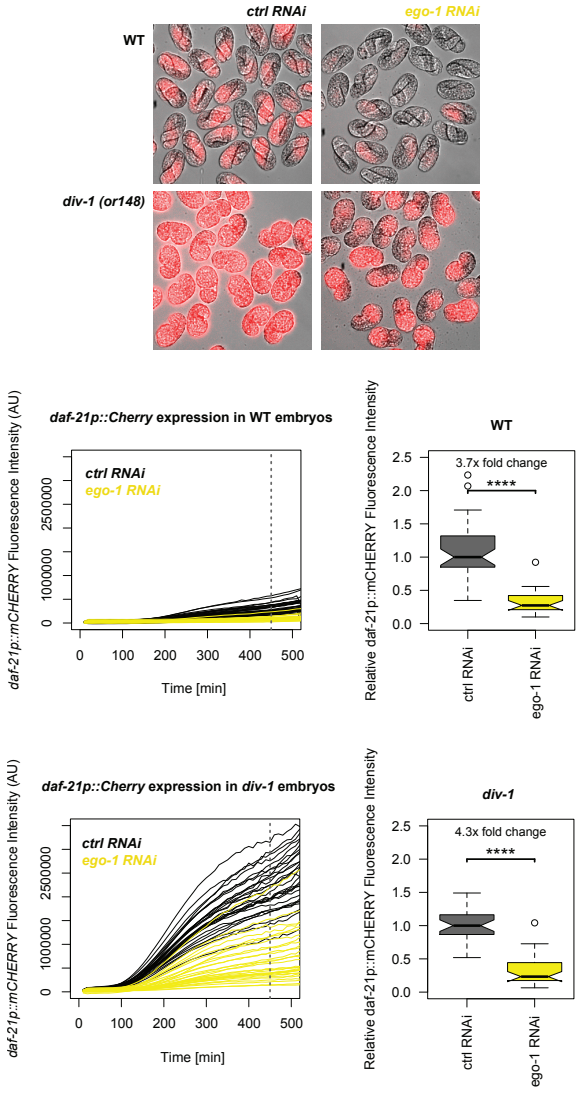

Figure 68. Transgene expression in *div-1 (or148)* and WT staged embryos extracted from worms fed with *ctrl* or *ego-1 (RNAi)*. Reduction in expression of *daf-21p::mCHERRY* is similar in *div-1* and WT backgrounds. Images show that *ego-1 (RNAi)* does not rescue the delay in embryonic development caused by *div-1 (or148)*. P values: **** p< 0.0001 (Wilcoxon rank test).

3.1.9 Reduced activity of CSR-1 pathway in early embryonic development rescues transgene over-expression triggered by replication stress

CSR-1 is an Argonaut protein known to act in the germline where it promotes transcription (Cecere et al., 2014), protects genes from epigenetic silencing (Seth et al., 2013) and provides transgenerational memory of germline gene expression (Conine et al., 2013). These functions have been proposed to emerge from a common mechanism based on CSR-1 bound small RNAs produced and transmitted through the germline. There is also evidence that the CSR-1 pathway stimulates expression of multi-copy transgene expression in somatic tissues (Fischer et al., 2013). Therefore, transgene suppression evoked by reduction in CSR-1 pathway could be taking place in the germline or during somatic differentiation. To distinguish between these two scenarios, we set up four parallel crosses between *div-1 (or148)* hermaphrodites and *daf-21p::GFP* (multi-copy) males (Figure 69). We knocked down *drh-3* using RNAi in one, both or none of the parents and measured the effect on transgene expression in the progeny. If the knockdown of *drh-3* affects production of licensing small RNAs targeting the transgene in the germline, then reducing DRH-3 activity in the male worms, which carry the transgene, should result in decreased expression in F1 progeny. However, we found no difference in transgene expression in such a scenario (Figure 69). Knocking down *drh-3* in the *div-1* mother and crossing it to *ctrl (RNAi)* fed males that supplied the transgene resulted in a potent reduction in transgene expression, similarly to when both parents where fed with *drh-3 (RNAi)* (Figure 69). The vast majority of proteins acting during early embryonic development are supplied maternally (through mRNA and protein present in the oocyte). Therefore, rather than changing the parentally supplied small RNA pools, the CSR-1 pathway appears to be modulating expression during embryonic development.

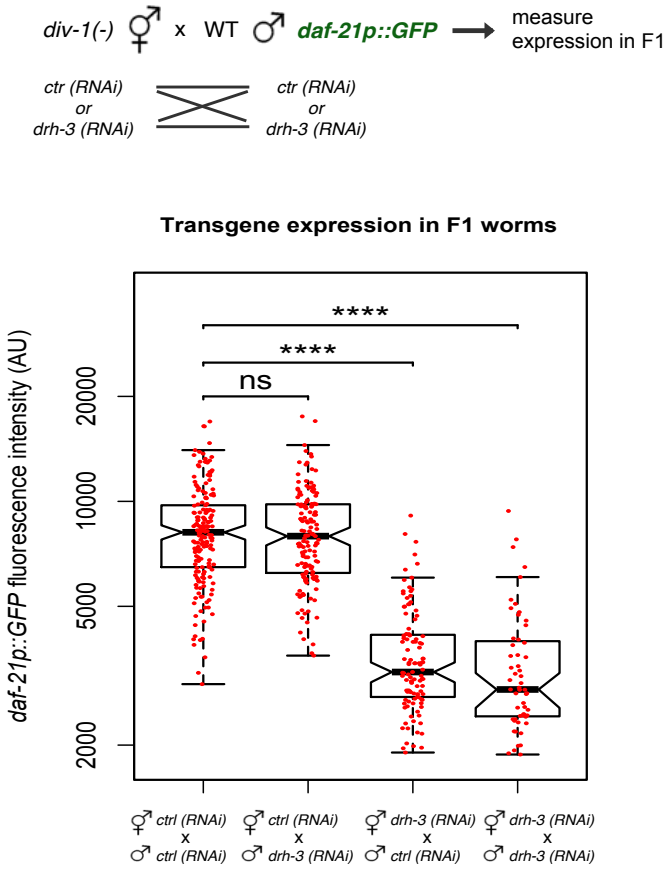

Figure 69. CSR-1 pathway acts during embryonic development to promote transgene expression. *div-1 (or148)* worms were fed with either *ctrl* or *drh-3 RNAi* and crossed to males carrying multicopy *daf-21p::GFP* transgene and fed with either *ctrl* or *drh-3 RNAi*. Expression of *daf-21p::GFP* was measured in the L1 progeny or each of the four combinations of parental treatments. P values: **** $p < 0.0001$, (Wilcoxon rank test). Y-axis in log scale.

How does the CSR-1 pathway regulate expression? One possibility is that it antagonizes heterochromatin on the transgene locus. In that case, depletion of CSR-1 pathway components in the triple mutant *mes-2, met-2, set-25* genetic background should have no or little effect on transgene expression as the canonical H3K27me3 and H3K9me1/2/3 are absent in these worms. We detected a significant decrease in transgene expression after knocking down two different elements of the CSR-1 pathway in the triple mutant background (Figure 70). However, the reduction was small with just a 1.4 fold reduction for *drh-3 (RNAi)* and 1.3 fold for *csr-1 (RNAi)*. This is in contrast to a strong reduction in expression detected in a wild-type or *div-1 (or148)* background after *ego-1 (RNAi)* (Figure 68). Therefore the CSR-1 pathway could be antagonizing maintenance of heterochromatin on the transgene during embryonic development.

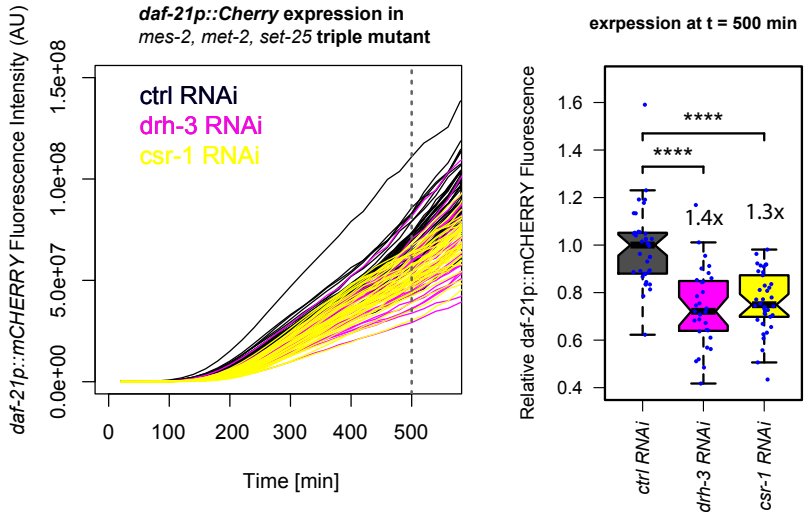

Figure 70. Time-lapse of *daf-21p::mCHERRY* expression in *mes-2, met-2, set-25* triple mutant staged embryos extracted from worms fed with *ctrl, drh-3 (RNAi) or csr-1 (RNAi)*. Reduction in expression caused by knockdown of CSR-1 pathway components is small (1.3 fold reduction for *csr-1 RNAi* and 1.4 reduction for *drh-3 RNAi*) but significant. P values: **** p< 0.0001 (Wilcoxon rank test).

As the CSR-1 pathway had been implicated in histone mRNA maturation (Avgousti et al., 2012), we tested whether interfering with this process in a different way would also result in transgene down-regulation. Using RNAi we knocked down *cdl-1*, which encodes the histone hairpin-binding protein required for core histone gene expression (Pettitt et al., 2002). We observed no reduction in GFP expression (Figure 71), suggesting that the effect on transgene expression caused by depletion of CSR-1 pathway is unlikely to be explained by reduced histone content.

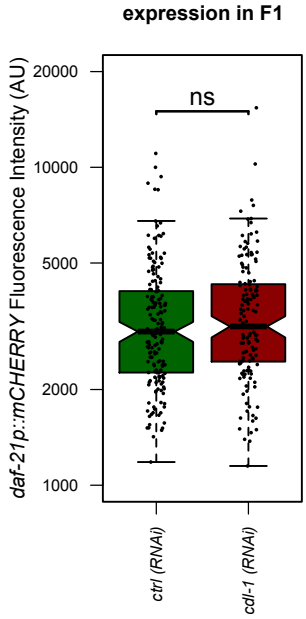

Figure 71. Depletion of *cdl-1* by RNAi does not result in decreased *daf-21p::mCHERRY* expression in *div-1 (or148)* worms. Expression was measured in progeny of *ctrl* or *cdl-1 (RNAi)* treated L1 worms synchronized by overnight hatch in L1. A short RNAi treatment (7 hours) of young adults was necessary as longer treatment resulted in very high penetrance of early embryonic lethality. Some embryonic lethality was also observed after 7h treatment, confirming that the RNAi knockdown was efficient. Y-axis in log scale.

4. Methods

4.1 Worm strains and culture conditions

All strains used in this study are listed in Supplementary Table 1. Bristol N2 strain was used as the wild type and all other strains used are derived from it. Worms where cultured using standard conditions (Stiernagle, 2006) using NGM plates seeded with *Escherichia coli* OP-50 strain and grown at 20 °C unless stated otherwise in the text. In the multi-generational experiments worms were propagated by washing the gravid worms gently off the plate on day 1 of adulthood and extracting their embryos using an alkaline hypochlorite solution (bleaching) (Stiernagle, 2006). Embryos where washed twice with M9 and plated on a fresh, OP-50 seeded NGM plate at an estimated concentration of 1000 embryos per plate.

4.2 Strain construction

The BCN1049 (*daf-21p::GFP*) and BCN1050 (*daf-21p::mCHERRY*) strains were generated by Alejandro Burga and are described in (Burga et al., 2011). The single copy *daf-21p::GFP* reporter was generated by Knudra Transgenicss using the same plasmid construct as the multi-copy BCN 1049 strain (Burga et al., 2011). All strains used in this study are listed in Table 5.

4.3 Worm sorting

To make the 'high' and 'low' sorted cohorts a large population of about 2000 synchronized L4 worms was grown on a single OP50-seeded 90mm NGM plate. Between 32 and 40 single worms were picked based on their extremely high or low fluorescence intensity relative to the population

average. These cohorts represented the parental P0 generation, the following generations where propagated without selecting for fluorescence.

4.4 Temperature shifts

For the temperature shift, embryos were extracted from gravid adults as described above, plated on seeded NGM plates and put inside a 25 °C incubator. Shifting the worms back from 25 to 20 °C was always done at L4/young adult stage and measurements were taken on the following day when the worms reached adulthood. As a reference, a synchronized, age-matched population grown continuously at 20 °C was used.

4.5 Crosses

All crosses where set-up with an approximately 2:1 ratio of males to L4 hermaphrodites, typically with about 50 hermaphrodites and 100 males. To generate feminized worms heterozygous for *daf-21p::mCHERRY* a strain carrying a loss of function mutation in *fog-2* gene was used. FOG-2 is essential for spermatogenesis in hermaphrodite, but has no apparent role in the males. Mutation in *fog-2* gene essentially turns hermaphrodites into females therefore ensuring that all progeny of *fog-2(-)* mothers comes from fertilization. *fog-2(-)* males carrying the *daf-21p::mCHERRY* transgene were crossed to *fog-2(-)* females and the F1 *daf-21p::mCHERRY(+/-)* females were then used for the experiment. The 'high' population was generated using *fog-2 (-)* daf-21p::mCHERRY males that came from a population grown at 25 °C for 3 generations while the 'low' population originated from worms grown constantly at 20 °C.

4.6 Imaging of adult worms

Hermaphrodite worms in their first day of adulthood were picked from a plate and dropped into a single well of a 96-well plate (Nunc, optical

bottom) filled with 90 µl of PBS, typically about 25 worms per well. 10 µl of 500mM sodium azide was then added to the well, which anesthetized the worms making them immobile. Worms were then manually separated from each other using an eyelash glued to a glass pipette and imaged within 30 minutes using a Leica DMI6000 B microscope equipped with 5x objective and Hamamatsu Orca Flash 4.0 digital camera and Lumen 200 metal arc lamp (Prior Scientific).

4.7 Measurement of fluorescent intensity of adult worms

Images were analyzed with Fiji/ImageJ software. Flat-field correction was done on both green and red channels by dividing by a background image of a well with no worms. The green channel captures the auto-fluorescence of worms and was used to identify the worms in images and create masks. The masks were then used to quantify mCHERRY intensity of fluorescence in each worm. For measurement of GFP intensity, DAPI channel was used to create masks from autofluorescence. Touching or overlapping worms as well as worms on edge of the image were discarded from analysis. Mean fluorescence intensity (Total Intensity/Area) was used as a measure of worm's brightness.

4.8 Time-lapse microscopy

To obtain embryos for time-lapse microscopy, 50 young gravid adult worms were selected and picked into 50 µl drop of PBS in a single well of a 3-well concavity slide. Worms were washed twice with 100 µl of PBS, then dissected with surgical needles releasing the embryos. 10 µl of 1:10 bleach solution (from 10 – 15 % stock) was added and after 20 seconds 50 µl of 5 % BSA in PBS was added which quenched the bleach solution. 4-cell stage embryos were selected based on morphology under a stereomicroscope and mouth-pipetted sequentially into the two neighboring wells, each containing 100 µl of PBS. Using a mouth-pipette,

washed embryos where transferred into a single well of a 96-well plate (Nunc, optical bottom) filled with 100 µl of PBS. To facilitate the imaging embryos where positioned near each other using a nose-hair pick. The procedure was done in parallel for 2 biological samples, each processed on a separate slide. The selection of 4-cell stage embryos was restrained to less than 10 minutes to ensure synchrony of embryos. Samples were imaged with a Leica DMI6000 B microscope equipped with Hamamatsu Orca Flash 4.0 digital camera and a motorized stage using a 10x objective. Images where acquired in Bright Field, GREEN and dsRED channel using with a frequency of 1/10 minutes. Data was analyzed in Fiji/ImageJ as described in (Burga et al., 2011).

4.9 Statistical Analysis

All data analysis on fluorescence intensity and corresponding plots were generating in R (version 2.15.3).

4.10 Immunofluorescence

Embryos from bleached worms were freeze-cracked on liquid nitrogen, fixed with MeOH for 5 min followed by PFA 1% for 2min. After three washes in PBS 0.25% Triton X-100 (PBS-T) slides were blocked in PBS-T 0.5% BSA before overnight incubation with primary antibody (H3K9me3 [#07-442, Millipore], H3K4me2 [#07-030, Millipore]. H3K27me3 [#07-449, Millipore], H3k36me3 [ab9050, Abcam])at 4°C. After three washes with PBS-T, slides were incubated for 2 hours with secondary antibody (Alexa-555 anti-rabbit; Invitrogen) at room temperature. After three washes in PBS-T, samples were either mounted in Fluoroshield with DAPI mounting medium (Sigma) or processed for DNA-FISH. Images were taken using a Leica SP5 confocal microscope.

4.11 DNA-FISH

For combined histone antibody and DNA-FISH we first carried out antibody staining as described above.DNA-FISH was performed as in (Bean et al., 2004). Briefly, slides were fixed in formaldehyde fixative (3.7% formaldehyde, 80mM HEPES buffer (pH6.9), 1.6 mM MgSO4, 0.8 mM EGTA) for 15 min, rinsed in distilled water for 5min and immersed in 3:1 methanol:glacial acetic acid for 15 min. Samples were allowed to air dry. Probes for mCHERRY DNA were produced using the DIG-Nick Transltion mix (Roche) and following the manufacturer's instructions. We applied the hybridization solution, consisting on labeled probe in 50% formamide, 2x saline sodium citrate (SSC) and 10% dextran sulfate, on each slide and denatured the samples at 80 °C for 10min. After an overnight hybridization at 37 °C, slides were washed in 50% formamide, 2x SSC at 37 °C for 15min, in 2xSSC at 37 °C for 7min and in PBS-T at room temperature for 5min. Samples were incubated with fluorescein-labeled antibody to digoxigenin (Roche) in PBS-T for 2h at 37 °C. After three washes in PBS-T samples were mounted in Fluoroshield with DAPI mounting medium (Sigma).

4.12 Single molecule FISH

Custom Stellaris® FISH Probes were designed against the GFP and endogenous daf-21 mRNA by utilizing the Stellaris® FISH Probe Designer (Biosearch Technologies, Inc., Petaluma, CA) available online at www.biosearchtech.com/stellarisdesigner. L4 stage BCN1049 and BCN1082 worms were hybridized with the GFP Stellaris FISH Probe set labeled with Quasar® dye and the daf-21 set labeled with CAL Fluor® Red 590 simultaneously, following the manufacturer's instructions available online at www.biosearchtech.com/stellarisprotocols. Formamide concentration in hybridization and wash buffer was 15% and the total concentration of pooled probes was 25 nM. Hybridization was performed

overnight at 30 C. Worms were imaged using an oil immersion 100x objective on a Leica DMI6000 B inverted microscope equipped with Hamamatsu Orca Flash 4.0 digital camera and a Lumen 200 metal arc lamp (Prior Scientific).

4.13 Preparation of RNA for the sequencing experiment

To prepare the 'High' and 'Low' daf-21p::mCHERRY populations for RNA extraction, P0 worms kept at 16 and 25 °C were transferred to 20 °C as L4s (12 worms/plate, 4 replicates per treatment). Four and a half days later, when much of the F1 progeny was gravid, the worms were bleached, washed 3x with M9 and the F2 larvae let to hatch o/n in M9 at 20 °C. L1 larvae were plated on 90mm NGM plates seeded with concentrated OP50 lawn and grown at 20 °C. 72 hours later the gravid worms were washed of the plates and RNA was extracted using TRIzol® extraction protocol [WORMBOOK CHAPTER].

