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REVIEW

A review of transgenerational epigenetics for RNAi, longevity, germline maintenance and olfactory imprinting in *Caenorhabditis elegans*

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ABSTRACT

Inheritance of acquired characteristics without changes in DNA sequence has been called transgenerational epigenetics. This review looks at studies that used the model system *Caenorhabditis elegans* to uncover mechanisms of transgenerational epigenetics in studies of RNA interference, studies of longevity, studies of germline continuity and a study on olfactory imprinting. In each case, researchers have uncovered critical roles for small RNAs and for Argonaute proteins. They have revealed several different genetic pathways that mediate RNA silencing of foreign RNA for a few or for many generations, as well as identifying a related pathway responsible for recognized self-generated RNAs. Together, these studies have greatly advanced our understanding of trangenerational epigenetics.

KEY WORDS: *C. elegans*, RNAi, Inherited

Introduction

The question of how much one generation affects the next has long been considered a cultural phenomenon, in which parents pass on the culture by teaching their children the rules of the society. An understanding of genetics has also contributed to the idea that traits and tendencies can be passed on through inheritance by offspring of specific DNA sequences from their parents. However, the recent explosion of research on transgenerational epigenetics has led to a greater understanding of how parental experience can alter gene expression, and thus phenotype, in one or more succeeding generations.

The nematode *Caenorhabditis elegans* offers an ideal model system in which to investigate transgenerational epigenetics (for reviews, see Cui and Han, 2007; Wenzel et al., 2011; Brasset and Chambeyron, 2013; Feng and Guang, 2013; Billi et al., 2013). With its sequenced genome, well-studied genetics and 3 day generation time, *C. elegans* offers opportunities to investigate mechanisms that mediate transgenerational epigenetics. In *C. elegans*, the major players in epigenetic regulation of the genome are transcription factors, post-translational histone modification and regulatory RNAs. A great deal of the research on transgenerational epigenetics in *C. elegans* has focused on small regulatory RNAs and histone modification. In mammals, chromatin is composed of nucleosomes that are made up of four highly conserved histones: H2A, H2B, H3 and H4 (Wenzel et al., 2011). In some cases, one or more of these core histones can be replaced by histone variants associated with a special function, such as H3.3, H2AZ or H2AX. When *C. elegans* core histones are compared with human core histones, there is greater than 80% identity in the amino acid sequence (Vanfleteren et al., 1989). The histones can be modified by a number of post-translational modification processes including methylation, acetylation, phosphorylation, ubiquitination, biotinylation, ADPribosylation and sumoylation. These modifications are associated with changes in both activation and repression of transcription. These post-translational modifications are added or taken off by a number of histone-modifying enzymes such as histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs) and histone demethylases (HDMs). A great deal of research has gone into mapping these histone modifications across the genome. From this work, differential distribution of these histone marks has been observed at both the transcription start sites and introns and/or exons of expressed rather than non-expressed genes. Within the nuclei of cells there are areas of transcriptionally active DNA (euchromatin) and areas of transcriptionally inactive DNA (heterochromatin). In *C. elegans*, electron microscopic studies have found that heterochromatin contains regions with high levels of repressive marks: di- and tri-methylated core histone H3 at lysine 9 and 27 (H3K9me2/3 and H3K27me3), while regions of the euchromatin are enriched for core histone H3 demethylated at lysine 4 (H3K4me2) (Wenzel et al., 2011). This pattern is similar to what has been found in *Drosophila* and mammals.

RNA interference (RNAi) was first described in *C. elegans* (Fire et al., 1998) as a gene silencing process produced by degradation of targeted mRNA by the introduction of a short, double-stranded RNA (dsRNA) from that gene. In the first description of RNAi, Fire and colleagues determined three basic characteristics of RNAi (Fire et al., 1998). These are: that the RNAi trigger is double stranded RNA, that the effects of RNAi are systemic and spread throughout the tissues, and that the effects of RNAi are heritable. These characteristics suggested that these far-reaching effects of RNAi must rely on some amplification mechanism triggered by dsRNA. Early evidence suggested that RNAi occurred post-transcriptionally in the cytoplasm because injection of dsRNA for promoter regions and for introns did not appear to have any effect (Fire et al., 1998). However, as studies identified genes critical for RNAi, and especially for transgenerational RNAi, there was increasing evidence for gene pathways mediating transcriptional silencing occurring in the nucleus (e.g. Guang et al., 2008).