4.14 Small RNA-seq library preparation and sequencing

1 microgram of total RNA was treated with Antarctic phosphatase (ref. M0289S, NEB) and subsequently with T4 PNK (3' phosphatase minus) (ref. M0236S, NEB) in order to make the 5' and 3' ends of the RNA available for adapter ligation. Samples were further processed using the TruSeq small RNA Sample Prep Kit (ref. RS-200-0012, Illumina) according to the manufacturer's protocol. Briefly, 3' adapters and subsequently 5' adapters were ligated to the RNA. cDNA was synthesized using reverse transcriptase (SuperScript II, ref. 18064-014, Invitrogen) and a specific primer (RNA RT Primer) complementary to the 3' RNA adapter. cDNA was further amplified by PCR using indexed adapters supplied in the kit. Finally, libraries were size selected using 6% Novex® TBE Gels (ref. EC6265BOX, Life Technologies). Fragments with insert

sizes of 18 to 36 bp were cut from the gel, and DNA was precipitated and eluted in 10 µl EB.

Final libraries were analyzed using Agilent DNA 1000 chip to estimate the quantity and check size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit (ref. KK4835, KapaBiosystems) prior to amplification with Illumina's cBot. Libraries were pooled and loaded at a concentration of 10 pM onto the flowcell, and were sequenced 1 x 50 on Illumina's HiSeq 2000.

4.15 Analysis of the small RNA sequencing data

We trimmed reads off the adaptor using cutadapt version 1.7.1 (Martin, 2011). Only reads with 15 nucleotides or longer and with an perfect match to the adaptor of at least 6 nucleotides were kept. The adaptor sequence was TGGAATTCTCGGGTGCCAAGGAACTCCAGTCAC, the default one for TruSeq Small RNA library preparation kits. Trimmed reads were collapsed to unique sequences keeping the counts of each molecule.

For microRNA identification we mapped reads against the miRBase (Griffiths-Jones et al., 2008) version 21 library of *C. elegans* microRNA hairpins using the miRaligner dependency of seqbuster (Pantano et al., 2010). Only exact matches to mature microRNAs in length and sequence composition were considered. After this step, we separated reads into different classes depending on their length and first nucleotide: 21U, 22G and 26G. The following analysis was done in parallel for the three subgroups of reads and the microRNA expression data.

We mapped reads using bowtie version 1.1.1 (Langmead et al., 2009) with the options -a - -best - -strata -v 0 to find only perfect matches of loci with the exact same mapping quality for each read. Reads were stored and post processed in bam format using SAMtools 0.1.19 (Li et al., 2009). The

reference custom genome used consisted of the *C. elegans* genome assembly WS235 from WormBase (Stein et al., 2001) plus an additional chromosome with the sequence of transgene vector. To identify targets of the different small RNA (sRNA) classes we assigned genes to the reads mapped antisense with the tool featureCounts (Liao et al., 2014) option -s 2 and the flag -M to include multimapping sequences. The annotation used is from Ensembl (Flicek et al., 2012) release 80.

Data scaling, normalization and tests for differential abundance of molecules was done with the DESeq2 package version 1.8.1 (Love et al., 2014) for R-3.2.0 (R Core Team 2015). To avoid biases due to specific small RNA (sRNA) class perturbations we scaled the data using the sequencing depth estimated by microRNA expression. Genes that show an enrichment of antisense molecules for any specific class below a p.value of 0.05 are considered significant. DESeq2 by default calculates significance using the Wald test and corrects for false discovery rate using Benjamini-Hochberg correction.

The profiles shown in the figures have been generated using the suite of utilities BEDtools 2.19.1 (Quinlan and Hall, 2010) and converted to BigWig (Kent et al., 2010). These profiles were loaded into IGV 2.3.57 (Robinson et al., 2011) to visualize the coverage over the integrated transgene.

Name	Genotype	Name	Genotype
N2	wild type	BCN1050	*crgIs1002[daf-21p::mCherry::unc-54 3'UTR; unc-119(+)]*
EU538	*div-1(or148) III*	BCN6101	*div-1(or148) III crgIs1002 IV*
WM158	*ergo-1(tm1860) V.*	BCN6115	*ergo-1(tm1860) V crgIs1002 IV*
YY158	*nrde-3(gg66) X.*	BCN6116	*nrde-3(gg66) X crgIs1002 IV*
YY538	*hrde-1(tm1200) III.*	BCN6108	*hrde-1(tm1200) III; crgIs1002 IV.*
MT17463	*set-25(n5021) III.*	BCN6105	*set-25(n5021) III crgIs1002 IV*
YY160	*nrde-1(gg88) III.*	BCN6106	*nrde-1(gg88) III crgIs1002 IV*
YY156	*nrde-2(gg95) II.*	BCN6107	*nrde-2(gg95) II crgIs1002 IV*
WM27	*rde-1(ne219) V.*	BCN6118	*rde-1(ne219) V crgIs1002 IV*
RB1025	*set-2(ok952) III.*	BCN6117	*set-2(ok952) III crgIs1002 IV*
ZR1	*rbr-2(tm1231) IV.*	BCN6119	*rbr-2(tm1231) IV crgIs1002 IV*
WM49	*rde-4(ne301) III.*	BCN6126	*rde-4(ne301) III crgIs1002 IV*
NL917	*mut-7(pk204) III.*	BCN6120	*mut-7(pk204) III crgIs1002 IV*
HC75	*ccIs4251 I; sid-1(qt2) V.*	BCN6124	*sid-1(qt2) V crgIs1002 IV*
MT16973	*met-1(n4337) I.*	BCN6111	*met-1(n4337) I crgIs1002 IV*
RB1304	*wdr-5.1(ok1417) III.*	BCN6121	*wdr-5.1(ok1417) III crgIs1002 IV*
BR3417	*spr-5(by134) I.*	BCN6113	*spr-5(by134) I crgIs1002 IV*
MT13293	*met-2(n4256) III.*	BCN6112	*met-2(n4256) III crgIs1002 IV*
SS186	*mes-2(bn11) unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II.*	BCN6129	*mes-2(bn11) unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II. crgIs1002 IV*
JK2663	*dpy-11(e224) mes-4(bn67) V/nT1 [unc-?(n754) let-? qIs50] (IV;V).*	BCN6128	*dpy-11(e224) mes-4(bn67) V/nT1 [unc-?(n754) let-? qIs50] (IV;V) crgIs1002 IV*
RB995	*hpl-2(ok916) III.*	BCN6122	*hpl-2(ok916) III crgIs1002 IV*
CB1301	*unc-54(e1301) I.*	BCN6131	*unc-54(e1301) I crgIs1002 IV*
CB4108	*fog-2(q71)V.*	BCN6136	*fog-2(q71) V crgIs1002 IV*
BCN6123	*met-2(n4256) set-25(n5021) III crgIs1002 IV*		
BCN6130	*mes-2(bn11) unc-4(e120)/mnC1 dpy-10(e128) unc-52(e444)II met-2(n4256) set-25(n5021) III crgIs1002 IV*		
BCN1049	*crgIs1004[daf-21p::GFP::unc- 54 3'UTR; unc-119(+)]*		
BCN1082	*crgIs1004[daf-21p::GFP::unc- 54 3'UTR; unc-119(+)]*		
TJ375	*gpIs1[hsp-16-2p::GFP]*		
KM267	*pKM1211(hsp-16.41::hlh-1 + pRF4 (rol-6))*		
MH1870	*kuIs54[sur-5::gfp]*		
JR667	*wIs51[SCMp::GFP + unc-119(+)].*		
CL2166	*dvIs19[pAF15(gst-4p::GFP::NLS)].*		
CF1553	*muIs84[pAD76(sod-3p::GFP)]*		
SJ4005	*zcIs4[hsp-4p::GFP] V*		

Table 5. C. elegans strains used in this study.

5. Discussion

5.1 The fading epigenetic memory of ancestral environment

5.1.1 Increased temperature triggers a heritable multigenerational change in somatic expression of a heterochromatic multi-copy transgene array

A growing body of evidence suggests that the phenotype of an organism is determined not only by its genotype and environment it experiences, but also by the epigenetic information inherited from its parents (Grossniklaus et al., 2013). In this work we used *C. elegans* as a model system to study the dynamics of transgenerational epigenetic inheritance of environmentally triggered change in gene expression and uncover the mechanisms that govern this process.

We showed that transiently raising the temperature of *C. elegans* development induces a heritable change in somatic expression of an integrated multi-copy transgene array detectable for multiple subsequent generations (Figures 10 and 11). Therefore, the level of the transgene expression is influenced by ancestral environment. Heritable, quantitative effects on somatic transgene expression were reported before in *C. elegans* (Arico et al., 2011; Rechavi et al., 2011; Sha and Fire, 2005), however these studies did not resort to a natural environmental trigger such as described here. Raising the temperature at which the worms develop for just one generation was sufficient to modify expression for at least seven subsequent generations (Figure 11). Prolonged exposure to high temperature progressively increased transgene expression for three subsequent generations before reaching a plateau (Figures 8 and 9) and

required 15 generations to reset upon return to the original environment (Figure 10). This means that the magnitude and transgenerational stability of the epigenetically inherited state depends on the duration of the environmental signal. (Schott et al., 2014) made a similar observation studying transgenerational changes in expression of endogenous genes in response to temperature change. An analogous built-up of epigenetic load was also documented in flies where heat shocking embryos for each successive generation increased transgenerational stability of a phenotype associated with depleted heterochromatin (Seong et al., 2011).

We did not observe a lasting multigenerational memory using a single copy version of the transgene (Figure 12), which suggests that the size and possibly the repetitive nature of the domain are important for long-term epigenetic stability. Large repetitive arrays are silenced in the *C. elegans* germline and become highly enriched in heterochromatin marks (Bessler et al., 2010; Jedrusik and Schulze, 2001; Kelly and Fire, 1998). In line with this, we observed no germline expression of the multi-copy *daf-21p::mCHERRY* and *daf-21p::GFP* transgenes that are controlled by promoter region of a germline-enriched *daf-21* gene (Figure 7, 17 and 18). We showed that the *daf-21::mCHERRY* is regulated by multiple heterochromatin components including putative H3K9 methyltransferases, chromodomain binding protein HPL-2 and the PRC2 complex (Figures 29, 30 and 39) in line with the heterochromatic nature of the transgene. Disruption of heterochromatic domains in response to high temperature was previously described in yeast (Kloc et al., 2008), flies (Gowen and Gay, 1934) and also in plants where it controls the onset of flowering in a process called vernalization (Baulcombe and Dean, 2014). Hence, temperature sensitivity might be a conserved property of heterochromatin.

We provide evidence for epigenetic transmission through both gametes (Figures 27 and 28). This is in contrast to a previous study that demonstrated that expression changes elicited by increased temperature in the ancestors are heritable through female gamete only (Schott et al., 2014). However, the authors investigated only endogenous transcripts present in the early embryo and therefore produced in the germline, whereas here we studied somatic expression of a transgenic array.

5.1.2 Germline nuclear RNAi and chromatin pathways establish the heritable state of somatic transgene expression

In *C. elegans* single copy transgenes can be stably silenced in the germline through activity of the nuclear RNAi pathway composed of siRNAs bound by an Argonaute HRDE-1 and nuclear components NRDE-1/2/4 (Ashe et al., 2012b; Luteijn et al., 2012; Shirayama et al., 2012). The silent state is propagated and requires chromatin components SET-25, SET-30 and HPL-2, which are though to act downstream from the nuclear RNAi machinery (Ashe et al., 2012b). We found that HRDE-1, NRDE-2 and SET-25 are required to keep the *daf-21p::mCHERRY* transgene partially repressed in somatic cells (Figures 29 and 30). We also showed that they are required for the multigenerational inheritance of temperature mediated changes in *daf-21p::mCHERRY* expression in somatic cells (Figures 32 and 33). In our experiments, depletion of maternally supplied SET-25, but not of HRDE-1 or NRDE-2, led to an immediate loss of expression differences in the progeny (Figure 38). Similarly, the temperature shift assay (Figure 32) and the sorting assay (Figure 35) demonstrated a requirement for SET-25 for transmission of altered expression states, whereas loss of HRDE-1 and NRDE-2 appears to have a more quantitative effect (Figures 32, 33, 35 and 36). Taken together our results demonstrate that the nuclear RNAi/chromatin pathway that controls the stable and heritable epigenetic silencing of single copy

transgenes in the germline is also required for epigenetic inheritance of quantitative changes in expression in somatic cells triggered by an environmental change.

5.1.3 Epigenetic inheritance of conflicting *cis* and *trans* signals determines the level of expression in the progeny

The inheritance of small RNAs acting in *trans* to silence gene expression in *C. elegans* is strongly supported by various studies (Alcazar et al., 2008; Rechavi et al., 2011; Sapetschnig et al., 2015). What is more controversial is whether the histone marks that are found downstream of the nuclear RNAi pathway, namely the H3K9me3 modification, can be passed across generations independently from the small RNAs and whether that can significantly affect expression. Our heterozygous crossbreeding experiment (Figure 45) supports the existence of both *cis* and *trans* modes of inheritance and moreover that they can have opposite outputs. Maternally supplied *trans*-acting small RNAs, which are more abundant in the 'high' *daf-21p::mCHERRY* worms (Figure 43) reduce the expression of paternally supplied *daf-21p::mCHERRY* transgene expression (Figure 45). This also demonstrates that the 'memory' of elevated expression in 'high' worms is not encoded in *trans* by the small RNA content. In support of the *cis* based mode of inheritance, we found that the *daf-21p::mCHERRY* transgene locus is depleted of H3K9me3 marks in the early embryos sired by 'high' *daf-21p::mCHERRY* parents (Figure 41). The transgene is transcriptionally inactive at that stage (Figures 14 and 15), which excludes that this difference is caused by co-transcriptional chromatin remodeling events and supports the role of H3K9me3 domain as transgenerational carrier of epigenetic information. Together, our results point towards a model where the state of expression of a somatically expressed transgene is encoded in *cis* by H3K9me3 on the locus, whereas *trans*-acting small RNAs that are inherited

independently from the locus modulate expression in each generation (Figure 50). This model explains the mechanism of inheritance of the environmentally modified trait, but also how the state is reset back to its original state in subsequent generations. Most studies that investigate epigenetic inheritance, especially in mammals, rely on correlative data. Our work demonstrates that an increased level of an epigenetic agent associated with silencing such as siRNAs might, paradoxically, be a hallmark of increased expression of their target.

5.2 Crossing the Weismann Barrier

5.2.1 Somatic expression of a multicopy transgene reinforces its repression in the germline in subsequent generations

In animals, changes in gene expression in somatic tissues that occur during an animal's life are normally considered to be incapable of influencing the next generation. Here we demonstrated that muscle-specific upregulation of the *daf-21p::mCHERRY* transgene during a single generation results in decreased expression of the transgene two generations later (Figure 47). Therefore somatic expression of a transgene influences its expression in subsequent generations. How is the signal communicated? Double stranded RNA produced in neuronal cells can travel to the germline through the SID-1 dsRNA channel where it can trigger transgenerationally stable silencing of a complementary sequence mediated by HRDE-1 (Devanapally et al., 2015). We reasoned that soma-triggered repression could participate in recovery of transgene expression state after exposure to high temperature. Indeed, *sid-1* mutant worms exhibit delayed transgenerational recovery of expression after a transient temperature increase (Figure 49). Hence, resetting of the epigenetic memory is reinforced by soma to germline communication mediated by the SID-1 dsRNA channel.

Combining the published data with our own observations leads to the conclusion that in *C. elegans* the Weismann barrier (Weismann, 1893) is permeable to epigenetic flow of information from the soma to germline mediated by dsRNA. These findings raise many important and interesting questions. Are all dsRNA molecules synthesised in somatic cells transported to the germline triggering germline silencing on the complementary loci? A recent study presented evidence for endogenous dsRNA complementary to 664 different sites distributed throughout the genome (Whipple et al., 2015). These sites primarily overlap with noncoding regions of coding genes and 31 % correspond to targets of endogenous siRNAs and to many stress response genes (Whipple et al., 2015). Perhaps these endogenous dsRNAs are involved in transgenerational control of expression that prepares the progeny for the stressful environment encountered by the parents.

It is also possible that soma to germline communication is a part of a protective system that keeps transposons and other repetitive elements repressed. In *A. thaliana*, retrotransposons are activated in the vegetative part of the gamete called a companion cell and this reinforces transposon methylation in the gamete (Ibarra et al., 2012) through an siRNA-based mechanism (Calarco et al., 2012). Similarly, expression of repetitive elements in somatic tissues of *C. elegans* could produce a substrate for promoting their repression in the germline. Germline nuclear RNAi participates in repression of LTR retrotransposons (Ni et al., 2014). Perhaps somatic expression of these elements provides a substrate for their silencing in the germline. It would be interesting to see if there is any evidence for mobilization of these transposons in the germline of *sid-1* mutant worms.

Worms might also use the SID-1 channel to 'buffer' somatic gene expression transgenerationally. Any somatic gene that becomes overexpressed could potentially be repressed in subsequent generation through an epigenetic mechanism if an anti-sense RNA molecule would concurrently be produced. Such anti-sense RNA should only be produced from repetitive loci such as that studied here. The extent and the function of the soma-to-germline epigenetic remodeling in *C. elegans* await further investigation.

To confirm that the heritable effect triggered by the *unc-54* allele is indeed due to mobile dsRNA, one would need to perform the same experiment in a *sid-1* mutant background. This should abolish the effect of somatic overexpression on the transgene activity in subsequent generations. Similarly, *hrde-1* and *set-25* mutants should also fail to reduce expression in generations following somatic overexpression. It would also be interesting to see if increasing *daf-21p::mCHERRY* expression in tissues other than muscle triggers a similar effect. Moreover, we have no evidence for production of dsRNA but it is likely given the repetitive and random way in which the transgenic arrays assemble. We still don't know whether the dsRNA produced in the soma is processed before the transport to the germline and why the somatic cells themselves do not silence the transgene (expression from the constitutive promoter might prevent full silencing in somatic cells).

5.2.2 Interaction between soma and germline generates an incoherent feed forward motif

In the progeny of an animal exposed to high temperature, the germline, which separates early on in embryonic development, inherits an H3K9me3 depleted heterochromatic state. In differentiated somatic cells reduced level of H3K9me3 inherited from the parent results in increased

transgene expression, which is likely accompanied by increased production of dsRNA. The dsRNA then travels to the germline where it promotes re-acquisition of the H3K9me3 mark, which will act to decrease expression of the transgene in subsequent generations. The interactions between chromatin in the zygote, germline and soma therefore form an incoherent feed forward loop (Figure 51) which is a network motif well known to result in a transient response to a stimulus (Mangan and Alon, 2003).

5.3 Stalled replication as a roadblock for epigenetic inheritance

5.3.1 Replication stress might interfere with inheritance of epigenetic marks

We showed that interfering with DNA replication during embryonic development results in de-repression of a multi-copy transgene. Several lines of evidence suggest that this effect is mediate via disruption of the inheritance of heterochromatin on the transgene locus. RNAi-mediated knockdown of the primase subunit DIV-1 in worm strains carrying various transgenic reporters has a larger effect on expression from multi-copy array. It is particularly evident for the *hsp-16.2p::GFP* heat-inducible reporter that shows a robust increase in expression after *div-1* knockdown in the multi-copy strain (Figure 52) but no elevated expression in the strain carrying a single copy reporter (Figure 53). Although we detected an increase in expression of the *daf-21p::GFP* single copy reporter (Figure 54) it was modest (1.79 fold change, p = 6.6e-05, Wilcoxon rank test) compared to the response of the multi-copy reporter (5.75 fold change, p-value = 1.6e-14, Wilcoxon rank test). This supports the notion that the replication fork stalling affects heterochromatin, since enrichment in heterochromatin marks is one of the key features of repetitive transgenes that distinguishes them from single

copy reporters. In this regard, it would interesting to test how other transgenes respond to inhibition of replication fork components and how does that correlates with their chromatin composition.

To search for the underlying mechanism we assayed a number of chromatin and RNAi component mutants for their ability to mount the *daf-21p::mCHERRY* transgene upregulation response when treated with *div-1 RNAi* (Figures 59 – 63). Mutation in no single RNAi or chromatin component abolished the effect on transgene expression caused by *div-1 (RNAi)* treatment. However, the mutant worms where genes encoding three histone-modifying enzymes *mes-2, met-2* and *set-25* were deleted exhibited a dampened response to *div-1 (RNAi)* treatment (Figures 62 and 63). This is consistent with the model that replication stress interferes with the inheritance of parental histone modifications to the daughter chromatin. Since the nucleosomes in *mes-2, met-2, set-25* triple mutants are devoid of the major heterochromatin marks H3K27me3 and H3K9me1/2/3, their loss at the replication fork stalling should have little effect on transgene expression, which is what we observed (Figure 62 and 63). The residual effect on transgene expression in these triple mutants caused by *div-1* (RNAi) (Figure 63) could be due to possible residual H3K27me3 present on the transgene, another heterochromatin mark or inappropriate incorporation of activation marks.

More direct evidence would be needed to claim that heterochromatin replication is compromised in *div-1 (RNAi)* or *div-1(or148)* mutant embryos. To demonstrate that chromatin really is affected one could perform chromatin immunoprecipitation using antibodies against the H3K27me3 and H3K9me3 marks followed by quantitative qPCR using primers complementary to *mCHERRY* using embryonic extracts from control and *div-1(RNAi)* treated samples. One could also quantify the

dynamics of the loss of heterochromatin marks over early embryonic divisions using immunohistochemistry in embryos lacking the enzyme necessary to replenish the marks after replication (Gaydos et al., 2014). If replication fork stalling really disrupts faithful copying of histone modifications from parental chromatin to the daughter strands the loss of heterochromatin marks over embryonic divisions would be accelerated.

The crossbreeding experiments (Figure 66) where we demonstrated that transgene upregulation caused by replication stress is heritable, also points to a heterochromatin-based mechanism. We know that the H3K9me3 content of the transgene chromatin is responsible for inheritance of expression levels. Therefore the H3K9me3 mark is likely depleted on the transgene locus in worms subjected to replicative stress. This is also supported by the fact that the heritability of the *div-1* triggered upregulation was suppressed in worms that had been previously depleted of H3K9me3 by means of high temperature (Figure 66). To confirm that the loss of heterochromatin repression caused by replicative stress is inherited through the same mechanism as the temperature-induced change one could cross the F1 males in (Figure 66) to *set-25* hermaphrodites and see if the expression differences in the progeny are indeed abolished.

Our data suggest that replication fork stalling during embryonic development results in disruption of mitotic inheritance of the heterochromatin state of the transgene. One of the proposed explanations for this phenomenon is that stalling the DNA polymerase complex can lead to its uncoupling from the helicase that unwinds the DNA double helix ahead of the replication fork. If the DNA polymerase cannot ‘keep up’ with the helicase it could result in an extended stretch of single-stranded DNA behind the helicase. The nucleosomes evicted from the DNA by the helicase (and associated proteins) would not be able to

reassemble on the daughter strands since single-stranded DNA is a poor substrate for nucleosome assembly. Hence, the epigenetic information would be lost (Sarkies and Sale, 2012). Such a loss of parental nucleosomes has been shown to occur in vertebrate DNA regions that are prone to forming secondary structures called G4-quadruplexes (Sarkies et al., 2010), particularly when combined with a loss of translesion DNA polymerase REV-1 that normally assists in efficient replication of those regions (Goodman and Woodgate, 2013). Interestingly, RNAi knockdown of *C. elegans* REV-1 homologue also resulted in de-repression of the *hsp-16.2p::GFP* multi-copy transgene (Table 3) and similarly leads to increased expression of the *daf-21p::mCHERRY* array (Kadri Reis, personal communication). Therefore it is possible that repetitive nature of integrated arrays makes them prone to create G4-quadruplexes or other ordered DNA structures that are particularly sensitive to loss of epigenetic information during a compromised DNA replication cycle.

In addition, an extended stretch of single stranded DNA that might form at the replication fork could act as a substrate for recruitment of RNA Pol II and spurious transcription (Mirkin and Mirkin, 2007). This could lead to eviction of heterochromatic nucleosomes from the chromatin as well as incorporation of H3.3 histone, which would favor a more open chromatin state (Frey et al., 2014; Ooi et al., 2006).

If the 'deficient nucleosome inheritance' model is wrong, what else could cause the transgene upregulation in *div-1* embryos? Perhaps it is due to disrupted 22G RNA-guided heterochromatin deposition, which (like in *S. pombe*) could take place during DNA replication. However our data argue against such a model because *nrde-1, nrde-2* and *hrde-1* mutants readily upregulate the transgene after knockdown of *div-1* by RNAi (Figures 59 and 61).

Another possibility is that depletion of DNA polymerase complex subunits leads not only to replication fork stalling, but also disrupts the relative timing of firing of replication origins. Eukaryotic genomes are known to replicate in phases, with the active, euchromatic regions replicating early in the S-phase and heterochromatin regions replicating late (Hiratani et al., 2008). It has been proposed that the local concentration of chromatin substrates might vary in time of the replication cycle (Lande-Diner et al., 2009). If the active DNA regions are replicated more slowly (for example as a result of DIV-1 depletion), this might lead to premature firing of the heterochromatin replication and substantial overlap between replication of active and repressive chromatin. It this way, heterochromatic regions might erroneously acquire properties of the active chromatin through activity of euchromatin-promoting factors present in the nucleoplasm due to still ongoing replication of active chromatin regions. In this context, one would expect that depletion of such chromatin activating agents would suppress the loss of heterochromatin during caused by delayed replication. This fits with our observation that inhibition of CRS-1 pathway components, which were proposed to participate in maintenance of active chromatin states (Cecere et al., 2014), rescues the upregulation of the transgene caused by *div-1(or148)* (Figures 67, 68 and 69). However, a distinction between early and late replicating regions have not been demonstrated in early embryonic cycles of *C. elegans* development, where the S-phase is short and might not allow for such temporal separation.

The different possible mechanisms described above are not necessarily mutually exclusive and it is likely that the truth lies somewhere in between these scenarios (or yet entirely elsewhere). However, our discovery that depletion of DNA polymerase component affects expression of a heterochromatic domain in a heritable fashion makes this

system an excellent substrate for further investigation in the research of mechanisms of epigenetic inheritance. We can now harness all the advantages of the *C. elegans* model system to gain a better understanding of how epigenetic states are maintained across cellular divisions and generations and how this is coupled to DNA replication.

5.3.2 Knockdown of RNAa pathway components rescues transgene over-expression triggered by replication stress

We found that the CSR-1 pathway, which includes a germline-expressed Argonaute CSR-1, RNA-dependent RNA Polymerase EGO-1 and a DEAD-box helicase DRH-3, participates in activation of the multi-copy transgene during embryonic development (Figures 67 and 69). Involvement of this pathway in control of somatic expression of multi-copy transgenes was previously reported (Fischer et al., 2013). Interestingly, by knocking down CSR-1 pathway components we were able to suppress the effect of replication stress on transgene expression (Figures 67 and 68). Therefore upregulation of a heterochromatic, multi-copy transgene triggered by replication stress might rely on the CSR-1 pathway components. Depleting CSR-1 or DRH-3 in *mes-2, met-2, set-25* triple mutants resulted only in a minor, 1.3 – 1.4 fold reduction in expression (Figure 70). It is therefore possible that both CSR-1 pathway and replication stress act through regulating heterochromatin on the transgene.

We could not eliminate the CSR-1 pathway entirely, since all of its components are essential for embryonic development (Claycomb et al., 2009), therefore our experiments relied on controlled RNAi knockdown experiments that did not result in complete loss of function phenotypes. We found that depletion of DRH-3 from the mother and delivery of the transgene from an unaffected father resulted in a robust decrease in

zygotic transgene activation (Figure 69). On the contrary, depleting DRH-3 in the father that delivered the transgene to the zygote had no effect on its expression (Figure 69). This suggests that CSR-1 components act in the early embryo to promote activation of the transgene. In the sequencing experiment we found a population antisense 22G RNAs targeting the *daf-21* promoter region (Figure 41B). We cannot exclude that some (or all) of these molecules originated from the endogenous *daf-21* gene, which is active in the germline (Figure 18). As such, these 22G RNAs might be CSR-1 bound and their inheritance could positively regulate expression of the transgene in *trans*. This would explain why depletion of DRH-3 in the maternal germline by RNAi affected the expression on paternally inherited transgene (Figure 69).

Our understanding of how the CSR-1 pathway affects the epigenetic state of the transgene is very limited. However, our experiments suggest that inhibiting CSR-1 could counteract the loss of epigenetic memory caused by replication stress. It would be interesting to see whether any endogenous CSR-1 targets are miss-expressed in worms affected by replication stress. Moreover, if the sterility observed in *div-1(or148)* mutant worms (Figure 56) is epigenetic in its cause, perhaps we could rescue this phenotype by inhibition of the CSR-1 pathway.

5.3 Ideas, speculations and open questions

5.3.1 Temperature change could have a direct effect on the germline chromatin

We showed that a single generation of growth at elevated temperature was sufficient to 'imprint' the transgene for higher expression in seven subsequent generations (Figure 11). We did not detect expression of the transgene in germline cells, even at 25 °C (Figure 7). Therefore, it might appear that the somatic response observed in the directly affected worms

is transferred through the germline to the next generation. Alternatively, the germline might respond to the temperature change independently of the soma. Transgene chromatin could be modified in the germline without any detectable transcriptional output and the effect would only be observed in the somatic cells of subsequent generations. In fact, preliminary results (*data not shown*) suggest a loss of H3K9me3 mark at the transgene locus in the gonads of animals developed at 25 °C for a single generation (shifted from 16 °C at embryonic stage). Several lines of evidence presented in this work suggest that elevated temperature has a direct effect on germline chromatin.

First, when the transgene was upregulated specifically in somatic cells, we did not observe an increased expression in the next generations (Figure 47). However, the somatic overexpression in our experiments was limited to muscle cells (Figure 48), therefore we cannot exclude that a signal born in a different tissue (such as intestine, pharynx, or neurons) in response to temperature mediates chromatin changes in the germline. It is important to mention here that van Oosten-Hawle et al. showed that the presence of a muscle specific *unc-54(e1301)* allele triggered an increase in transcriptional multi-copy *daf-21p::GFP* transgene expression in other tissues apart from body wall muscle namely in intestine, pharynx and canal body(van Oosten-Hawle et al., 2013). They interpreted these results as evidence for trans-tissue chaperone signaling: misfolded UNC-54 protein transcribed from the *e1301* allele in the muscle tissue triggers upregulation of the chaperone that is communicated to other tissues achieving an organismal-level of response. We observed no upregulation of the *daf-21p::mCHERRY* transgene in tissues other than muscle in *unc-54(e1301)* worms (Figure 48). In fact, pharyngeal expression appeared suppressed compared to animals with the transgene in wild-type genetic background (Figure 48). Since the *unc-54(e1301)* allele is temperature

sensitive, it is possible that (van Oosten-Hawle et al., 2013) subjected the worms to 25 °C in order to facilitate identification of the homozygous mutants when creating the *unc-54(e1301), daf-21p::GFP* strain. This would cause a transgenerationally stable upregulation of the transgene in non-muscle tissues which would still be detectable in future generations grown at low temperature.

Another reason to believe that germline can respond to temperature independently from the soma is that the absence of SID-1, which constitutes a route for soma-to-germline communication (Devanapally et al., 2015), did not abolish the transgenerational memory of increased expression (Figure 49). However, the SID-1 channel does appear to be important for the recovery of the expression after environmental perturbation (Figure 49). Mutation of *sid-1* prevents transport of dsRNA from soma to germline, but other molecules transported through different routes could be mediating the response.

Multiple aspects of germ line physiology and development in *C. elegans* are known to be sensitive to environmental changes. Conserved signaling pathways including Insulin/IGF, TGFβ, TOR, AMPK and nuclear hormone receptor pathways mediate this sensitivity (Hubbard et al., 2013). It would be interesting to test if inhibiting any of these pathways interferes with any aspect of the transgenerational dynamics of heterochromatic transgene expression. Moreover, response to a rapid temperature changes are known to be coordinated by the nervous system. For example, the heat shock response in somatic cells of *C. elegans* is mediated by the activity of AFD neurons (Prahlad et al., 2008). Repeating our temperature assay in worms carrying the *daf-21p::mCHERRY* transgene and a loss-of-function mutation affecting the AFD would reveal

whether the transgenerational effect is dependent of thermal sensation arising from the AFD neurons.

5.3.2 What is the direct effect of increased temperature?

Negative effects of increased temperature on the maintenance of heterochromatic states in animals have been observed in yeast (Kloc et al., 2008), flies (Gowen and Gay, 1934) and is well known in plants, where it controls the flowering time in a process of vernalization (Baulcombe and Dean, 2014). We do not now which molecular step in the process of heterochromatin establishment or maintenance is sensitive to temperature. We envision several possible routes through which the transgene upregulation might be triggered including increased activity of its promoter, decreased efficiency of RNAi silencing and lower fidelity of nucleosome inheritance during DNA replication.

It is possible that increased temperature is causing heritable changes in transgene chromatin described in this work through a transcription-dependent process. The *daf-21* promoter drives expression of *C. elegans* homologue of HSP90 chaperone, which is activated by misfolded proteins (van Oosten-Hawle et al., 2013) and therefore to be more strongly activated at higher temperatures. This could lead to transcriptionally coupled remodeling of the transgene chromatin in the germline through eviction of heterochromatic nucleosome or recruitment of histone-modifying enzymes (Venkatesh and Workman, 2015).

Most of the molecular steps involved in heterochromatin assembly happen during DNA replication (Moazed, 2011) and increased temperature could interfere with one or multiple stages. In *S. pombe* RNAi mediated deposition of heterochromatin marks takes place during DNA replication (Kloc et al., 2008) and was suggested to be less efficient at higher

temperature (Kloc et al., 2008) but the molecular basis for this is unknown. Since RNAi mediated heterochromatin assembly is highly sensitive to the prior presence of the H3K9me3 at the locus (Shanker et al., 2010), it is possible that the apparent decrease of RNAi-dependent silencing at higher temperature is due to a deficiency in RNAi-independent chromatin replication (Audergon et al., 2015; Ragunathan et al., 2015).

It would be interesting to track the expression of the *daf-21p::mCHERRY* transgene at different temperatures immediately after loss of HRDE-1. Perhaps at 16 °C inheritance of expression is less reliant on HRDE-1, and SET-25 alone would be sufficient to maintain the transgene in a repressed state for a considerable number of generations. Like in *S. pombe* (Allshire and Ekwall, 2015; Audergon et al., 2015), an H3K9me3 demethylase could be interfering with the inheritance process. A putative H3K9me3 demethylase JMJD-2 was shown to largely suppress progressive fertility defects caused by mutation in *spr-5* (Greer et al., 2014). A similar effect was observed for a chromodomain-containing protein EAP-1 (Greer et al., 2014). Inhibition of JMJD-2 and EAP-1 could therefore increase transgenerational stability of H3K9me3 domains and promote their inheritance.

Another possibility is that one of the proteins involved in the pathway could be downregulated, misfolded or modified at higher temperature. Temperature-triggered post-translational modification could change the properties of a key element of heterochromatin impairing its function as is the case in the ATF-2 depended heterochromatin formation in *D. melanogaster* (Seong et al., 2011). Moreover, at higher temperature siRNA targeting could be less efficient due to weakening of the base pairing between the small RNA molecule and the target transcript. This

could be tested in an *in vitro* assay using a reconstituted minimal system containing a known amount of siRNAs loaded into Argonaute and target RNA. One would then need to measure how efficiently do the target mRNA molecules co-precipitate with the Argonaute depending on the temperature. In addition, a sensitive spectroscopy technique that can quantify relative abundance of single and double stranded RNA could also be applied. Yet, if in *C. elegans* temperature interferes with heterochromatin assembly due to inefficiency of the nuclear RNAi pathway, mutants that are defective in nuclear RNAi are expected to exhibit no further increase in transgene expression when developed at higher temperature. In our assay, all tested mutants (including nuclear RNAi components *nrde-2* and *hrde-1* as well as the downstream chromatin component *set-25*) mounted a robust temperature response (Figure 32, 33, 34). The only exception was the *wdr-5.1* mutant, where the increase was still substantial, albeit not significant.

In *S. pombe*, is RNAi- independent inheritance of H3K9me3 is insufficient for effective long term silencing, even in the absence of the H3K9me3 demethylase (Audergon et al., 2015; Ragunathan et al., 2015). These studies show that the mark can be propagated across cellular divisions independently from RNAi. In embryonic divisions of *C. elegans* heterochromatic nucleosomes containing the H3K27me3 mark appears to be inherited to the daughter strands (Gaydos et al., 2014). It would be interesting to test if the same dynamics would be observed using an antibody detecting H3K9me3.

Conceivably, elevated temperature could interfere with the passive HMT-independent passage of parental nucleosomes to daughter chromatin, simply through increasing entropy and diffusion. To demonstrate this, one would need to use maternally deficient HMT embryos (lacking either

PRC2 or SET-25) and quantify the rate of decay of paternally delivered heterochromatic nucleosomes (using an appropriate antibody) over embryonic divisions at different temperatures. This model also predicts, that if all heterochromatin marks were absent, elevated temperature would have no additional effect on transgene expression. In this respect it would be interesting to see if the *mes-2, met-2, set-25* triple-mutant worms, which lack H3K27me3 and H3K9me1/2/3 marks, loose the ability to increase transgene expression when shifted from low to high temperature. Such a model also implies that the active marks such as H3K36me3 that are propagated in the early embryos would also be lost, which could affect gene expression and physiology of the developed animal.

The inefficiency of RNAi-independent heterochromatin inheritance at high temperature could be the reason why the small RNA components become important for fertility when worms are grown at 25, rather than at 20 °C (Buckley et al., 2012). In plants, it was demonstrated that siRNAs are necessary to restore the silencing on a retrotransposons that is activated by heat stress (Ito et al., 2011). If siRNA pathway is compromised multiple novel insertions of the retrotransposon are detected in the progeny of stressed plants (Ito et al., 2011). DNA replication is prone to errors and hence organisms evolved a large array of repair mechanisms (Sancar et al., 2004). Similarly, small RNAs might function as epigenetic repair mechanisms that correct errors in replication of heterochromatic state. The silencing 22G RNAs that associate in *C. elegans* with HRDE-1 would counteract post-replicative heterochromatin loss; whereas the CSR-1 associated licensing 22G RNAs would fix any errors in replication of epigenetically active state and protect it from encroaching heterochromatin domains.

5.3.3 The role of other histone marks in epigenetic inheritance of somatic expression levels

Our experiments show that mutating enzymes that regulate methylation states different than H3K9me3 also intereferes with inheritance of an elevated expression state triggered by temperature (Figure 33). This includes an H3K4me3 demethylase *spr-5*, putative H3K9me2 methyltransferase *met-2*, and H3K36me3 methyltransferase *met-1*. One reason for the apparent lack of transgenerational memory triggered by temperature in these mutants could be that the transgene is already in a de-repressed state, characterized by low H3K9me3. Consistently, in *spr-5* worms H3K9me3 is at a much lower level compared to wild-type worms (Greer et al., 2014). Similarly, mass spectroscopy analysis of *met-2* mutant worms revealed a strong depletion of H3K9me3 mark (Towbin et al., 2012). Immunofluorescence experiments could be performed to test this idea.