A large number of studies in *C. elegans* have investigated mechanisms of RNAi (for reviews, see Billi et al., 2013; Fischer, 2010; Grishok, 2005; Feng and Guang, 2013), and many of the mechanisms that have been identified are highly conserved throughout phylogeny. The classic trigger for RNAi is dsRNA, which is cleaved into primary short interfering RNA (siRNA) by DICER, a conserved RNase III enzyme (Zamore et al., 2000;

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List of abbreviations

CSR-1	chromosome segregation and RNAi deficient
dsRNA	double-stranded RNA
miRNA	micro RNAs
piRNA	PIWI-interacting RNA
PIWI	P-element-induced wimpy testis
RdRP	RNA-dependent RNA polymerase
RNAa	RNA-induced epigenetic gene activation
RNAe	RNA-induced epigenetic silencing
RNAi	RNA interference
siRNA	short interfering RNA (endo-, endogenous; exo-, exogenous)
WAGO	worm Argonaute protein

Bernstein et al., 2001). Closer examination of the RNAs made during RNAi showed that in addition to the primary siRNAs there was an abundance of secondary siRNAs produced by RNA-dependent RNA polymerase (RdRP) that direct the silencing to target sequences (Sijen et al., 2001). These secondary siRNA pathways involve a number of Argonaute (AGO) proteins (a catalytic component of the RNA-induced silencing complex, RISC). These and other studies have led to our current understanding of one genetic pathway for RNAi in hermaphrodite worms, as seen in Fig. 1. The cellular machinery (DICER) that is responsible for siRNAs is also responsible for the production of micro RNAs (miRNA); and, like siRNA, the miRNAs function with Argonaute family proteins (Ambros, 2001). The first miRNAs to be described were *lin-4* (Lee et al., 1993) and *let-7* (Reinhart et al., 2000), two miRNAs that interact in the heterochronic pathway that determines the timing of development in *C. elegans*. These studies showed that miRNAs negatively regulated their target mRNAs by binding to target sites in the 3′ UTRs. Since the original discovery of RNAi in *C. elegans* (Fire et al., 1998), and the discovery of miRNAs (Lee et al., 1993; Reinhart et al., 2000), the field of small RNAs has exploded on to the genetic scene (for review, see Vella and Slack, 2005). In *C. elegans*, thousands of unique endogenous small RNAs have been identified; these small RNAs fall into three classes, based on size and function: (1) siRNAs (endo-siRNAs for endogenous RNAs and exo-siRNAs for exogenous RNAs), (2) miRNAs and (3) PIWI-interacting RNAs (where PIWI is P-element-induced wimpy testis; piRNAs) (Billi et al., 2013). All of these small RNAs interact with Argonaute effector proteins to recognize specific RNA transcripts and through partial or perfect complementarity regulate their expression, most commonly though inhibition. *Caenorhabditis elegans* has 25 different Argonautes that are grouped into three clades: the Argonaute-like proteins, the PIWI-like proteins and a worm-specific clade of Argonautes that are called the WAGO (worm Argonaute) proteins (Kelly, 2014). The different types of small RNAs are defined according to their length, structure, nucleotide composition and modifications, and the specific Argonaute proteins to which they bind (Feng and Guang, 2013). The siRNAs are 22–26 nucleotide sequences involved in RNAi, or silencing of RNA transcripts. The exo-siRNAs are RNAs induced by long, double-stranded exogenous RNA that originates from virus-derived RNA, cellular transfections, microinjections or feeding bacteria expressing dsRNA. The endo-siRNAs target RNAs produced by the worm genome itself, and influence gene expression by the degradation of a transcript, by translational inhibition and by modification of chromatin (Kelly, 2014). The miRNAs are 22 nucleotide RNAs, divided into a number of families, that play key roles in regulating gene expression during development, differentiation, oncogenesis and antiviral responses by upregulation or downregulation of expression of other RNAs (Claycomb, 2012;