Another confounding factor is the partially penetrant sterility and general fecundity defects that manifest in some of these mutants when they are grown at 25 °C. This has been reported for *spr-5* (Katz et al., 2009), *met-2* (Checchi and Engebrecht, 2011), *nrde-2* (Buckley et al., 2012) and also *hrde-1* (Buckley et al., 2012). Our own observation of these mutant strains after exposure to 25 °C confirmed these defects, although we did not record or quantify it. This phenomenon could result in selection and bias the results. As an example, consider a heterogeneous population of *spr-5* mutant worms grown at 20 °C. It has been shown that at 25 °C the H3K9me3 mark is depleted progressively with each generation (Greer et al., 2014). Individual worms will vary in terms of how close they are to sterility. This should be correlated with the global extent of H3K9me3 loss, which in turn is decisive for the transgene expression level. Therefore, if we shift our population from 20 to 25 °C, the brighter worms

are expected to become sterile or produce much less progeny. This will select for the worms at the lower end of expression distribution to contribute disproportionally to the next generation. It might explain why in the F1 and F2 generations, the median expression is lower in the population whose parents developed at 25 °C in *spr-5* and *met-2* mutants (Figure 33). To if this really is the case, one could pre-sort 'high' and 'low' mutant worms (as in Figure 35) and perform the temperature shift assay in both groups in parallel while measuring expression and fecundity in each group. Therefore, various histone methyl-transferases could be essential for inheritance of epigenetic state of *daf-21p::mCHERRY* transgene through indirect effect on H3K9me3 mark. However, at this stage we cannot exclude that H3K36me3, H3K9me2 or H3K4me3 methyl marks are somehow directly involved in establishment or maintenance of environmentally induced heritable epigenetic state studied here.

How about the other major heterochromatin mark - H3K27me3? Does it play any role on in the transgenerational dynamics of gene expression in response to temperature? On first sight it appears not. Crossing 'high' and 'low' male worms to *mes-2* mutant worms did not abolish the difference between the populations neither reduced it (Figure 39). In fact, although expression in both groups was increased, it did so more prominently in the progeny of 'high' worms. The fold change, calculated as the ratio of the medians of F1 'high' and F1 'low' populations was 1.85 for the control and 2.77 for *mes-2*. We did not observe such a "magnifying" response for any other mutants tested this way (Figure 38). How can we interpret this result? One possibility is that in high worms, which have lost much of the H3K9me3 at the transgene locus due to elevated temperature, repression becomes more dependent on H3K27me3. Although this type of redundancy between these two marks was not reported in *C. elegans*, the H3K9me2 mark can functionally replace H3K27me3 in maintaining the X

chromosome repressed in the male germline (Gaydos et al., 2014). Quantitative fluorescent imaging or chromatin immuno-precipitation using antibodies against H3K27me3 could show whether this type of heterochromatin compensation really takes place.

5.3.3 A delay between siRNA production and acquisition of H3K9me3 marks is likely important for transgenerational expression dynamics

Evidence for the delay between the appearance of 22G RNAs and acquisition of H3K9me3 marks has been provided by (Buckley et al., 2012; Gu et al., 2012) and is described in the introduction section of this work. Our data support the existence of the delay, such that it is necessary to explain why the transgene requires multiple generations to recover the expression level despite a readily elevated 22G RNA content in the early generations after environmental perturbation. In fact this delay was first noted by Burton et al. who exposed GFP expressing worms to dsRNA and noted : "*siRNAs are detectable before the appearance of H3K9me marks, suggesting that chromatin marks are not directly inherited but, rather, reestablished in inheriting progeny. Interestingly, H3K9me marks appear more prominently in inheriting progeny than in animals directly exposed to dsRNA, suggesting that germ-line transmission of silencing signals may enhance the efficiency of siRNA-directed H3K9me"* (Burton et al., 2011). Perhaps siRNA mediated H3K9me3 targeting can only happen at a specific stage of the developmental cycle such as the early embryonic divisions. As is the case for the mammalian germline, *C. elegans* embryonic germ cells and early blastomeres could also be particularly prone to large changes in epigenetic composition. Such a limited temporal window of action would explain the gradual, multi-generational effect of elevated 22G RNAs on the *daf-21p::mCHERRY* transgene expression. A quantitative immunohistochemistry approach could provide a temporal

profile of H3K9me3 changes in germline germ cells and early somatic blastomeres and could provide support for the above hypothesis.

5.3.4 A model for transgenerational epigenetic inheritance and resetting based on two incoherent feed-forward loops

We propose the following integrated model for epigenetic regulation of a multi-copy heterochromatic locus and its inheritance (Figure 72). The model explains expression dynamics of somatic expression after environmental perturbation and makes several testable predictions. In the germline, the H3K9me3 chromatin marks deposited by SET-25 on the transgene locus are propagated mitotically and meiotically across generations maintaining a relatively steady level across the germline cycle. The antisense 22G RNAs engage the nuclear RNAi machinery to deposit H3K9me3 at the transgene locus. These HRDE-1 bound 22G RNAs can engage in amplification (Sapetschnig et al., 2015), which counteracts their gradual dilution over multiple generations. Additionally, somatic expression of the repetitive transgene might provide substrate for the nuclear RNAi silencing pathway, through SID-1 mediated supply of double stranded RNA. If environmental conditions are stable for a long time, the system will reach equilibrium and therefore a steady state of expression across generations. In animals whose ancestors experience high temperature we observed depleted H3K9me3 at the transgene (Figure 41). This is translated into higher expression of the transgene in somatic cells for multiple generations (Figure 10). This increased transgene expression is accompanied by higher level of 22G RNAs (Figure 42), which we show have silencing properties (Figure 45). We propose that the elevated somatic expression increases the amount of dsRNA transported to the germline through the SID-1 channel, which provides template for production of 22G RNAs in the germline and facilitate recovery of a repressed state (Figure 51). Repeating the sequencing experiment

described in (Figure 42) in *sid-1* mutants could reveal if the increase of 22G RNA population in 'high' worms is due to soma-germline transport of dsRNA.

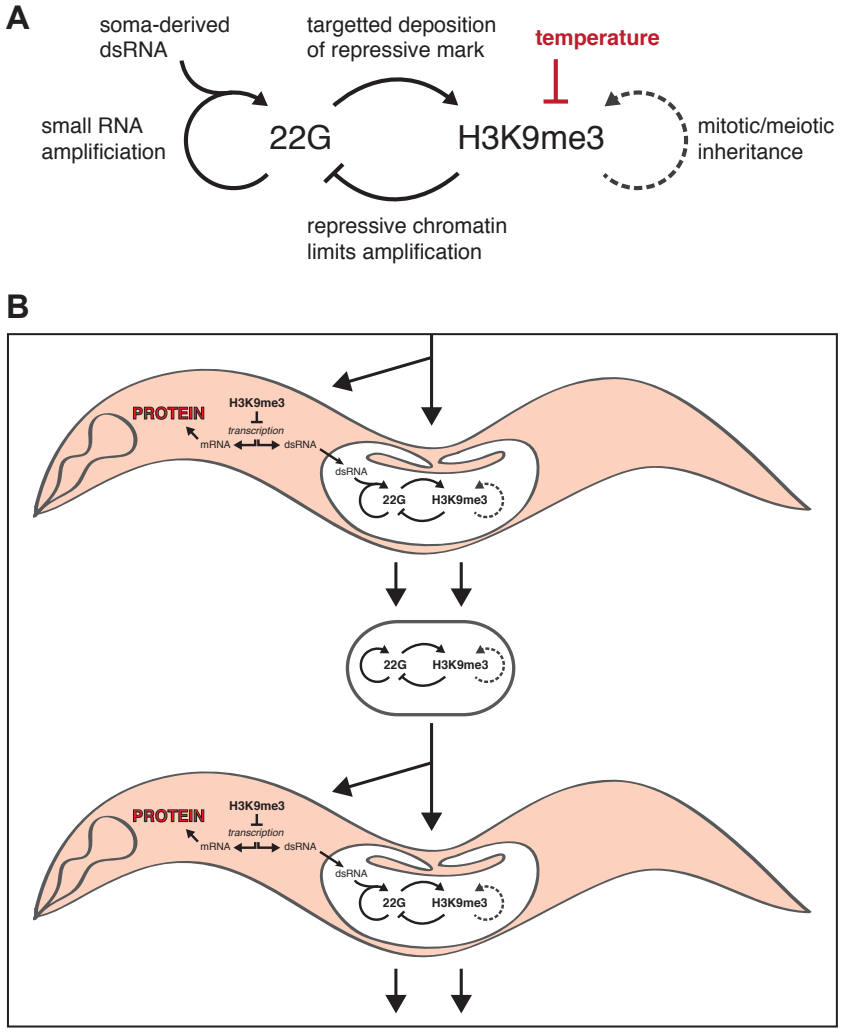

Figure 72. Proposed integrated model for transgenerational propagation of epigenetic state on a repetitive transgene array in *C. elegans*.

The type of interaction suggested here can be described as an incoherent feed forward loop (Mangan and Alon, 2003), where the same input (decreased H3K9me3 in the germline of P0) generates an activating signal that affects the output in a positive way (increased expression of transgene in subsequent generation due to decreased H3K9me3 inherited from parents) and an inhibitory signal that has a negative impact on the same output (increased soma-to-germline repression) (Figure 51). Examination of this motif reveals an inherent delay in transmission of the two signals. Loss of the H3K9me3 in the germline of P0 or in the early development of F1 will directly lead to increased somatic expression in F1 generation. This increased expression will then generate the silencing signal that communicates to the germline and reduces expression in the next generation. Therefore the somatic inhibitory arm of this feedforward motif will cause an observable effect on transgene expression only in F2 generation.

An alternative and not mutually exclusive explanation could be that H3K9me3 marks on the target locus that generate repressive heterochromatic environment limit production of the transcript that serves as a template for RNA-dependent RNA Polymerase that is required for 22G RNA amplification (Aoki et al., 2007; Sijen et al., 2007). In this case, loss of repressive H3K9me3 from the transgene due to temperature or replicative stress would result in increased transcriptional activity at the locus providing more templates for 22G siRNA production. This would create a negative feedback on the 22G RNAs pathway and could explain why the level of 22G RNAs is lower in *daf-21p::mCHERRY* 'low' animals (Figure 42). The intergenerational delay between the small RNAs and the deposition of H3K9me3 described in previous studies (Buckley et al., 2012; Gu et al., 2012) would provide the delay necessary to generate an incoherent feed forward motif that would exhibit the delayed recovery

response that we observed in our experiment (Figure 73). To test if such a negative feedback really exist, one could compare the content of 22G RNAs targeting the transgene in *set-25* and wild type worms. Loss of H3K9me3, which is observed in animals lacking SET-25 (Towbin et al., 2012), should result in increased amount of 22G RNAs. The two proposed incoherent feed-forward loops could act in parallel to mediate the transgenerational dynamics of the germline repression of repetitive elements like the transgene studied here.

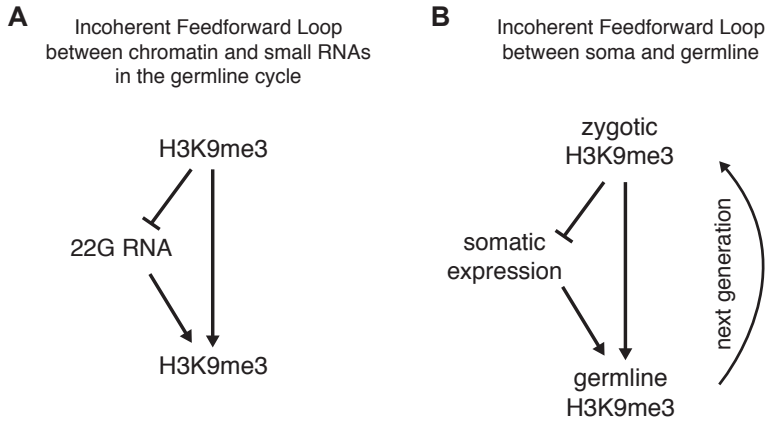

Figure 73. Two incoherent feedforward loops (IFFL) govern transgenerational dynamics of transgene expression. (A) We propose that the H3K9me3 state of the transgene is inherited throughout the germline cycle mitotically and meiotically. 22G RNAs guide deposition of H3K9me3 to the locus. Increased H3K9me3 reduces transcription at the locus depleting the template for 22G RNA amplification and therefore reducing their pool. Therefore loss of H3K9me3 leads to increase of 22G RNAs that restore the mark in the next generation (B) The amount of H3K9me3 on the transgene in the zygote is inherited through the germline to the next generation. High H3K9me3 decreases somatic expression of the transgene. Somatic expression stimulates the nuclear RNAi pathway in the germline through supply of mobile dsRNAs, which results in deposition of additional H3K9me3 in on the locus leading to dampened expression in the following generation.

5.3.5 Consequences of stochastic epi-mutations during embryonic development

Large changes in the epigenetic makeup of the genome occur in early embryonic development making it particularly sensitive to unwanted epigenetic errors (Heard and Martienssen, 2014). The rapid succession of coordinated cellular divisions leaves little time for correcting errors in propagation of epigenetic marks. Moreover, a heritable epigenetic alteration that happens in a single cell in one of the first cycles of divisions has the potential to affect multiple tissues of the developed organism. Stochastic errors in the propagation of heterochromatin state (epi-mutations) during embryonic development can have different consequence for the affected P0 generation and its F1 progeny depending on the identity of the affected cell. In *Gedankenexperiments* that use the transgenerational propagation of a repressed heterochromatic state in *C. elegans* as a model system we outline four possible scenarios for the phenotypic outcome of such stochastic 'epi-mutation' on the affected generation and its progeny (Figure 74). For example, perturbation of the repressed state in the one-cell zygote will be propagated throughout the whole lineage including the germline, which will lead to inheritance of the de-repressed state. Upregulation will be observed in the affected P0 generation as well as its progeny (Figure 74A). If an epi-mutation occurs in the germline lineage after separation from the soma it will have no effect on somatic expression in the P0 generation, but will manifest in the F1 progeny through increased somatic expression (Figure 74B). On the contrary a stochastic error in transmission of the repressed state occurring in the somatic lineage will lead to upregulation of the locus in affected P0 animal, but since the germline was not affected, such an event would not lead to increased expression in the F1 progeny (Figure 74D). In fact, negative feedback acting from soma to germline could result in increased

repression of the locus leading to abnormally low expression in the F1 progeny.

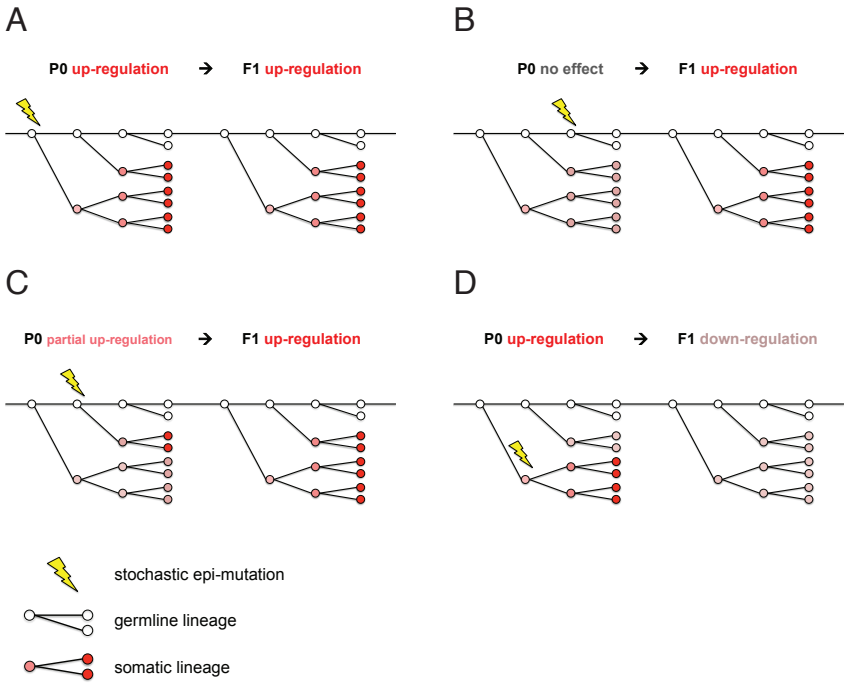

Figure 74. Consequences of stochastic errors (epi-mutations) during propagation of heterochromatin state depend on the identity of the affected cell. (A) Repressed state is perturbed in the zygote, therefore all cells of the organism are affected, including the germline that propagates the de-repressed state to the next generations and soma that exhibits increased expression from the locus (B) An epi-mutation that occurs only in the germ cells has no effect on somatic expression in the P0 generation, but will manifest in the F1 progeny through increased somatic expression. (C) An epi-mutation occurring in the second embryonic division of the germline lineage. Some somatic cells and all of the germline cells of P0 are affected leading to upregulation of the locus only in part of somatic tissues of P0 but in all somatic tissues of F1. (D) An epi-mutation occurring in somatic blastomere results in de-repression of the locus in P0 soma. Since P0 germline is not affected, the F1 generation does not exhibit upregulation. Due to the negative feedback from soma to germline in P0, the F1 generation experiences down-regulation of the locus.

5.3.6 Is there a functional role for transgenerational inheritance of chromatin state in response to temperature?

Given the remarkable multigenerational stability of the epigenetic change triggered by environment described in this thesis one cannot help but wonder about the possible functional role for such a mechanism and whether it could provide the animal with any selective advantage. A study based on a simulation demonstrated that in a situation when the environment that changes relatively slow compared to generation time and direct maternal and environmental cues are unreliable an incomplete resetting of epigenetic memory could have adaptive value for the organism (English et al., 2015). Since *C. elegans* is a terrestrial nematode it is acutely affected by fluctuations in the ambient temperature that include high frequency changes due to daytime cycle as well as slower fluctuations dictated by changing weather and seasons. Relying on ancestral epigenetic cues could therefore allow the developing worm to match its physiology to the average temperature it will experience in its life. The mechanism of transgenerational control of heterochromatin repression described here could therefore serve as a 'thermostat' that tunes the chromatin compaction across generations adjusting it to a gradually changing environment.

5. References

(1998). Genome sequence of the nematode C. elegans: a platform for investigating biology. Science *282*, 2012-2018.

Abe, M., Tsai, S.Y., Jin, S.G., Pfeifer, G.P., and Szabo, P.E. (2011). Sex-specific dynamics of global chromatin changes in fetal mouse germ cells. PloS one *6*, e23848.

Alcazar, R.M., Lin, R., and Fire, A.Z. (2008). Transmission dynamics of heritable silencing induced by double-stranded RNA in Caenorhabditis elegans. Genetics *180*, 1275-1288.

Allshire, R.C., and Ekwall, K. (2015). Epigenetic Regulation of Chromatin States in Schizosaccharomyces pombe. Cold Spring Harbor perspectives in biology *7*.

Alon, U. (2007). Network motifs: theory and experimental approaches. Nature reviews Genetics *8*, 450-461.

Altun, Z.F., Herndon, L.A., Crocker, C., Lints, R. and Hall, D.H. (2015). WormAtlas.

Andersen, E.C., and Horvitz, H.R. (2007). Two C. elegans histone methyltransferases repress lin-3 EGF transcription to inhibit vulval development. Development *134*, 2991-2999.

Anway, M.D., Cupp, A.S., Uzumcu, M., and Skinner, M.K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science *308*, 1466-1469.

Aoki, K., Moriguchi, H., Yoshioka, T., Okawa, K., and Tabara, H. (2007). In vitro analyses of the production and activity of secondary small interfering RNAs in C. elegans. The EMBO journal *26*, 5007-5019.

Aparicio, O.M., Billington, B.L., and Gottschling, D.E. (1991). Modifiers of position effect are shared between telomeric and silent mating-type loci in S. cerevisiae. Cell *66*, 1279-1287.

Aran, D., Toperoff, G., Rosenberg, M., and Hellman, A. (2011). Replication timing-related and gene body-specific methylation of active human genes. Human molecular genetics *20*, 670-680.

Arico, J.K., Katz, D.J., van der Vlag, J., and Kelly, W.G. (2011). Epigenetic patterns maintained in early Caenorhabditis elegans embryos can be established by gene activity in the parental germ cells. PLoS genetics *7*, e1001391.

Arteaga-Vazquez, M.A., and Chandler, V.L. (2010). Paramutation in maize: RNA mediated trans-generational gene silencing. Current opinion in genetics & development *20*, 156-163.

Ashe, A., Sapetschnig, A., Weick, E.-M., Mitchell, J., Bagijn, Marloes P., Cording, Amy C., Doebley, A.-L., Goldstein, Leonard D., Lehrbach, Nicolas J., Le Pen, J.*, et al.* (2012a). piRNAs Can Trigger a Multigenerational Epigenetic Memory in the Germline of C. elegans. Cell *150*, 88-99.