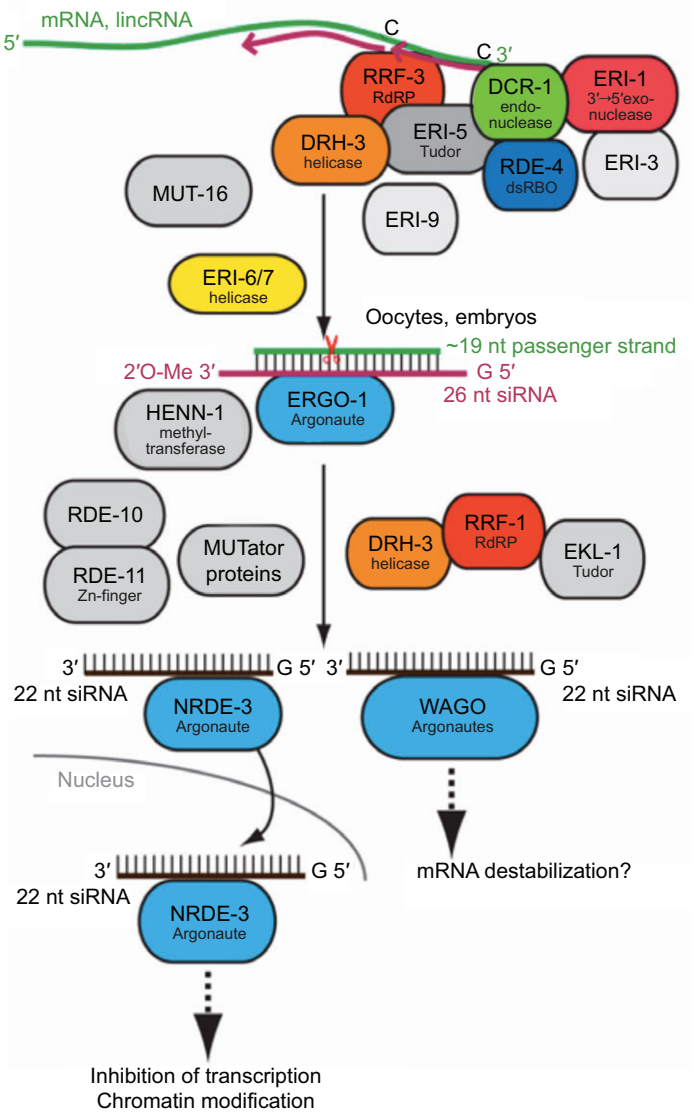

Fig. 1. Example of nuclear and cytoplasmic RNA interference (RNAi) pathways: ERGO-1 26G siRNA pathways. The templates for 26G short interfering RNA (siRNA) generation are ~500 mRNAs and long intervening non-coding RNAs (lincRNAs). A complex containing an RNA-dependent RNA polymerase (RdRP) and DCR-1 generates 26G siRNAs in both oocytes and embryos. These 26G siRNAs associate with the Argonaute protein ERGO-1. Silencing occurs in the cytoplasm through association with WAGO (worm Argonaute) class Argonautes; in the nucleus, silencing is mediated through interaction with the Argonaute NRDE-3 (nuclear RNAi defective-3) (modified from Billi et al., 2013). nt, nucleotide.

Kelly, 2014). piRNAs are 21 and 22 nucleotide, small germline-expressed RNAs that play roles in germline development and transposon silencing to protect genomic integrity during gametogenesis, and have been linked to transgenerational silencing (Claycomb, 2012; Kelly, 2014). Studies of the mechanisms of action of miRNA, siRNA and piRNA have shown that they vary greatly in biogenesis mechanisms, protein co-factors and effector function (Billi et al., 2013). A large amount of cellular machinery is dedicated to RNAi; there are different gene pathways involved in cytoplasmic RNAi and nuclear RNAi (Fig. 1) as well as different pathways for somatic and germline cells (Fig. 2) (Billi et al., 2013). What differentiates the cytoplasmic and the nuclear siRNA pathways in somatic cells is the Argonaute that is recruited: if the 22 nucleotide

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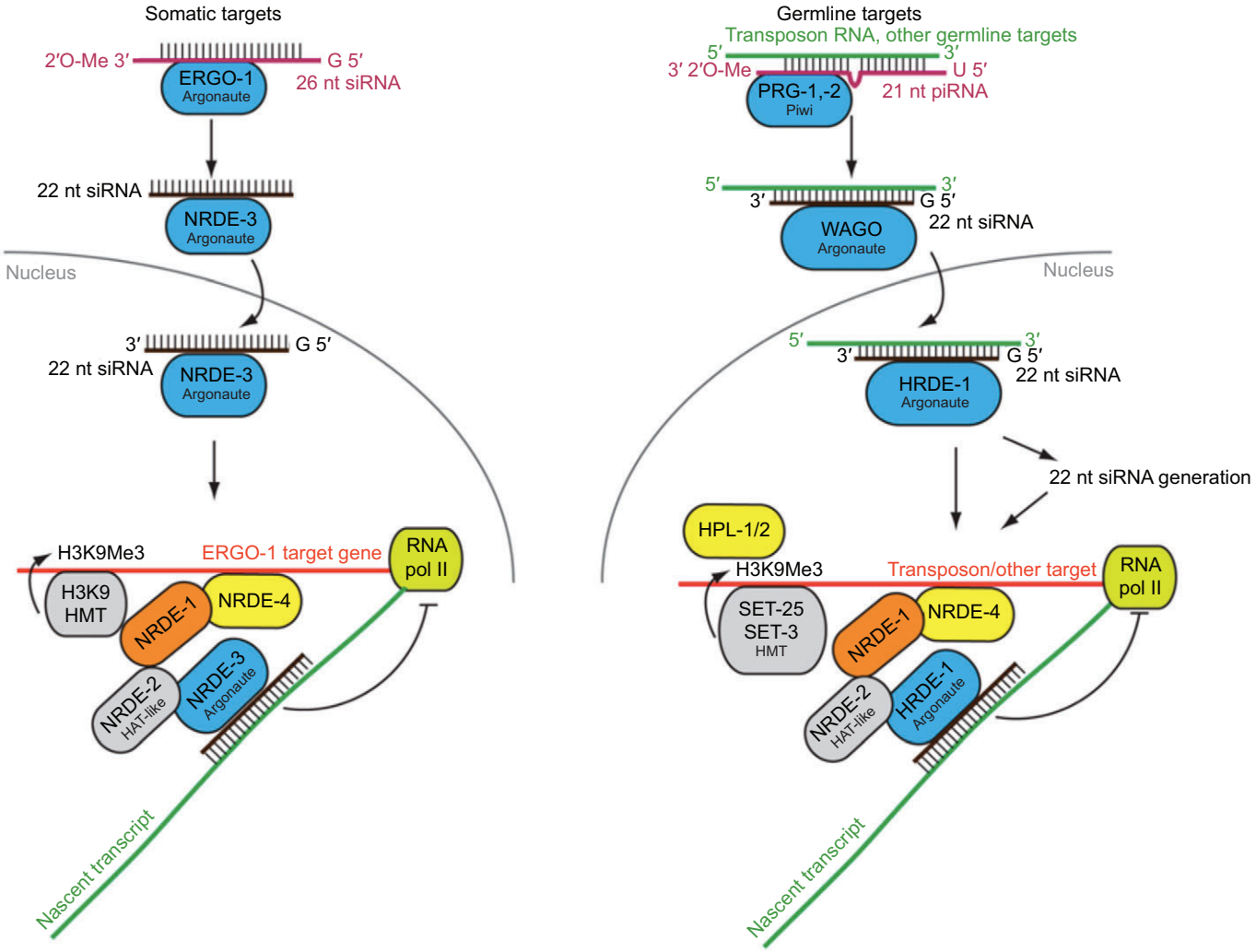