Ashe, A., Sapetschnig, A., Weick, E.M., Mitchell, J., Bagijn, M.P., Cording, A.C., Doebley, A.L., Goldstein, L.D., Lehrbach, N.J., Le Pen, J.*, et al.* (2012b). piRNAs can trigger a multigenerational epigenetic memory in the germline of C. elegans. Cell *150*, 88-99.

Asikainen, S., Heikkinen, L., Wong, G., and Storvik, M. (2008). Functional characterization of endogenous siRNA target genes in Caenorhabditis elegans. BMC genomics *9*, 270.

Audergon, P.N., Catania, S., Kagansky, A., Tong, P., Shukla, M., Pidoux, A.L., and Allshire, R.C. (2015). Epigenetics. Restricted epigenetic inheritance of H3K9 methylation. Science *348*, 132-135.

Avgousti, D.C., Palani, S., Sherman, Y., and Grishok, A. (2012). CSR-1 RNAi pathway positively regulates histone expression in C. elegans. The EMBO journal *31*, 3821-3832.

Bagijn, M.P., Goldstein, L.D., Sapetschnig, A., Weick, E.M., Bouasker, S., Lehrbach, N.J., Simard, M.J., and Miska, E.A. (2012). Function, targets, and evolution of Caenorhabditis elegans piRNAs. Science *337*, 574-578.

Barriere, A., and Felix, M.A. (2005). Natural variation and population genetics of Caenorhabditis elegans. WormBook : the online review of C elegans biology, 1-19.

Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Batista, P.J., Ruby, J.G., Claycomb, J.M., Chiang, R., Fahlgren, N., Kasschau, K.D., Chaves, D.A., Gu, W., Vasale, J.J., Duan, S.*, et al.* (2008). PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in C. elegans. Molecular cell *31*, 67-78.

Baulcombe, D.C., and Dean, C. (2014). Epigenetic regulation in plant responses to the environment. Cold Spring Harbor perspectives in biology *6*, a019471.

Bean, C.J., Schaner, C.E., and Kelly, W.G. (2004). Meiotic pairing and imprinted X chromatin assembly in Caenorhabditis elegans. Nature genetics *36*, 100-105.

Begasse, M.L., and Hyman, A.A. (2011). The first cell cycle of the Caenorhabditis elegans embryo: spatial and temporal control of an asymmetric cell division. Results and problems in cell differentiation *53*, 109-133.

Beisson, J., and Sonneborn, T.M. (1965). CYTOPLASMIC INHERITANCE OF THE ORGANIZATION OF THE CELL CORTEX IN PARAMECIUM AURELIA. Proceedings of the National Academy of Sciences of the United States of America *53*, 275-282.

Bender, L.B., Cao, R., Zhang, Y., and Strome, S. (2004). The MES-2/MES-3/MES-6 complex and regulation of histone H3 methylation in C. elegans. Current biology : CB *14*, 1639-1643.

Berger, S.L., Kouzarides, T., Shiekhattar, R., and Shilatifard, A. (2009). An operational definition of epigenetics. Genes & development *23*, 781-783.

Bessler, J.B., Andersen, E.C., and Villeneuve, A.M. (2010). Differential localization and independent acquisition of the H3K9me2 and H3K9me3 chromatin modifications in the Caenorhabditis elegans adult germ line. PLoS genetics *6*, e1000830.

Billi, A.C., Fischer, S.E., and Kim, J.K. (2014). Endogenous RNAi pathways in C. elegans. WormBook : the online review of C elegans biology, 1-49.

Blewitt, M., and Whitelaw, E. (2013). The use of mouse models to study epigenetics. Cold Spring Harbor perspectives in biology *5*, a017939.

Blewitt, M.E., Vickaryous, N.K., Paldi, A., Koseki, H., and Whitelaw, E. (2006). Dynamic reprogramming of DNA methylation at an epigenetically sensitive allele in mice. PLoS genetics *2*, e49.

Bonne-Andrea, C., Wong, M.L., and Alberts, B.M. (1990). In vitro replication through nucleosomes without histone displacement. Nature *343*, 719-726.

Boulin, T., Etchberger, J.F., and Hobert, O. (2006). Reporter gene fusions. WormBook : the online review of C elegans biology, 1-23.

Boyes, J., and Bird, A. (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. Cell *64*, 1123-1134.

Brand, A.H., Breeden, L., Abraham, J., Sternglanz, R., and Nasmyth, K. (1985). Characterization of a "silencer" in yeast: a DNA sequence with properties opposite to those of a transcriptional enhancer. Cell *41*, 41-48.

Brennecke, J., Aravin, A.A., Stark, A., Dus, M., Kellis, M., Sachidanandam, R., and Hannon, G.J. (2007). Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell *128*, 1089-1103.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Brink, R.A. (1956). A Genetic Change Associated with the R Locus in Maize Which Is Directed and Potentially Reversible. Genetics *41*, 872-889.

Brzeski, J., and Brzeska, K. (2011). The maze of paramutation: a rough guide to the puzzling epigenetics of paramutation. Wiley interdisciplinary reviews RNA *2*, 863-874.

Buckley, B.A., Burkhart, K.B., Gu, S.G., Spracklin, G., Kershner, A., Fritz, H., Kimble, J., Fire, A., and Kennedy, S. (2012). A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature *489*, 447-451.

Burga, A., Casanueva, M.O., and Lehner, B. (2011). Predicting mutation outcome from early stochastic variation in genetic interaction partners. Nature *480*, 250-253.

Burggren, W.W. (2015). Dynamics of epigenetic phenomena: intergenerational and intragenerational phenotype 'washout'. The Journal of experimental biology *218*, 80-87.

Burkhart, K.B., Guang, S., Buckley, B.A., Wong, L., Bochner, A.F., and Kennedy, S. (2011). A pre-mRNA-associating factor links endogenous siRNAs to chromatin regulation. PLoS genetics *7*, e1002249.

Burton, A., and Torres-Padilla, M.E. (2014). Chromatin dynamics in the regulation of cell fate allocation during early embryogenesis. Nature reviews Molecular cell biology *15*, 723-734.

Burton, N.O., Burkhart, K.B., and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America *108*, 19683-19688.

Calarco, J.P., Borges, F., Donoghue, M.T., Van Ex, F., Jullien, P.E., Lopes, T., Gardner, R., Berger, F., Feijo, J.A., Becker, J.D.*, et al.* (2012). Reprogramming of DNA methylation in pollen guides epigenetic inheritance via small RNA. Cell *151*, 194-205.

Cantone, I., and Fisher, A.G. (2013). Epigenetic programming and reprogramming during development. Nature structural & molecular biology *20*, 282-289.

Capowski, E.E., Martin, P., Garvin, C., and Strome, S. (1991). Identification of grandchildless loci whose products are required for normal germ-line development in the nematode Caenorhabditis elegans. Genetics *129*, 1061-1072.

Carone, B.R., Fauquier, L., Habib, N., Shea, J.M., Hart, C.E., Li, R., Bock, C., Li, C., Gu, H., Zamore, P.D.*, et al.* (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. Cell *143*, 1084-1096.

Carrozza, M.J., Li, B., Florens, L., Suganuma, T., Swanson, S.K., Lee, K.K., Shia, W.J., Anderson, S., Yates, J., Washburn, M.P.*, et al.* (2005). Histone H3 methylation by Set2 directs deacetylation of coding regions by Rpd3S to suppress spurious intragenic transcription. Cell *123*, 581-592.

Cavalli, G., and Paro, R. (1998). The Drosophila Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis. Cell *93*, 505-518.

Cecere, G., Hoersch, S., O'Keeffe, S., Sachidanandam, R., and Grishok, A. (2014). Global effects of the CSR-1 RNA interference pathway on the transcriptional landscape. Nature structural & molecular biology *21*, 358-365.

Champagne, F.A., and Curley, J.P. (2009). Epigenetic mechanisms mediating the long-term effects of maternal care on development. Neuroscience and biobehavioral reviews *33*, 593-600.

Chandler, V.L., and Walbot, V. (1986). DNA modification of a maize transposable element correlates with loss of activity. Proceedings of the National Academy of Sciences of the United States of America *83*, 1767-1771.

Checchi, P.M., and Engebrecht, J. (2011). Caenorhabditis elegans Histone Methyltransferase MET-2 Shields the Male X Chromosome from Checkpoint Machinery and Mediates Meiotic Sex Chromosome Inactivation. PLoS genetics *7*, e1002267.

Chedin, F. (2011). The DNMT3 family of mammalian de novo DNA methyltransferases. Progress in molecular biology and translational science *101*, 255-285.

Cheng, T.H., and Gartenberg, M.R. (2000). Yeast heterochromatin is a dynamic structure that requires silencers continuously. Genes & development *14*, 452-463.

Claycomb, J.M., Batista, P.J., Pang, K.M., Gu, W., Vasale, J.J., van Wolfswinkel, J.C., Chaves, D.A., Shirayama, M., Mitani, S., Ketting, R.F.*, et al.* (2009). The Argonaute CSR-1 and its 22G-RNA cofactors are required for holocentric chromosome segregation. Cell *139*, 123-134.

Coe, E.H. (1959). A REGULAR AND CONTINUING CONVERSION-TYPE PHENOMENON AT THE B LOCUS IN MAIZE. Proceedings of the National Academy of Sciences of the United States of America *45*, 828-832.

Comb, M., and Goodman, H.M. (1990). CpG methylation inhibits proenkephalin gene expression and binding of the transcription factor AP-2. Nucleic acids research *18*, 3975-3982.

Conine, C.C., Batista, P.J., Gu, W., Claycomb, J.M., Chaves, D.A., Shirayama, M., and Mello, C.C. (2010). Argonautes ALG-3 and ALG-4 are required for spermatogenesis-specific 26G-RNAs and thermotolerant sperm in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America *107*, 3588-3593.

Conine, C.C., Moresco, J.J., Gu, W., Shirayama, M., Conte, D., Jr., Yates, J.R., 3rd, and Mello, C.C. (2013). Argonautes promote male fertility and provide a paternal memory of germline gene expression in C. elegans. Cell *155*, 1532-1544.

Cooper, S., Dienstbier, M., Hassan, R., Schermelleh, L., Sharif, J., Blackledge, Neil P., De Marco, V., Elderkin, S., Koseki, H., Klose, R.*, et al.* (2014). Targeting Polycomb to Pericentric Heterochromatin in Embryonic Stem Cells Reveals a Role for H2AK119u1 in PRC2 Recruitment. Cell reports *7*, 1456-1470.

Correa, R.L., Steiner, F.A., Berezikov, E., and Ketting, R.F. (2010). MicroRNA-directed siRNA biogenesis in Caenorhabditis elegans. PLoS genetics *6*, e1000903.

Cortijo, S., Wardenaar, R., Colome-Tatche, M., Gilly, A., Etcheverry, M., Labadie, K., Caillieux, E., Hospital, F., Aury, J.M., Wincker, P.*, et al.* (2014). Mapping the epigenetic basis of complex traits. Science *343*, 1145-1148.

Couteau, F., Guerry, F., Muller, F., and Palladino, F. (2002). A heterochromatin protein 1 homologue in Caenorhabditis elegans acts in germline and vulval development. EMBO reports *3*, 235-241.

Craig, J.M. (2005). Heterochromatin--many flavours, common themes. BioEssays : news and reviews in molecular, cellular and developmental biology *27*, 17-28.

Cutter, A.R., and Hayes, J.J. (2015). A brief review of nucleosome structure. FEBS letters.

Darwin, C. (1868). The variation of animals and plants under domestication, 1st edn (London: John Murray).

Darwin, C. (1869). On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life., 6th edn (London: John Murray).

Darwin, E. (1794). Zoonomia; or the Laws of Organic Life.

Das, P.P., Bagijn, M.P., Goldstein, L.D., Woolford, J.R., Lehrbach, N.J., Sapetschnig, A., Buhecha, H.R., Gilchrist, M.J., Howe, K.L., Stark, R., *et al.* (2008). Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress Tc3 transposon mobility in the Caenorhabditis elegans germline. Molecular cell *31*, 79-90.

de Moor, C.H., Meijer, H., and Lissenden, S. (2005). Mechanisms of translational control by the 3' UTR in development and differentiation. Seminars in cell & developmental biology *16*, 49-58.

De Paoli, E., Dorantes-Acosta, A., Zhai, J., Accerbi, M., Jeong, D.H., Park, S., Meyers, B.C., Jorgensen, R.A., and Green, P.J. (2009). Distinct extremely abundant siRNAs associated with cosuppression in petunia. RNA *15*, 1965-1970.

De Rubertis, F., Kadosh, D., Henchoz, S., Pauli, D., Reuter, G., Struhl, K., and Spierer, P. (1996). The histone deacetylase RPD3 counteracts genomic silencing in Drosophila and yeast. Nature *384*, 589-591.

DeChiara, T.M., Robertson, E.J., and Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. Cell *64*, 849-859.

Del Rizzo, P.A., and Trievel, R.C. (2014). Molecular basis for substrate recognition by lysine methyltransferases and demethylases. Biochimica et biophysica acta *1839*, 1404-1415.

Demerec, M., and Slizynska, H. (1937). Mottled White 258-18 of Drosophila Melanogaster. Genetics *22*, 641-649.

Dennis, E.S., and Brettell, R.I. (1990). DNA methylation of maize transposable elements is correlated with activity. Philosophical transactions of the Royal Society of London Series B, Biological sciences *326*, 217-229.

Dernburg, A.F., Zalevsky, J., Colaiacovo, M.P., and Villeneuve, A.M. (2000). Transgene-mediated cosuppression in the C. elegans germ line. Genes & development *14*, 1578-1583.

Detlefsen, J.A. (1925). THE INHERITANCE OF ACQUIRED CHARACTERS, Vol 5.

Devanapally, S., Ravikumar, S., and Jose, A.M. (2015). Double-stranded RNA made in C. elegans neurons can enter the germline and cause transgenerational gene silencing. Proceedings of the National Academy of Sciences of the United States of America *112*, 2133-2138.

Dias, B.G., and Ressler, K.J. (2014). Parental olfactory experience influences behavior and neural structure in subsequent generations. Nature neuroscience *17*, 89-96.

Dillon, N., and Festenstein, R. (2002). Unravelling heterochromatin: competition between positive and negative factors regulates accessibility. Trends in genetics : TIG *18*, 252-258.

Doench, J.G., Petersen, C.P., and Sharp, P.A. (2003). siRNAs can function as miRNAs. Genes & development *17*, 438-442.

Edgar, L.G., Wolf, N., and Wood, W.B. (1994). Early transcription in Caenorhabditis elegans embryos. Development *120*, 443-451.

Eggleston, A.K., O'Neill, T.E., Bradbury, E.M., and Kowalczykowski, S.C. (1995). Unwinding of nucleosomal DNA by a DNA helicase. The Journal of biological chemistry *270*, 2024-2031.

Eissenberg, J.C., James, T.C., Foster-Hartnett, D.M., Hartnett, T., Ngan, V., and Elgin, S.C. (1990). Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. Proceedings of the National Academy of Sciences of the United States of America *87*, 9923-9927.

Encalada, S.E., Martin, P.R., Phillips, J.B., Lyczak, R., Hamill, D.R., Swan, K.A., and Bowerman, B. (2000). DNA replication defects delay cell division and disrupt cell polarity in early Caenorhabditis elegans embryos. Developmental biology *228*, 225-238.

English, S., Pen, I., Shea, N., and Uller, T. (2015). The information value of non-genetic inheritance in plants and animals. PloS one *10*, e0116996.

Eun, C., Lorkovic, Z.J., Sasaki, T., Naumann, U., Matzke, A.J., and Matzke, M. (2012). Use of forward genetic screens to identify genes required for RNA-directed DNA methylation in Arabidopsis thaliana. Cold Spring Harbor symposia on quantitative biology *77*, 195-204.

Ezenwa, V.O., Gerardo, N.M., Inouye, D.W., Medina, M., and Xavier, J.B. (2012). Microbiology. Animal behavior and the microbiome. Science *338*, 198-199.

Feinbaum, R., and Ambros, V. (1999). The timing of lin-4 RNA accumulation controls the timing of postembryonic developmental events in Caenorhabditis elegans. Developmental biology *210*, 87-95.

Feng, S., Jacobsen, S.E., and Reik, W. (2010). Epigenetic reprogramming in plant and animal development. Science *330*, 622-627.

Filion, G.J., van Bemmel, J.G., Braunschweig, U., Talhout, W., Kind, J., Ward, L.D., Brugman, W., de Castro, I.J., Kerkhoven, R.M., Bussemaker, H.J., *et al.* (2010). Systematic protein location mapping reveals five principal chromatin types in Drosophila cells. Cell *143*, 212-224.

Filippakopoulos, P., and Knapp, S. (2014). Targeting bromodomains: epigenetic readers of lysine acetylation. Nature reviews Drug discovery *13*, 337-356.

Finch, J.T., Lutter, L.C., Rhodes, D., Brown, R.S., Rushton, B., Levitt, M., and Klug, A. (1977). Structure of nucleosome core particles of chromatin. Nature *269*, 29-36.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Fischer, R.L. (1918). The Correlation between Relatives on the Supposition of Mendelian Inheritance. Philosophical Transactions of the Royal Society of Edinburgh *52*, 399-433.

Fischer, S.E., Butler, M.D., Pan, Q., and Ruvkun, G. (2008). Trans-splicing in C. elegans generates the negative RNAi regulator ERI-6/7. Nature *455*, 491-496.

Fischer, S.E., Montgomery, T.A., Zhang, C., Fahlgren, N., Breen, P.C., Hwang, A., Sullivan, C.M., Carrington, J.C., and Ruvkun, G. (2011). The ERI-6/7 helicase acts at the first stage of an siRNA amplification pathway that targets recent gene duplications. PLoS genetics *7*, e1002369.

Fischer, S.E., Pan, Q., Breen, P.C., Qi, Y., Shi, Z., Zhang, C., and Ruvkun, G. (2013). Multiple small RNA pathways regulate the silencing of repeated and foreign genes in C. elegans. Genes & development *27*, 2678-2695.

Fischer, T., Cui, B., Dhakshnamoorthy, J., Zhou, M., Rubin, C., Zofall, M., Veenstra, T.D., and Grewal, S.I. (2009). Diverse roles of HP1 proteins in heterochromatin assembly and functions in fission yeast. Proceedings of the National Academy of Sciences of the United States of America *106*, 8998-9003.

Fisher, R.A., and Bennett, J.H. (1930). The Genetical Theory of Natural Selection: A Complete Variorum Edition (OUP Oxford).

Flicek, P., Amode, M.R., Barrell, D., Beal, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fairley, S., Fitzgerald, S., *et al.* (2012). Ensembl 2012. Nucleic acids research *40*, D84-90.

Francis, N.J., Follmer, N.E., Simon, M.D., Aghia, G., and Butler, J.D. (2009). Polycomb proteins remain bound to chromatin and DNA during DNA replication in vitro. Cell *137*, 110-122.

Frey, A., Listovsky, T., Guilbaud, G., Sarkies, P., and Sale, J.E. (2014). Histone H3.3 is required to maintain replication fork progression after UV damage. Current biology : CB *24*, 2195-2201.

Fridmann-Sirkis, Y., Stern, S., Elgart, M., Galili, M., Zeisel, A., Shental, N., and Soen, Y. (2014). Delayed development induced by toxicity to the host can be inherited by a bacterial-dependent, transgenerational effect. Frontiers in genetics *5*, 27.

Frøkjær-Jensen, C., Davis, M.W., Hopkins, C.E., Newman, B., Thummel, J.M., Olesen, S.-P., Grunnet, M., and Jorgensen, E.M. (2008). Single copy insertion of transgenes in C. elegans. Nature genetics *40*, 1375-1383.

Fu, Y., Luo, G.Z., Chen, K., Deng, X., Yu, M., Han, D., Hao, Z., Liu, J., Lu, X., Dore, L.C.*, et al.* (2015). N(6)-methyldeoxyadenosine marks active transcription start sites in chlamydomonas. Cell *161*, 879-892.