Fig. 2. Nuclear RNAi pathways required for transgenerational silencing. Two pathways, silencing somatic targets and germline targets, converge on the same NRDE proteins but require distinct nuclear Argonaute proteins: cytoplasmic secondary 22G siRNAs associate with NRDE-3 in the soma and with HRDE-1 (heritable RNAi defective 1) in the germline. The Argonaute:siRNA complex translocates to the nucleus where it recruits NRDE-2 and NRDE-1 to the nascent pre-mRNA. NRDE-1 also associates with chromatin in a NRDE-4-dependent manner. NRDE-1 and NRDE-4 promote histone H3K9 trimethylation by histone methyltransferases and thus inhibit transcription. In addition, the NRDE proteins inhibit RNA polymerase II. Nuclear RNAi is heritable across generations: silencing signals, most likely siRNAs generated in the germline of the parent, are inherited and direct H3K9 trimethylation in the offspring (modified from Billi et al., 2013).

siRNA sequence associates with WAGO-type Argonautes, then the target mRNA is destabilized in the cytoplasm. If the 22 nucleotide siRNA associates with the NRDE-3 Argonaute, then it is shuttled into the nucleus where it participates in pathways that silence their targets in the cytoplasm through inhibition of transcription and/or chromatin remodelling (see Fig. 1). Many tens of genes have been found to play roles in a large number of RNAi pathways; in this review, I have attempted to highlight a few of the key players in transgenerational inheritance of RNAi. A more comprehensive discussion of the genes implicated in a large number of RNAi pathways can be found in other reviews (e.g. Billi, 2013).

Early studies

Grishok et al. (Grishok et al., 2000) screened for genes critical for the inheritance of RNAi and discovered two classes of genes involved in this. The first class comprises genes required for the production of the heritable component of RNAi: *rde-1* (an Argonaute member of the PAZ-PIWI protein family) and *rde-4* (dsRNA binding protein). The second class of genes were not needed for the initiation of RNAi but were required for the response to the heritable RNAi component: *rde-2*, which has a homolog in *Caenorhabditis briggsae* but no homolog in other animals, and *mut-7*, which is probably an exoribonuclease.

Results from many studies indicated that RNAi could last into the F1 or F2 generations; however, Vastenhouw et al. (Vastenhouw et al., 2006) showed that a single exposure to RNAi in P0 could induce silencing of the targeted gene that lasted for more than 20 generations. This inheritance did not happen in all offspring; only about 30% of them inherited the phenotype. The original finding was with RNAi of the gene *ceh-20* expressed in the germline; subsequently, they tested 171 other genes and found similar results for 13 of them. Thus, the transgenerational inheritance of RNA was not universal but occurred only for a subset of genes. Later studies showed that the majority of the genes for which there was transgenerational RNAi were genes expressed in the germline. In contrast to this, transgenerational silencing was not often observed for genes expressed in somatic cells (Billi et al., 2013). Further studies showed that distinct but converging pathways mediate

transgenerational silencing of genes in somatic cells versus germline cells (Fig. 2). First-generation silencing depends on the classic RNAi genes *rde-1* and *rde-4*; however, these genes are not part of the inheritance mechanism. In the candidate screen for genes required for the transgenerational effect, Vastenhouw et al. (Vastenhouw et al., 2006) identified four genes that, when knocked out, abolished inheritance. These genes are all involved in chromatin remodelling: *had-4* (a class II histone deacetylase), K03D10.4 (a histone acetyltransferase of the MYST family), *isw-1* (homologue of the yeast chromatin remodelling ATPase ISW1) and *mrg-1* (a chromo-domain protein). These findings suggest that the inheritance of the RNAi-induced phenotypes was occurring via silencing at the transcriptional level. Further evidence for this hypothesis was that culturing worms in the presence of trichostatin A, a histone deacetylase, eliminated the silencing effect. In a second experiment, Vastenhouw et al. (Vastenhouw et al., 2006) used animals expressing a single copy of the green fluorescent protein (GFP) gene under the control of a germline-specific promoter and fed them dsRNA homologous to the GFP sequence. As the worms developed, any that did not express GFP were transferred to new plates. All of the F1 unexposed offspring showed reduced GFP expression compared with the parent that was exposed to RNAi. As the generations increased, not all offspring showed the effect; however, the authors were able to detect some animals with reduced GFP expression for over 80 generations!

Guang et al. (Guang et al., 2008) used chemical mutagenesis to screen for mutations that produced worms that, in response to dsRNA, retained the ability to silence RNAs in the cytoplasm, but failed to silence RNAs localized to the nucleus. In this screen, they identified a number of alleles of an Argonaute protein called nuclear RNAi defective-3 (*nrde-3*). In wild-type worms, NRDE-1::GFP fusion product was localized to the nucleus of cells. In strains of worms with mutations in genes known to be involved in siRNA (i.e. *eri-1*, *ergo-1*, *mut-7*, *rde-4* or *mut 2*), NRDE-1::GFP fusion product was observed in the cytoplasm rather than the nucleus. Guang et al. (Guang et al., 2008) demonstrated that, in the cytoplasm, NRDE-3 binds siRNAs generated by RdRPs and transports them into the nucleus. Burton et al. (Burton et al., 2011) showed that for multigenerational silencing in response to dsRNA, NRDE-3 functions in a nuclear RNA pathway with NRDE-1, NRDE-2 and NRDE-4 to regulate gene expression by inhibiting RNA polymerase II during transcription. In addition, NRDE-2 is critical for enrichment of H3K9 methylation at sites targeted by the RNAi. Together, members of this NRDE nuclear pathway mediate heritable expression of siRNAs and heritable H3K9me marks in progeny after parental exposure to dsRNA.

The germline

The germline is an important site of epigenetic activity, both for the development of the individual and for transmission of epigenetic regulation across generations. Germlines are considered 'immortal cell lines' as a germline is a continuous cell lineage that connects all generations. In *C. elegans*, mutations in many of the genes implicated in transgenerational epigenetics (e.g. *prg-1*, *hrde-1*, *nrde-1*, *spr-5*) result in progressive sterility over multiple generations, ultimately leading to a 'mortal' germline phenotype. Epigenetic changes that occur in the germline have the potential to be carried across generations. The germline has regulatory mechanisms that initiate, maintain and terminate epigenetic transmission. The germline is established and develops during embryogenesis, after which cells in the germline switch from mitotic cell division to meiotic cell division to produce gametes. Development of the gametes is accompanied by a great deal of epigenetic programming that, if stabilized, has the potential to impact developmental events in subsequent generations. As a result of the original epigenetic programming of cells to make the gamete, there follows a phase of heightened epigenetic 'reprogramming' events in the gamete pronuclei after fertilization. These reprogramming events are necessary for normal development to occur. Disruption of this reprogramming process is lethal, suggesting that some of the epigenetic content of the original gamete is detrimental to survival and that epigenetic modification is necessary to 'reset' the genome for proper development to occur. This reprogramming is not a random process: some epigenetic marks are maintained, while others are removed. Much of the epigenetic regulation of germline cells is the result of a balance of methylation and demethylation of histone H3; for example, methylation at lysines 4, 9, 27 and 36 (H3K4me, H3K9me, H3K27me and H3K27me, respectively) are most closely associated with heritable states of transcriptional activity. Methylation of H3K4 and H3K6 is usually associated with transcriptional activation, while methylation of H3K9 and H3K27 is associated with heritable transcriptional repression. In addition, methylation at one site can influence methylation at one or more other sites, leading to an interrelated network. In the *C. elegans* genome, two H3K36 methyltransferases have been identified; they encode the MET-1 and MES-4 enzymes that regulate all H3K36 methylation.