Furuhashi, H., and Kelly, W.G. (2010). The epigenetics of germ-line immortality: lessons from an elegant model system. Development, growth & differentiation *52*, 527-532.

Furuhashi, H., Takasaki, T., Rechtsteiner, A., Li, T., Kimura, H., Checchi, P.M., Strome, S., and Kelly, W.G. (2010). Trans-generational epigenetic regulation of C. elegans primordial germ cells. Epigenetics & chromatin *3*, 15.

Gao, M., and Arkov, A.L. (2013). Next generation organelles: structure and role of germ granules in the germline. Molecular reproduction and development *80*, 610-623.

Garrigues, J.M., Sidoli, S., Garcia, B.A., and Strome, S. (2015). Defining heterochromatin in C. elegans through genome-wide analysis of the heterochromatin protein 1 homolog HPL-2. Genome research *25*, 76-88.

Gassmann, R., Rechtsteiner, A., Yuen, K.W., Muroyama, A., Egelhofer, T., Gaydos, L., Barron, F., Maddox, P., Essex, A., Monen, J.*, et al.* (2012). An inverse relationship to germline transcription defines centromeric chromatin in C. elegans. Nature *484*, 534-537.

Gavazzo, P., Vergani, L., Mascetti, G.C., and Nicolini, C. (1997). Effects of histone acetylation on chromatin structure. Journal of cellular biochemistry *64*, 466-475.

Gayatri, S., and Bedford, M.T. (2014). Readers of histone methylarginine marks. Biochimica et biophysica acta *1839*, 702-710.

Gaydos, L.J., Rechtsteiner, A., Egelhofer, T.A., Carroll, C.R., and Strome, S. (2012). Antagonism between MES-4 and Polycomb repressive complex 2

promotes appropriate gene expression in C. elegans germ cells. Cell reports *2*, 1169-1177.

Gaydos, L.J., Wang, W., and Strome, S. (2014). Gene repression. H3K27me and PRC2 transmit a memory of repression across generations and during development. Science *345*, 1515-1518.

Gent, J.I., Lamm, A.T., Pavelec, D.M., Maniar, J.M., Parameswaran, P., Tao, L., Kennedy, S., and Fire, A.Z. (2010). Distinct phases of siRNA synthesis in an endogenous RNAi pathway in C. elegans soma. Molecular cell *37*, 679-689.

Gerstein, M.B., Lu, Z.J., Van Nostrand, E.L., Cheng, C., Arshinoff, B.I., Liu, T., Yip, K.Y., Robilotto, R., Rechtsteiner, A., Ikegami, K.*, et al.* (2010). Integrative analysis of the Caenorhabditis elegans genome by the modENCODE project. Science *330*, 1775-1787.

Girton, J.R., and Johansen, K.M. (2008). Chromatin structure and the regulation of gene expression: the lessons of PEV in Drosophila. Advances in genetics *61*, 1-43.

Gold, M., Hurwitz, J., and Anders, M. (1963). The enzymatic methylation of RNA and DNA. Biochemical and biophysical research communications *11*, 107-114.

Goodman, M.F., and Woodgate, R. (2013). Translesion DNA polymerases. Cold Spring Harbor perspectives in biology *5*, a010363.

Gowen, J.W., and Gay, E.H. (1934). Chromosome Constitution and Behavior in Eversporting and Mottling in Drosophila Melanogaster. Genetics *19*, 189-208.

Greenstein, D. (2005). Control of oocyte meiotic maturation and fertilization. WormBook : the online review of C elegans biology, 1-12.

Greer, E.L., Beese-Sims, S.E., Brookes, E., Spadafora, R., Zhu, Y., Rothbart, S.B., Aristizabal-Corrales, D., Chen, S., Badeaux, A.I., Jin, Q.*, et al.* (2014). A histone methylation network regulates transgenerational epigenetic memory in C. elegans. Cell reports *7*, 113-126.

Greer, E.L., Blanco, M.A., Gu, L., Sendinc, E., Liu, J., Aristizabal-Corrales, D., Hsu, C.H., Aravind, L., He, C., and Shi, Y. (2015). DNA Methylation on N(6)-Adenine in C. elegans. Cell *161*, 868-878.

Greer, E.L., Maures, T.J., Ucar, D., Hauswirth, A.G., Mancini, E., Lim, J.P., Benayoun, B.A., Shi, Y., and Brunet, A. (2011). Transgenerational epigenetic inheritance of longevity in Caenorhabditis elegans. Nature *479*, 365-371.

Greer, E.L., and Shi, Y. (2012). Histone methylation: a dynamic mark in health, disease and inheritance. Nature reviews Genetics *13*, 343-357.

Grewal, S.I., and Elgin, S.C. (2002). Heterochromatin: new possibilities for the inheritance of structure. Current opinion in genetics & development *12*, 178-187.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., and Enright, A.J. (2008). miRBase: tools for microRNA genomics. Nucleic acids research *36*, D154-158.

Grishok, A., Sinskey, J.L., and Sharp, P.A. (2005). Transcriptional silencing of a transgene by RNAi in the soma of C. elegans. Genes & development *19*, 683-696.

Grishok, A., Tabara, H., and Mello, C.C. (2000). Genetic requirements for inheritance of RNAi in C. elegans. Science *287*, 2494-2497.

Grossniklaus, U., Kelly, W.G., Ferguson-Smith, A.C., Pembrey, M., and Lindquist, S. (2013). Transgenerational epigenetic inheritance: how important is it? Nature reviews Genetics *14*, 228-235.

Gruenbaum, Y., Stein, R., Cedar, H., and Razin, A. (1981). Methylation of CpG sequences in eukaryotic DNA. FEBS letters *124*, 67-71.

Gruneberg, H. (1936). GREY-LETHAL, A NEW MUTATION IN THE HOUSE MOUSE. Journal of Heredity *27*, 105-109.

Gu, S.G., Pak, J., Guang, S., Maniar, J.M., Kennedy, S., and Fire, A. (2012). Amplification of siRNA in Caenorhabditis elegans generates a transgenerational sequence-targeted histone H3 lysine 9 methylation footprint. Nature genetics *44*, 157-164.

Gu, W., Shirayama, M., Conte, D., Jr., Vasale, J., Batista, P.J., Claycomb, J.M., Moresco, J.J., Youngman, E.M., Keys, J., Stoltz, M.J., *et al.* (2009). Distinct argonaute-mediated 22G-RNA pathways direct genome surveillance in the C. elegans germline. Molecular cell *36*, 231-244.

Guang, S., Bochner, A.F., Burkhart, K.B., Burton, N., Pavelec, D.M., and Kennedy, S. (2010). Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. Nature *465*, 1097-1101.

Guest, W.C., Plotkin, S.S., and Cashman, N.R. (2011). Toward a mechanism of prion misfolding and structural models of PrP(Sc): current knowledge and future directions. Journal of toxicology and environmental health Part A *74*, 154-160.

Guibert, S., Forne, T., and Weber, M. (2012). Global profiling of DNA methylation erasure in mouse primordial germ cells. Genome research *22*, 633-641.

Guo, H., Zhu, P., Yan, L., Li, R., Hu, B., Lian, Y., Yan, J., Ren, X., Lin, S., Li, J., *et al.* (2014). The DNA methylation landscape of human early embryos. Nature *511*, 606-610.

Guo, X., Zhang, R., Wang, J., and Lu, R. (2013). Antiviral RNA silencing initiated in the absence of RDE-4, a double-stranded RNA binding protein, in Caenorhabditis elegans. Journal of virology *87*, 10721-10729.

Guz, J., Foksinski, M., and Olinski, R. (2010). [Mechanism of DNA methylation and demethylation--its role in control of genes expression]. Postepy biochemii *56*, 7-15.

Ha, M., and Kim, V.N. (2014). Regulation of microRNA biogenesis. Nature reviews Molecular cell biology *15*, 509-524.

Hackett, J.A., Sengupta, R., Zylicz, J.J., Murakami, K., Lee, C., Down, T.A., and Surani, M.A. (2013). Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. Science *339*, 448-452.

Haldane, J.B.S. (1934). A Mathematical Theory of Natural and Artificial Selection Part X. Some Theorems on Artificial Selection. Genetics *19*, 412-429.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Hammoud, S.S., Nix, D.A., Zhang, H., Purwar, J., Carrell, D.T., and Cairns, B.R. (2009). Distinctive chromatin in human sperm packages genes for embryo development. Nature *460*, 473-478.

Han, T., Manoharan, A.P., Harkins, T.T., Bouffard, P., Fitzpatrick, C., Chu, D.S., Thierry-Mieg, D., Thierry-Mieg, J., and Kim, J.K. (2009). 26G endo-siRNAs regulate spermatogenic and zygotic gene expression in Caenorhabditis elegans. Proceedings of the National Academy of Sciences of the United States of America *106*, 18674-18679.

Hansen, J.C. (2006). Linking genome structure and function through specific histone acetylation. ACS chemical biology *1*, 69-72.

Hashimoto, H., Liu, Y., Upadhyay, A.K., Chang, Y., Howerton, S.B., Vertino, P.M., Zhang, X., and Cheng, X. (2012). Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. Nucleic acids research *40*, 4841-4849.

Hayes, J.J., and Lee, K.M. (1997). In vitro reconstitution and analysis of mononucleosomes containing defined DNAs and proteins. Methods *12*, 2-9.

Heard, E., and Martienssen, R.A. (2014). Transgenerational epigenetic inheritance: myths and mechanisms. Cell *157*, 95-109.

Heitz, E. (1928). Das heterochromatin der moose. I Jahrb Wiss Botanik, 762–818

Hellman, A., and Chess, A. (2007). Gene body-specific methylation on the active X chromosome. Science *315*, 1141-1143.

Hiratani, I., Ryba, T., Itoh, M., Yokochi, T., Schwaiger, M., Chang, C.W., Lyou, Y., Townes, T.M., Schubeler, D., and Gilbert, D.M. (2008). Global reorganization of replication domains during embryonic stem cell differentiation. PLoS biology *6*, e245.

Ho, J.W., Jung, Y.L., Liu, T., Alver, B.H., Lee, S., Ikegami, K., Sohn, K.A., Minoda, A., Tolstorukov, M.Y., Appert, A.*, et al.* (2014). Comparative analysis of metazoan chromatin organization. Nature *512*, 449-452.

Holdeman, R., Nehrt, S., and Strome, S. (1998). MES-2, a maternal protein essential for viability of the germline in Caenorhabditis elegans, is homologous to a Drosophila Polycomb group protein. Development *125*, 2457-2467.

Holliday, R. (1987). The inheritance of epigenetic defects. Science *238*, 163-170.

Holoch, D., and Moazed, D. (2015). RNA-mediated epigenetic regulation of gene expression. Nature reviews Genetics *16*, 71-84.

Hong, E.J., Villen, J., Gerace, E.L., Gygi, S.P., and Moazed, D. (2005). A cullin E3 ubiquitin ligase complex associates with Rik1 and the Clr4 histone H3-K9 methyltransferase and is required for RNAi-mediated heterochromatin formation. RNA biology *2*, 106-111.

Hu, C.W., Chen, J.L., Hsu, Y.W., Yen, C.C., and Chao, M.R. (2015). Trace analysis of methylated and hydroxymethylated cytosines in DNA by isotope-dilution LC-MS/MS: first evidence of DNA methylation in Caenorhabditis elegans. The Biochemical journal *465*, 39-47.

Hubbard, E.J., and Greenstein, D. (2005). Introduction to the germ line. WormBook : the online review of C elegans biology, 1-4.

Hubbard, E.J., Korta, D.Z., and Dalfo, D. (2013). Physiological control of germline development. Advances in experimental medicine and biology *757*, 101-131.

Hublitz, P., Albert, M., and Peters, A.H. (2009). Mechanisms of transcriptional repression by histone lysine methylation. The International journal of developmental biology *53*, 335-354.

Huxley, J. (1942). Evolution: The Modern Synthesis (John Wiley & Sons).

Ibarra, C.A., Feng, X., Schoft, V.K., Hsieh, T.F., Uzawa, R., Rodrigues, J.A., Zemach, A., Chumak, N., Machlicova, A., Nishimura, T.*, et al.* (2012). Active DNA demethylation in plant companion cells reinforces transposon methylation in gametes. Science *337*, 1360-1364.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature *403*, 795-800.

Ingouff, M., Hamamura, Y., Gourgues, M., Higashiyama, T., and Berger, F. (2007). Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Current biology : CB *17*, 1032-1037.

Iqbal, K., Tran, D.A., Li, A.X., Warden, C., Bai, A.Y., Singh, P., Wu, X., Pfeifer, G.P., and Szabo, P.E. (2015). Deleterious effects of endocrine disruptors are corrected in the mammalian germline by epigenome reprogramming. Genome biology *16*, 59.

Ishimi, Y., Ohba, Y., Yasuda, H., and Yamada, M. (1981). The interaction of H1 histone with nucleosome core. Journal of biochemistry *89*, 1881-1888.

Ito, H., Gaubert, H., Bucher, E., Mirouze, M., Vaillant, I., and Paszkowski, J. (2011). An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress. Nature *472*, 115-119.

Ito, S., D'Alessio, A.C., Taranova, O.V., Hong, K., Sowers, L.C., and Zhang, Y. (2010). Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature *466*, 1129-1133.

Jackson, R.J., and Standart, N. (2007). How do microRNAs regulate gene expression? Science's STKE : signal transduction knowledge environment *2007*, re1.

James, T.C., and Elgin, S.C. (1986). Identification of a nonhistone chromosomal protein associated with heterochromatin in Drosophila melanogaster and its gene. Molecular and cellular biology *6*, 3862-3872.

Jarosz, D.F., Lancaster, A.K., Brown, J.C., and Lindquist, S. (2014). An evolutionarily conserved prion-like element converts wild fungi from metabolic specialists to generalists. Cell *158*, 1072-1082.

Jedrusik, M.A., and Schulze, E. (2001). A single histone H1 isoform (H1.1) is essential for chromatin silencing and germline development in Caenorhabditis elegans. Development *128*, 1069-1080.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

Johannes, F., Porcher, E., Teixeira, F.K., Saliba-Colombani, V., Simon, M., Agier, N., Bulski, A., Albuisson, J., Heredia, F., Audigier, P.*, et al.* (2009). Assessing the impact of transgenerational epigenetic variation on complex traits. PLoS genetics *5*, e1000530.

Johnson, C.L., and Spence, A.M. (2011). Epigenetic licensing of germline gene expression by maternal RNA in C. elegans. Science *333*, 1311-1314.

Johnson, L.M., Fisher-Adams, G., and Grunstein, M. (1992). Identification of a non-basic domain in the histone H4 N-terminus required for repression of the yeast silent mating loci. The EMBO journal *11*, 2201-2209.

Jose, A.M., Smith, J.J., and Hunter, C.P. (2009). Export of RNA silencing from C. elegans tissues does not require the RNA channel SID-1. Proceedings of the National Academy of Sciences of the United States of America *106*, 2283-2288.

Joshi, P.M., Riddle, M.R., Djabrayan, N.J., and Rothman, J.H. (2010). Caenorhabditis elegans as a model for stem cell biology. Developmental dynamics : an official publication of the American Association of Anatomists *239*, 1539-1554.

Juang, B.T., Gu, C., Starnes, L., Palladino, F., Goga, A., Kennedy, S., and L'Etoile, N.D. (2013). Endogenous nuclear RNAi mediates behavioral adaptation to odor. Cell *154*, 1010-1022.

Kanoh, J., Sadaie, M., Urano, T., and Ishikawa, F. (2005). Telomere binding protein Taz1 establishes Swi6 heterochromatin independently of RNAi at telomeres. Current biology : CB *15*, 1808-1819.

Kashtan, N., Itzkovitz, S., Milo, R., and Alon, U. (2004). Topological generalizations of network motifs. Physical review E, Statistical, nonlinear, and soft matter physics *70*, 031909.

Katan-Khaykovich, Y., and Struhl, K. (2011). Splitting of H3-H4 tetramers at transcriptionally active genes undergoing dynamic histone exchange. Proceedings of the National Academy of Sciences of the United States of America *108*, 1296-1301.

Kato, H., Goto, D.B., Martienssen, R.A., Urano, T., Furukawa, K., and Murakami, Y. (2005). RNA polymerase II is required for RNAi-dependent heterochromatin assembly. Science *309*, 467-469.

Katz, D.J., Edwards, T.M., Reinke, V., and Kelly, W.G. (2009). A C. elegans LSD1 demethylase contributes to germline immortality by reprogramming epigenetic memory. Cell *137*, 308-320.

Kelly, W.G. (2014). Transgenerational epigenetics in the germline cycle of Caenorhabditis elegans. Epigenetics & chromatin *7*, 6.

Kelly, W.G., and Fire, A. (1998). Chromatin silencing and the maintenance of a functional germline in Caenorhabditis elegans. Development *125*, 2451-2456.

Kelly, W.G., Schaner, C.E., Dernburg, A.F., Lee, M.H., Kim, S.K., Villeneuve, A.M., and Reinke, V. (2002). X-chromosome silencing in the germline of C. elegans. Development *129*, 479-492.

Kelly, W.G., Xu, S., Montgomery, M.K., and Fire, A. (1997). Distinct requirements for somatic and germline expression of a generally expressed Caernorhabditis elegans gene. Genetics *146*, 227-238.

Kennedy, S., Wang, D., and Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. Nature *427*, 645-649.

Kent, W.J., Zweig, A.S., Barber, G., Hinrichs, A.S., and Karolchik, D. (2010). BigWig and BigBed: enabling browsing of large distributed datasets. Bioinformatics *26*, 2204-2207.

Keogh, M.C., Kurdistani, S.K., Morris, S.A., Ahn, S.H., Podolny, V., Collins, S.R., Schuldiner, M., Chin, K., Punna, T., Thompson, N.J.*, et al.* (2005). Cotranscriptional set2 methylation of histone H3 lysine 36 recruits a repressive Rpd3 complex. Cell *123*, 593-605.

Kerr, S.C., Ruppersburg, C.C., Francis, J.W., and Katz, D.J. (2014). SPR-5 and MET-2 function cooperatively to reestablish an epigenetic ground state during passage through the germ line. Proceedings of the National Academy of Sciences of the United States of America *111*, 9509-9514.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G., and Plasterk, R.H. (1999). Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell *99*, 133-141.

Ketting, R.F., and Plasterk, R.H. (2000). A genetic link between co-suppression and RNA interference in C. elegans. Nature *404*, 296-298.

Kiani, J., Grandjean, V., Liebers, R., Tuorto, F., Ghanbarian, H., Lyko, F., Cuzin, F., and Rassoulzadegan, M. (2013). RNA-mediated epigenetic heredity requires the cytosine methyltransferase Dnmt2. PLoS genetics *9*, e1003498.

Kim, D., Kwon, Y.K., and Cho, K.H. (2008). The biphasic behavior of incoherent feed-forward loops in biomolecular regulatory networks. BioEssays : news and reviews in molecular, cellular and developmental biology *30*, 1204-1211.

Kim, H.S., Choi, E.S., Shin, J.A., Jang, Y.K., and Park, S.D. (2004). Regulation of Swi6/HP1-dependent heterochromatin assembly by cooperation of components of the mitogen-activated protein kinase pathway and a histone deacetylase Clr6. The Journal of biological chemistry *279*, 42850-42859.

Kim, S., Spike, C., and Greenstein, D. (2013). Control of oocyte growth and meiotic maturation in Caenorhabditis elegans. Advances in experimental medicine and biology *757*, 277-320.

Kloc, A., Zaratiegui, M., Nora, E., and Martienssen, R. (2008). RNA interference guides histone modification during the S phase of chromosomal replication. Current biology : CB *18*, 490-495.

Kouzarides, T. (1999). Histone acetylases and deacetylases in cell proliferation. Current opinion in genetics & development *9*, 40-48.

Koziol, M.J., and Rinn, J.L. (2010). RNA traffic control of chromatin complexes. Current opinion in genetics & development *20*, 142-148.

Krauss, V., and Reuter, G. (2011). DNA methylation in Drosophila--a critical evaluation. Progress in molecular biology and translational science *101*, 177-191.

Kueng, S., Oppikofer, M., and Gasser, S.M. (2013). SIR proteins and the assembly of silent chromatin in budding yeast. Annual review of genetics *47*, 275-306.

Lamarck, J. (1809). Philosophie zoologique, ou exposition des considérations relatives à l'histoire naturelle des animaux.

Lande-Diner, L., Zhang, J., and Cedar, H. (2009). Shifts in replication timing actively affect histone acetylation during nucleosome reassembly. Molecular cell *34*, 767-774.

Lane, N., Dean, W., Erhardt, S., Hajkova, P., Surani, A., Walter, J., and Reik, W. (2003). Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis (New York, NY : 2000) *35*, 88-93.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S.L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology *10*, R25.

Lapierre, L.R., and Hansen, M. (2012). Lessons from C. elegans: signaling pathways for longevity. Trends in endocrinology and metabolism: TEM *23*, 637-644.

Lee, H.C., Gu, W., Shirayama, M., Youngman, E., Conte, D., Jr., and Mello, C.C. (2012a). C. elegans piRNAs mediate the genome-wide surveillance of germline transcripts. Cell *150*, 78-87.

Lee, I., Hendrix, A., Kim, J., Yoshimoto, J., and You, Y.J. (2012b). Metabolic rate regulates L1 longevity in C. elegans. PloS one *7*, e44720.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. The EMBO journal *23*, 4051-4060.

Lehner, B., Tischler, J., and Fraser, A.G. (2006). RNAi screens in Caenorhabditis elegans in a 96-well liquid format and their application to the systematic identification of genetic interactions. Nature protocols *1*, 1617-1620.

Lewis, E.B. (1950). The phenomenon of position effect. Advances in genetics *3*, 73-115.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. Bioinformatics *25*, 2078-2079.

Li, T., and Kelly, W.G. (2011). A role for Set1/MLL-related components in epigenetic regulation of the Caenorhabditis elegans germ line. PLoS genetics *7*, e1001349.

Li, X., Ito, M., Zhou, F., Youngson, N., Zuo, X., Leder, P., and Ferguson-Smith, A.C. (2008). A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. Developmental cell *15*, 547-557.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics *30*, 923-930.

Liou, G.G., Tanny, J.C., Kruger, R.G., Walz, T., and Moazed, D. (2005). Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. Cell *121*, 515-527.

Lisch, D. (2012). Regulation of transposable elements in maize. Current opinion in plant biology *15*, 511-516.

Lister, R., Mukamel, E.A., Nery, J.R., Urich, M., Puddifoot, C.A., Johnson, N.D., Lucero, J., Huang, Y., Dwork, A.J., Schultz, M.D*., et al.* (2013). Global epigenomic reconfiguration during mammalian brain development. Science *341*, 1237905.

Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M*., et al.* (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature *462*, 315-322.

Liu, T., Rechtsteiner, A., Egelhofer, T.A., Vielle, A., Latorre, I., Cheung, M.-S., Ercan, S., Ikegami, K., Jensen, M., Kolasinska-Zwierz, P*., et al.* (2011). Broad chromosomal domains of histone modification patterns in C. elegans. Genome research *21*, 227-236.

Liu, W.H., Roemer, S.C., Port, A.M., and Churchill, M.E. (2012). CAF-1-induced oligomerization of histones H3/H4 and mutually exclusive interactions with Asf1 guide H3/H4 transitions among histone chaperones and DNA. Nucleic acids research *40*, 11229-11239.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome biology *15*, 550.

Lu, P.Y., Xie, F., and Woodle, M.C. (2005). In vivo application of RNA interference: from functional genomics to therapeutics. Advances in genetics *54*, 117-142.

Lu, X., Wontakal, S.N., Emelyanov, A.V., Morcillo, P., Konev, A.Y., Fyodorov, D.V., and Skoultchi, A.I. (2009). Linker histone H1 is essential for Drosophila development, the establishment of pericentric heterochromatin, and a normal polytene chromosome structure. Genes & development *23*, 452-465.

Lumey, L.H., Stein, A.D., and Susser, E. (2011). Prenatal famine and adult health. Annual review of public health *32*, 237-262.

Lussana, F., Painter, R.C., Ocke, M.C., Buller, H.R., Bossuyt, P.M., and Roseboom, T.J. (2008). Prenatal exposure to the Dutch famine is associated with a preference for fatty foods and a more atherogenic lipid profile. The American journal of clinical nutrition *88*, 1648-1652.

Luteijn, M.J., van Bergeijk, P., Kaaij, L.J., Almeida, M.V., Roovers, E.F., Berezikov, E., and Ketting, R.F. (2012). Extremely stable Piwi-induced gene silencing in Caenorhabditis elegans. The EMBO journal *31*, 3422-3430.

MacDonald, V.E., and Howe, L.J. (2009). Histone acetylation: where to go and how to get there. Epigenetics : official journal of the DNA Methylation Society *4*, 139-143.

Mangan, S., and Alon, U. (2003). Structure and function of the feed-forward loop network motif. Proceedings of the National Academy of Sciences of the United States of America *100*, 11980-11985.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. 2011 *17*.

Masatsugu, T., and Yamamoto, K. (2009). Multiple lysine methylation of PCAF by Set9 methyltransferase. Biochemical and biophysical research communications *381*, 22-26.

Masumoto, H., Nakato, R., Kanemaki, M., Shirahige, K., and Hachinohe, M. (2011). The inheritance of histone modifications depends upon the location in the chromosome in Saccharomyces cerevisiae. PloS one *6*, e28980.

Mattick, J.S. (2003). Challenging the dogma: the hidden layer of non-protein-coding RNAs in complex organisms. BioEssays : news and reviews in molecular, cellular and developmental biology *25*, 930-939.

McClintock, B. (1961). Some Parallels Between Gene Control Systems in Maize and in Bacteria. The American naturalist *95*, 265-277.

McEwan, D.L., Weisman, A.S., and Hunter, C.P. (2012). Uptake of extracellular double-stranded RNA by SID-2. Molecular cell *47*, 746-754.

Meehan, R., Lewis, J., Cross, S., Nan, X., Jeppesen, P., and Bird, A. (1992). Transcriptional repression by methylation of CpG. Journal of cell science Supplement *16*, 9-14.

Meister, P., Schott, S., Bedet, C., Xiao, Y., Rohner, S., Bodennec, S., Hudry, B., Molin, L., Solari, F., Gasser, S.M.*, et al.* (2011). Caenorhabditis elegans Heterochromatin protein 1 (HPL-2) links developmental plasticity, longevity and lipid metabolism. Genome biology *12*, R123.

Mendenhall, A.R., Tedesco, P.M., Taylor, L.D., Lowe, A., Cypser, J.R., and Johnson, T.E. (2012). Expression of a single-copy hsp-16.2 reporter predicts life span. The journals of gerontology Series A, Biological sciences and medical sciences *67*, 726-733.

Mette, M.F., Aufsatz, W., van der Winden, J., Matzke, M.A., and Matzke, A.J. (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. The EMBO journal *19*, 5194-5201.

Mirkin, E.V., and Mirkin, S.M. (2007). Replication Fork Stalling at Natural Impediments. Microbiology and Molecular Biology Reviews : MMBR *71*, 13-35.

Mirouze, M., Lieberman-Lazarovich, M., Aversano, R., Bucher, E., Nicolet, J., Reinders, J., and Paszkowski, J. (2012). Loss of DNA methylation affects the recombination landscape in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America *109*, 5880-5885.

Miyoshi, K., Tsukumo, H., Nagami, T., Siomi, H., and Siomi, M.C. (2005). Slicer function of Drosophila Argonautes and its involvement in RISC formation. Genes & development *19*, 2837-2848.

Moazed, D. (2001). Enzymatic activities of Sir2 and chromatin silencing. Current opinion in cell biology *13*, 232-238.

Moazed, D. (2011). Mechanisms for the inheritance of chromatin states. Cell *146*, 510-518.

Morgan, C.P., and Bale, T.L. (2011). Early prenatal stress epigenetically programs dysmasculinization in second-generation offspring via the paternal

lineage. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 11748-11755.

Morgan, H.D., Sutherland, H.G., Martin, D.I., and Whitelaw, E. (1999). Epigenetic inheritance at the agouti locus in the mouse. Nature genetics *23*, 314-318.

Motamedi, M.R., Verdel, A., Colmenares, S.U., Gerber, S.A., Gygi, S.P., and Moazed, D. (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell *119*, 789-802.

Mottus, R., Sobel, R.E., and Grigliatti, T.A. (2000). Mutational analysis of a histone deacetylase in Drosophila melanogaster: missense mutations suppress gene silencing associated with position effect variegation. Genetics *154*, 657-668.

Muller, H.J. (1930). Types of visible variations induced by X-rays inDrosophila. Journ of Genetics *22*, 299-334.

Nakamura, T., Liu, Y.J., Nakashima, H., Umehara, H., Inoue, K., Matoba, S., Tachibana, M., Ogura, A., Shinkai, Y., and Nakano, T. (2012). PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. Nature *486*, 415-419.

Nanney, D.L. (1958). Epigenetic Control Systems. Proceedings of the National Academy of Sciences of the United States of America *44*, 712-717.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. The Plant cell *2*, 279-289.

Nelsen, E.M., Frankel, J., and Jenkins, L.M. (1989). Non-genic inheritance of cellular handedness. Development *105*, 447-456.

Ni, J.Z., Chen, E., and Gu, S.G. (2014). Complex coding of endogenous siRNA, transcriptional silencing and H3K9 methylation on native targets of germline nuclear RNAi in C. elegans. BMC genomics *15*, 1157.

Nicholas, W.L., Dougherty, E.C., and Hansen, E.L. (1959). AXENIC CULTIVATION OF CAENORHARDITIS BRIGGSAE (NEMATODA: RHABDITIDAE) WITH CHEMICALLY UNDEFINED SUPPLEMENTS; COMPARATIVE STUDIES WITH RELATED NEMATODES*. Annals of the New York Academy of Sciences *77*, 218-236.

Nilsson, E.E., and Skinner, M.K. (2015). Environmentally induced epigenetic transgenerational inheritance of disease susceptibility. Translational research : the journal of laboratory and clinical medicine *165*, 12-17.

Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D., and Grewal, S.I. (2004). RITS acts in cis to promote RNA interference-mediated transcriptional and post-transcriptional silencing. Nature genetics *36*, 1174-1180.

Novick, A., and Weiner, M. (1957). ENZYME INDUCTION AS AN ALL-OR-NONE PHENOMENON. Proceedings of the National Academy of Sciences of the United States of America *43*, 553-566.

Oegema, K., and Hyman, A.A. (2006). Cell division. WormBook : the online review of C elegans biology, 1-40.

Oliver, B., Parisi, M., and Clark, D. (2002). Gene expression neighborhoods. Journal of biology *1*, 4.

Onishi, M., Liou, G.G., Buchberger, J.R., Walz, T., and Moazed, D. (2007). Role of the conserved Sir3-BAH domain in nucleosome binding and silent chromatin assembly. Molecular cell *28*, 1015-1028.

Ooi, S.L., Priess, J.R., and Henikoff, S. (2006). Histone H3.3 variant dynamics in the germline of Caenorhabditis elegans. PLoS genetics *2*, e97.

Ost, A., Lempradl, A., Casas, E., Weigert, M., Tiko, T., Deniz, M., Pantano, L., Boenisch, U., Itskov, P.M., Stoeckius, M.*, et al.* (2014). Paternal diet defines offspring chromatin state and intergenerational obesity. Cell *159*, 1352-1364.

Padilla, P.A., Garcia, A.M., Ladage, M.L., and Toni, L.S. (2014). Caenorhabditis elegans: an old genetic model can learn new epigenetic tricks. Integrative and comparative biology *54*, 52-60.

Painter, R.C., Osmond, C., Gluckman, P., Hanson, M., Phillips, D.I., and Roseboom, T.J. (2008). Transgenerational effects of prenatal exposure to the Dutch famine on neonatal adiposity and health in later life. BJOG : an international journal of obstetrics and gynaecology *115*, 1243-1249.

Pantano, L., Estivill, X., and Marti, E. (2010). SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets, reveals ubiquitous miRNA modifications in human embryonic cells. Nucleic acids research *38*, e34.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Muller, P.*, et al.* (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86-89.

Pavelec, D.M., Lachowiec, J., Duchaine, T.F., Smith, H.E., and Kennedy, S. (2009). Requirement for the ERI/DICER complex in endogenous RNA interference and sperm development in Caenorhabditis elegans. Genetics *183*, 1283-1295.

Petruk, S., Sedkov, Y., Johnston, D.M., Hodgson, J.W., Black, K.L., Kovermann, S.K., Beck, S., Canaani, E., Brock, H.W., and Mazo, A. (2012). TrxG and PcG proteins but not methylated histones remain associated with DNA through replication. Cell *150*, 922-933.

Pettitt, J., Crombie, C., Schumperli, D., and Muller, B. (2002). The Caenorhabditis elegans histone hairpin-binding protein is required for core histone gene expression and is essential for embryonic and postembryonic cell division. Journal of cell science *115*, 857-866.

Phillips, C.M., Montgomery, T.A., Breen, P.C., and Ruvkun, G. (2012). MUT-16 promotes formation of perinuclear mutator foci required for RNA silencing in the C. elegans germline. Genes & development *26*, 1433-1444.

Pirrotta, V. (2002). Silence in the germ. Cell *110*, 661-664.

Prahlad, V., Cornelius, T., and Morimoto, R.I. (2008). Regulation of the Cellular Heat Shock Response in Caenorhabditis elegans by Thermosensory Neurons. Science (New York, NY) *320*, 811-814.

Prusiner, S.B. (1998). Prions. Proceedings of the National Academy of Sciences of the United States of America *95*, 13363-13383.

Ptashne, M. (2007). On the use of the word 'epigenetic'. Current biology : CB *17*, R233-236.

Ptashne, M. (2013). Faddish stuff: epigenetics and the inheritance of acquired characteristics. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *27*, 1-2.

Ptashne, M., and Gann, A. (2002). Genes and Signals (Cold Spring Harbor Laboratory Press).

Puschendorf, M., Terranova, R., Boutsma, E., Mao, X., Isono, K., Brykczynska, U., Kolb, C., Otte, A.P., Koseki, H., Orkin, S.H.*, et al.* (2008). PRC1 and Suv39h specify parental asymmetry at constitutive heterochromatin in early mouse embryos. Nature genetics *40*, 411-420.

Quenneville, S., Verde, G., Corsinotti, A., Kapopoulou, A., Jakobsson, J., Offner, S., Baglivo, I., Pedone, P.V., Grimaldi, G., Riccio, A.*, et al.* (2011). In embryonic stem cells, ZFP57/KAP1 recognize a methylated hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. Molecular cell *44*, 361-372.

Quinlan, A.R., and Hall, I.M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics *26*, 841-842.

Radford, E.J., Ito, M., Shi, H., Corish, J.A., Yamazawa, K., Isganaitis, E., Seisenberger, S., Hore, T.A., Reik, W., Erkek, S.*, et al.* (2014). In utero effects. In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. Science *345*, 1255903.

Radman-Livaja, M., Liu, C.L., Friedman, N., Schreiber, S.L., and Rando, O.J. (2010). Replication and active demethylation represent partially overlapping mechanisms for erasure of H3K4me3 in budding yeast. PLoS genetics *6*, e1000837.

Ragunathan, K., Jih, G., and Moazed, D. (2015). Epigenetics. Epigenetic inheritance uncoupled from sequence-specific recruitment. Science *348*, 1258699.

Raj, A., van den Bogaard, P., Rifkin, S.A., van Oudenaarden, A., and Tyagi, S. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. Nature methods *5*, 877-879.

Rassoulzadegan, M., Grandjean, V., Gounon, P., Vincent, S., Gillot, I., and Cuzin, F. (2006). RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. Nature *441*, 469-474.

Rechavi, O., Houri-Ze'evi, L., Anava, S., Goh, W.S., Kerk, S.Y., Hannon, G.J., and Hobert, O. (2014). Starvation-induced transgenerational inheritance of small RNAs in C. elegans. Cell *158*, 277-287.

Rechavi, O., Minevich, G., and Hobert, O. (2011). Transgenerational inheritance of an acquired small RNA-based antiviral response in C. elegans. Cell *147*, 1248-1256.

Rechtsteiner, A., Ercan, S., Takasaki, T., Phippen, T.M., Egelhofer, T.A., Wang, W., Kimura, H., Lieb, J.D., and Strome, S. (2010). The histone H3K36 methyltransferase MES-4 acts epigenetically to transmit the memory of germline gene expression to progeny. PLoS genetics *6*, e1001091.

Reinhart, B.J., and Bartel, D.P. (2002). Small RNAs correspond to centromere heterochromatic repeats. Science *297*, 1831.

Reinhart, B.J., Slack, F.J., Basson, M., Pasquinelli, A.E., Bettinger, J.C., Rougvie, A.E., Horvitz, H.R., and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901-906.

Reinke, V., Gil, I.S., Ward, S., and Kazmer, K. (2004). Genome-wide germline-enriched and sex-biased expression profiles in Caenorhabditis elegans. Development *131*, 311-323.

Remy, J.J. (2010). Stable inheritance of an acquired behavior in Caenorhabditis elegans. Current biology : CB *20*, R877-878.

Richmond, T.J., Finch, J.T., Rushton, B., Rhodes, D., and Klug, A. (1984). Structure of the nucleosome core particle at 7 A resolution. Nature *311*, 532-537.

Robert, V.J., Sijen, T., van Wolfswinkel, J., and Plasterk, R.H. (2005). Chromatin and RNAi factors protect the C. elegans germline against repetitive sequences. Genes & development *19*, 782-787.

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nature biotechnology *29*, 24-26.

Robson, G.C. (1936). The variation of animals in nature, by G.C. Robson ... and O.W. Richards ... with 2 coloured plates and 30 illustrations in the text (London, New York [etc.]: Longmans, Green and co).

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H., and Bartel, D.P. (2006). Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell *127*, 1193-1207.

Rusche, L.N., Kirchmaier, A.L., and Rine, J. (2003). The establishment, inheritance, and function of silenced chromatin in Saccharomyces cerevisiae. Annual review of biochemistry *72*, 481-516.

Ruthenburg, A.J., Allis, C.D., and Wysocka, J. (2007). Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. Molecular cell *25*, 15-30.

Sadaie, M., Kawaguchi, R., Ohtani, Y., Arisaka, F., Tanaka, K., Shirahige, K., and Nakayama, J. (2008). Balance between distinct HP1 family proteins controls heterochromatin assembly in fission yeast. Molecular and cellular biology *28*, 6973-6988.

Sancar, A., Lindsey-Boltz, L.A., Unsal-Kacmaz, K., and Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annual review of biochemistry *73*, 39-85.

Sanchez-Romero, M.A., Cota, I., and Casadesus, J. (2015). DNA methylation in bacteria: from the methyl group to the methylome. Current opinion in microbiology *25*, 9-16.

Santos, F., Hendrich, B., Reik, W., and Dean, W. (2002). Dynamic reprogramming of DNA methylation in the early mouse embryo. Developmental biology *241*, 172-182.

Sapetschnig, A., Sarkies, P., Lehrbach, N.J., and Miska, E.A. (2015). Tertiary siRNAs mediate paramutation in C. elegans. PLoS genetics *11*, e1005078.

Sarkies, P., Ashe, A., Le Pen, J., McKie, M.A., and Miska, E.A. (2013). Competition between virus-derived and endogenous small RNAs regulates gene expression in Caenorhabditis elegans. Genome research *23*, 1258-1270.

Sarkies, P., and Miska, E.A. (2014). Small RNAs break out: the molecular cell biology of mobile small RNAs. Nature reviews Molecular cell biology *15*, 525-535.

Sarkies, P., Reams, C., Simpson, L.J., and Sale, J.E. (2010). Epigenetic instability due to defective replication of structured DNA. Molecular cell *40*, 703-713.

Sarkies, P., and Sale, J.E. (2012). Propagation of histone marks and epigenetic memory during normal and interrupted DNA replication. Cellular and molecular life sciences : CMLS *69*, 697-716.

Saunders, A., Core, L.J., and Lis, J.T. (2006). Breaking barriers to transcription elongation. Nature reviews Molecular cell biology *7*, 557-567.

Saze, H. (2008). Epigenetic memory transmission through mitosis and meiosis in plants. Seminars in cell & developmental biology *19*, 527-536.

Schaner, C.E., Deshpande, G., Schedl, P.D., and Kelly, W.G. (2003). A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies. Developmental cell *5*, 747-757.

Schierenberg, E. (2006). Embryological variation during nematode development. WormBook : the online review of C elegans biology, 1-13.

Schneider, S., Kaufmann, W., Buesen, R., and van Ravenzwaay, B. (2008). Vinclozolin--the lack of a transgenerational effect after oral maternal exposure during organogenesis. Reprod Toxicol *25*, 352-360.

Schoft, V.K., Chumak, N., Mosiolek, M., Slusarz, L., Komnenovic, V., Brownfield, L., Twell, D., Kakutani, T., and Tamaru, H. (2009). Induction of RNA-directed DNA methylation upon decondensation of constitutive heterochromatin. EMBO reports *10*, 1015-1021.

Schott, D., Yanai, I., and Hunter, C.P. (2014). Natural RNA interference directs a heritable response to the environment. Scientific reports *4*, 7387.

Schotta, G., Ebert, A., and Reuter, G. (2003). SU(VAR)3-9 is a conserved key function in heterochromatic gene silencing. Genetica *117*, 149-158.

Schuettengruber, B., and Cavalli, G. (2009). Recruitment of polycomb group complexes and their role in the dynamic regulation of cell fate choice. Development *136*, 3531-3542.

Seervai, R.N., and Wessel, G.M. (2013). Lessons for inductive germline determination. Molecular reproduction and development *80*, 590-609.

Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., and Reik, W. (2012). The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. Molecular cell *48*, 849-862.

Sengupta, P., and Samuel, A.D. (2009). Caenorhabditis elegans: a model system for systems neuroscience. Current opinion in neurobiology *19*, 637-643.

Seong, K.H., Li, D., Shimizu, H., Nakamura, R., and Ishii, S. (2011). Inheritance of stress-induced, ATF-2-dependent epigenetic change. Cell *145*, 1049-1061.

Serio, T.R., and Lindquist, S.L. (2000). Protein-only inheritance in yeast: something to get [PSI+]-ched about. Trends in cell biology *10*, 98-105.

Seth, M., Shirayama, M., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2013). The C. elegans CSR-1 argonaute pathway counteracts epigenetic silencing to promote germline gene expression. Developmental cell *27*, 656-663.

Sha, K., and Fire, A. (2005). Imprinting capacity of gamete lineages in Caenorhabditis elegans. Genetics *170*, 1633-1652.

Shanker, S., Job, G., George, O.L., Creamer, K.M., Shaban, A., and Partridge, J.F. (2010). Continuous requirement for the Clr4 complex but not RNAi for centromeric heterochromatin assembly in fission yeast harboring a disrupted RITS complex. PLoS genetics *6*, e1001174.

Sharif, J., and Koseki, H. (2011). Recruitment of Dnmt1 roles of the SRA protein Np95 (Uhrf1) and other factors. Progress in molecular biology and translational science *101*, 289-310.

She, X., Xu, X., Fedotov, A., Kelly, W.G., and Maine, E.M. (2009). Regulation of heterochromatin assembly on unpaired chromosomes during Caenorhabditis elegans meiosis by components of a small RNA-mediated pathway. PLoS genetics *5*, e1000624.

Shih, J.D., and Hunter, C.P. (2011). SID-1 is a dsRNA-selective dsRNA-gated channel. RNA *17*, 1057-1065.

Shilatifard, A. (2012). The COMPASS family of histone H3K4 methylases: mechanisms of regulation in development and disease pathogenesis. Annual review of biochemistry *81*, 65-95.

Shirayama, M., Seth, M., Lee, H.C., Gu, W., Ishidate, T., Conte, D., Jr., and Mello, C.C. (2012). piRNAs initiate an epigenetic memory of nonself RNA in the C. elegans germline. Cell *150*, 65-77.

Shore, D., and Nasmyth, K. (1987). Purification and cloning of a DNA binding protein from yeast that binds to both silencer and activator elements. Cell *51*, 721-732.

Shorter, J., and Lindquist, S. (2005). Prions as adaptive conduits of memory and inheritance. Nature reviews Genetics *6*, 435-450.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465-476.

Sijen, T., and Plasterk, R.H. (2003). Transposon silencing in the Caenorhabditis elegans germ line by natural RNAi. Nature *426*, 310-314.

Sijen, T., Steiner, F.A., Thijssen, K.L., and Plasterk, R.H. (2007). Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. Science *315*, 244-247.

Simmer, F., Tijsterman, M., Parrish, S., Koushika, S.P., Nonet, M.L., Fire, A., Ahringer, J., and Plasterk, R.H. (2002). Loss of the putative RNA-directed RNA polymerase RRF-3 makes C. elegans hypersensitive to RNAi. Current biology : CB *12*, 1317-1319.

Slotkin, R.K., and Martienssen, R. (2007). Transposable elements and the epigenetic regulation of the genome. Nature reviews Genetics *8*, 272-285.

Smith, Z.D., Chan, M.M., Humm, K.C., Karnik, R., Mekhoubad, S., Regev, A., Eggan, K., and Meissner, A. (2014). DNA methylation dynamics of the human preimplantation embryo. Nature *511*, 611-615.

Sogo, J.M., Stahl, H., Koller, T., and Knippers, R. (1986). Structure of replicating simian virus 40 minichromosomes. The replication fork, core histone segregation and terminal structures. Journal of molecular biology *189*, 189-204.

Spofford, J.B. (1967). Single-locus modification of position-effect variegation in Drosophila melanogaster. I. White variegation. Genetics *57*, 751-766.

Stein, L., Sternberg, P., Durbin, R., Thierry-Mieg, J., and Spieth, J. (2001). WormBase: network access to the genome and biology of Caenorhabditis elegans. Nucleic acids research *29*, 82-86.

Steiner, F.A., Okihara, K.L., Hoogstrate, S.W., Sijen, T., and Ketting, R.F. (2009). RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in Caenorhabditis elegans. Nature structural & molecular biology *16*, 207-211.

Sternberg, P.W., and Han, M. (1998). Genetics of RAS signaling in C. elegans. Trends in genetics : TIG *14*, 466-472.

Stiernagle, T. (2006). Maintenance of C. elegans. WormBook : the online review of C elegans biology, 1-11.

Stoeckius, M., Grun, D., and Rajewsky, N. (2014). Paternal RNA contributions in the Caenorhabditis elegans zygote. The EMBO journal *33*, 1740-1750.

Strahl, B.D., and Allis, C.D. (2000). The language of covalent histone modifications. Nature *403*, 41-45.

Strahl, B.D., Grant, P.A., Briggs, S.D., Sun, Z.W., Bone, J.R., Caldwell, J.A., Mollah, S., Cook, R.G., Shabanowitz, J., Hunt, D.F.*, et al.* (2002). Set2 is a nucleosomal histone H3-selective methyltransferase that mediates transcriptional repression. Molecular and cellular biology *22*, 1298-1306.

Strome, S., Powers, J., Dunn, M., Reese, K., Malone, C.J., White, J., Seydoux, G., and Saxton, W. (2001). Spindle dynamics and the role of gamma-tubulin in early Caenorhabditis elegans embryos. Molecular biology of the cell *12*, 1751-1764.

Strome, S., and Wood, W.B. (1983). Generation of asymmetry and segregation of germ-line granules in early C. elegans embryos. Cell *35*, 15-25.

Stuwe, E., Toth, K.F., and Aravin, A.A. (2014). Small but sturdy: small RNAs in cellular memory and epigenetics. Genes & development *28*, 423-431.

Sulston, J.E. (1976). Post-embryonic development in the ventral cord of Caenorhabditis elegans. Philosophical transactions of the Royal Society of London Series B, Biological sciences *275*, 287-297.

Sulston, J.E. (2003). C. elegans: the cell lineage and beyond. Bioscience reports *23*, 49-66.

Sulston, J.E., and Horvitz, H.R. (1977). Post-embryonic cell lineages of the nematode, Caenorhabditis elegans. Developmental biology *56*, 110-156.

Sulston, J.E., Schierenberg, E., White, J.G., and Thomson, J.N. (1983). The embryonic cell lineage of the nematode Caenorhabditis elegans. Developmental biology *100*, 64-119.

Swygert, S.G., and Peterson, C.L. (2014). Chromatin dynamics: interplay between remodeling enzymes and histone modifications. Biochimica et biophysica acta *1839*, 728-736.

Szyf, M. (2015). Nongenetic inheritance and transgenerational epigenetics. Trends in molecular medicine *21*, 134-144.

Szyf, M., and Detich, N. (2001). Regulation of the DNA methylation machinery and its role in cellular transformation. Progress in nucleic acid research and molecular biology *69*, 47-79.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell *99*, 123-132.

Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell *109*, 861-871.

Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L.M., Liu, D.R., Aravind, L.*, et al.* (2009). Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science *324*, 930-935.

Tauffenberger, A., and Parker, J.A. (2014). Heritable transmission of stress resistance by high dietary glucose in Caenorhabditis elegans. PLoS genetics *10*, e1004346.

Thiel, G., Lietz, M., and Hohl, M. (2004). How mammalian transcriptional repressors work. European journal of biochemistry / FEBS *271*, 2855-2862.

Thomas, J.O. (1984). The higher order structure of chromatin and histone H1. Journal of cell science Supplement *1*, 1-20.

Till, S., Lejeune, E., Thermann, R., Bortfeld, M., Hothorn, M., Enderle, D., Heinrich, C., Hentze, M.W., and Ladurner, A.G. (2007). A conserved motif in Argonaute-interacting proteins mediates functional interactions through the Argonaute PIWI domain. Nature structural & molecular biology *14*, 897-903.

Timmons, L., Court, D.L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene *263*, 103-112.

Tobi, E.W., Goeman, J.J., Monajemi, R., Gu, H., Putter, H., Zhang, Y., Slieker, R.C., Stok, A.P., Thijssen, P.E., Muller, F.*, et al.* (2014). DNA methylation signatures link prenatal famine exposure to growth and metabolism. Nature communications *5*, 5592.

Towbin, B.D., Gonzalez-Aguilera, C., Sack, R., Gaidatzis, D., Kalck, V., Meister, P., Askjaer, P., and Gasser, S.M. (2012). Step-wise methylation of histone H3K9 positions heterochromatin at the nuclear periphery. Cell *150*, 934-947.

Tsai, H.Y., Chen, C.C., Conte, D., Jr., Moresco, J.J., Chaves, D.A., Mitani, S., Yates, J.R., 3rd, Tsai, M.D., and Mello, C.C. (2015). A ribonuclease coordinates siRNA amplification and mRNA cleavage during RNAi. Cell *160*, 407-419.

Updike, D., and Strome, S. (2010). P granule assembly and function in Caenorhabditis elegans germ cells. J Androl *31*, 53-60.

Updike, D.L., Knutson, A.K., Egelhofer, T.A., Campbell, A.C., and Strome, S. (2014). Germ-granule components prevent somatic development in the C. elegans germline. Current biology : CB *24*, 970-975.

Updike, D.L., and Strome, S. (2009). A genomewide RNAi screen for genes that affect the stability, distribution and function of P granules in Caenorhabditis elegans. Genetics *183*, 1397-1419.

Vakoc, C.R., Sachdeva, M.M., Wang, H., and Blobel, G.A. (2006). Profile of histone lysine methylation across transcribed mammalian chromatin. Molecular and cellular biology *26*, 9185-9195.

Valinezhad Orang, A., Safaralizadeh, R., and Kazemzadeh-Bavili, M. (2014). Mechanisms of miRNA-Mediated Gene Regulation from Common Downregulation to mRNA-Specific Upregulation. International journal of genomics *2014*, 970607.

van de Werken, C., van der Heijden, G.W., Eleveld, C., Teeuwssen, M., Albert, M., Baarends, W.M., Laven, J.S.E., Peters, A.H.F.M., and Baart, E.B. (2014). Paternal heterochromatin formation in human embryos is H3K9/HP1 directed and primed by sperm-derived histone modifications. Nature communications *5*, 5868.

van Oosten-Hawle, P., Porter, R.S., and Morimoto, R.I. (2013). Regulation of organismal proteostasis by transcellular chaperone signaling. Cell *153*, 1366-1378.

Vasale, J.J., Gu, W., Thivierge, C., Batista, P.J., Claycomb, J.M., Youngman, E.M., Duchaine, T.F., Mello, C.C., and Conte, D., Jr. (2010). Sequential rounds of RNA-dependent RNA transcription drive endogenous small-RNA biogenesis in the ERGO-1/Argonaute pathway. Proceedings of the National Academy of Sciences of the United States of America *107*, 3582-3587.

Vastenhouw, N.L., Brunschwig, K., Okihara, K.L., Muller, F., Tijsterman, M., and Plasterk, R.H. (2006). Gene expression: long-term gene silencing by RNAi. Nature *442*, 882.

Veenendaal, M.V., Painter, R.C., de Rooij, S.R., Bossuyt, P.M., van der Post, J.A., Gluckman, P.D., Hanson, M.A., and Roseboom, T.J. (2013). Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. BJOG : an international journal of obstetrics and gynaecology *120*, 548-553.

Venkatesh, S., and Workman, J.L. (2015). Histone exchange, chromatin structure and the regulation of transcription. Nature reviews Molecular cell biology *16*, 178-189.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S.I., and Moazed, D. (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science *303*, 672-676.

Verdel, A., Vavasseur, A., Le Gorrec, M., and Touat-Todeschini, L. (2009). Common themes in siRNA-mediated epigenetic silencing pathways. The International journal of developmental biology *53*, 245-257.

Verdone, L., Agricola, E., Caserta, M., and Di Mauro, E. (2006). Histone acetylation in gene regulation. Briefings in functional genomics & proteomics *5*, 209-221.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I., and Martienssen, R.A. (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science *297*, 1833-1837.

Waddington, C.H. (1940). The genetic control of wing development inDrosophila. Journ of Genetics *41*, 75-113.

Waddington, C.H. (1942a). Canalization of development and the inheritance of acquired characters. Nature, 563-565.

Waddington, C.H. (1942b). The epigenotype. Endevour *1*, 18-20.

Wang, Y., and Jia, S. (2009). Degrees make all the difference: the multifunctionality of histone H4 lysine 20 methylation. Epigenetics : official journal of the DNA Methylation Society *4*, 273-276.

Wedeles, C.J., Wu, M.Z., and Claycomb, J.M. (2013). Protection of germline gene expression by the C. elegans Argonaute CSR-1. Developmental cell *27*, 664-671.

Wedeles, C.J., Wu, M.Z., and Claycomb, J.M. (2014). Silent no more: Endogenous small RNA pathways promote gene expression. Worm *3*, e28641.

Weismann, A. (1893). The Germ-plasm: a theory of heredity. Translated by W. Newton Parker and Harriet Rönnfeldt (New York: Scribner).

Whipple, J.M., Youssef, O.A., Aruscavage, P.J., Nix, D.A., Hong, C., Johnson, W.E., and Bass, B.L. (2015). Genome-wide profiling of the C. elegans dsRNAome. RNA *21*, 786-800.

Whitelaw, E. (2015). Disputing Lamarckian epigenetic inheritance in mammals. Genome biology *16*, 60.

Wienholds, E., Kloosterman, W.P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H.R., Kauppinen, S., and Plasterk, R.H. (2005).

MicroRNA expression in zebrafish embryonic development. Science *309*, 310-311.

Wigler, M., Levy, D., and Perucho, M. (1981). The somatic replication of DNA methylation. Cell *24*, 33-40.

Wilkins, C., Dishongh, R., Moore, S.C., Whitt, M.A., Chow, M., and Machaca, K. (2005). RNA interference is an antiviral defence mechanism in Caenorhabditis elegans. Nature *436*, 1044-1047.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science *295*, 2456-2459.

Wood, A.J., and Oakey, R.J. (2006). Genomic imprinting in mammals: emerging themes and established theories. PLoS genetics *2*, e147.

Wright, S. (1931). Evolution in Mendelian Populations. Genetics *16*, 97-159.

Wykes, S.M., and Krawetz, S.A. (2003). The structural organization of sperm chromatin. The Journal of biological chemistry *278*, 29471-29477.

Xiao, Y., Bedet, C., Robert, V.J., Simonet, T., Dunkelbarger, S., Rakotomalala, C., Soete, G., Korswagen, H.C., Strome, S., and Palladino, F. (2011). Caenorhabditis elegans chromatin-associated proteins SET-2 and ASH-2 are differentially required for histone H3 Lys 4 methylation in embryos and adult germ cells. Proceedings of the National Academy of Sciences of the United States of America *108*, 8305-8310.

Xu, L., Paulsen, J., Yoo, Y., Goodwin, E.B., and Strome, S. (2001). Caenorhabditis elegans MES-3 is a target of GLD-1 and functions epigenetically in germline development. Genetics *159*, 1007-1017.

Xu, M., Long, C., Chen, X., Huang, C., Chen, S., and Zhu, B. (2010). Partitioning of histone H3-H4 tetramers during DNA replication-dependent chromatin assembly. Science *328*, 94-98.

Yamaguchi, S., Hong, K., Liu, R., Inoue, A., Shen, L., Zhang, K., and Zhang, Y. (2013). Dynamics of 5-methylcytosine and 5-hydroxymethylcytosine during germ cell reprogramming. Cell research *23*, 329-339.

Yang, H., Zhang, Y., Vallandingham, J., Li, H., Florens, L., and Mak, H.Y. (2012). The RDE-10/RDE-11 complex triggers RNAi-induced mRNA degradation by association with target mRNA in C. elegans. Genes & development *26*, 846-856.

Yankulov, K. (2013). Dynamics and stability: epigenetic conversions in position effect variegation. Biochemistry and cell biology = Biochimie et biologie cellulaire *91*, 6-13.

Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.C., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell *127*, 747-757.

Youngson, N.A., and Whitelaw, E. (2008). Transgenerational epigenetic effects. Annual review of genomics and human genetics *9*, 233-257.

Yuan, S., Oliver, D., Schuster, A., Zheng, H., and Yan, W. (2015). Breeding scheme and maternal small RNAs affect the efficiency of transgenerational inheritance of a paramutation in mice. Scientific reports *5*, 9266.

Zanetti, S., and Puoti, A. (2013). Sex determination in the Caenorhabditis elegans germline. Advances in experimental medicine and biology *757*, 41-69.

Zhang, G., Huang, H., Liu, D., Cheng, Y., Liu, X., Zhang, W., Yin, R., Zhang, D., Zhang, P., Liu, J.*, et al.* (2015). N(6)-methyladenine DNA modification in Drosophila. Cell *161*, 893-906.

Zhang, H., Gao, L., Anandhakumar, J., and Gross, D.S. (2014). Uncoupling transcription from covalent histone modification. PLoS genetics *10*, e1004202.

Zhou, B.R., Feng, H., Kato, H., Dai, L., Yang, Y., Zhou, Y., and Bai, Y. (2013). Structural insights into the histone H1-nucleosome complex. Proceedings of the National Academy of Sciences of the United States of America *110*, 19390-19395.

Zhuang, J.J., Banse, S.A., and Hunter, C.P. (2013a). The nuclear argonaute NRDE-3 contributes to transitive RNAi in Caenorhabditis elegans. Genetics *194*, 117-131.

Zhuang, J.J., Banse, S.A., and Hunter, C.P. (2013b). The Nuclear Argonaute NRDE-3 Contributes to Transitive RNAi in Caenorhabditis elegans. Genetics *194*, 117-131.

Zhuang, J.J., and Hunter, C.P. (2012). The Influence of Competition Among Small RNA Pathways on Development. Genes *3*.