Caenorhabditis elegans met-1 is analogous to yeast SET2 in that activity seems to be co-transcriptional (Kelly, 2014). Studies of *met-1* suggest it is not essential for survival as homozygous *met-1* worms are fertile (although they show some somatic defects). Anderson and Horvitz (Andersen and Horvitz, 2007) showed that mutations in *met-1* do not lead to strong phenotypes; however, *met-1* in combination with mutations in an H3K9 methyltransferase, *met-2*, have progressively higher numbers of sterile offspring in each generation, leading to a mortal germline phenotype. In contrast, *mes-4* activity in the germ cells is critical for germline development. MES-4 is related to mammalian SET nuclear receptor binding proteins NSD1/2. Maternally supplied MES-4 is sufficient for the development of the germline but not for fertility in the next generation. Research from the Strome lab (Bender et al., 2004; Bender et al., 2006; Rechsteiner et. al., 2010; Gaydos et al., 2012) has shown that MES-4 is critical in the embryonic and very early post-embryonic germline, but not needed in adults to produce functional gametes. This is because, in the embryonic germ cells, MES-4 retains the H3K36 methylation patterns of the parent, and this pattern is required for early germline development. In *mes-4* mutants, levels of H3K36 methylation decrease progressively across cell divisions. The pattern of methylation of H3K36me3 is produced in the adult germline by MET-1, and its maintenance in the developing embryo requires MES-4. When MES-4 is absent from the parental cells, the gamete's MET-1-dependent methylation patterns are quickly diluted by DNA replication. Thus, the role of the methytransferase MES-4 is to maintain patterns of methylation to provide a transgenerational inheritance of H3K36 methylation patterns in the germline, which are essential for germ cell function.

The H3K36 methylation pattern maintained by MES-4 works antagonistically with MES-2/-3/-6 proteins, homologues of the polycomb repression complex 2 (PRC2), that produce H3K27 methylation. Mutations in *mes-2*, *mes-3* or *mes-6* have similar phenotypes to *mes-4* with maternal-effect sterility (Bender et al., 2004). Evidence for the antagonistic relationship between *mes-4* and *mes-2*/*-3*/*-6* was seen in ChIP-chip analyses (Gaydos et al., 2012)

where depletion of H3K36me in germline-expressed genes in *mes-4* mutant embryos led to an intrusion of H3K27me into these genes from nearby regions. H3K4 methylation is usually associated with gene activation. Using genes that demethylate H3K4, Katz et al. (Katz et al., 2009) showed that epigenetic patterns of demethylation can be stably inherited through the germline for multiple generations, and mutations in *spr-5*, an H3K4 demethylase, lead to a 'mortal' germline.

A number of different complex sets of proteins regulate H3K4me2 and H3K4me3 in all cells of embryonic *C. elegans*. These COMPASS (complex proteins associated with Set1; also labelled Set1-like or mixed-lineage leukaemia, MLL) protein complexes are similar to those found in mammals and contain many homologous genes (i.e. Wdr5, Ash2l, RbBP5, Cfp1 and Dpy30). In the early embryo, H3K4 methylation requires Set1/MLL activity, but appears to be independent of transcription. Li and Kelly (Li and Kelly, 2011) reported that worms carrying mutations in MLL components first show a decrease in the number of post-embryonic stem cells; however, over repeated generations they begin to show a partially mortal germline.

Arico et al. (Arico et al., 2011) showed that the H3K4 methylation patterns in the adult worm are transmitted to their offspring. At fertilization a complex cascade of events occurs, including a large influx of histone H3.3 into all chromosomes. After the first cell division all chromosomes become notably enriched in H3K4 histone H3 unmodified at K4. The methylation pattern of H3K4 on the chromosomes is primarily maintained with enriched H3K4me3 on the autosomes and the maternal X chromosome and grossly depleted in the paternal X chromosome. Thus, the maternal pattern predominates. A genome-wide analysis of the methylation profile of the embryonic germline indicated that the H3K4me profile matches the profile of H3K4me in the gamete, reflecting the same pattern in the parental germ cell. Thus, the methylation patterns of H3K4 that are originally established by transcription are inherited from the adult germline of one generation to the embryonic germline of the next generation.

Buckley et al. (Buckley et al., 2012) carried out a screen for genes that are critical for transgenerational GFP silencing in the germ cells. They used a GFP transgene and screened for worms that silenced the transgene (eliminated expression of GFP) in response to RNAi exposure, but that did not show silencing in subsequent generations. In this way they found an Argonaute protein that associates with siRNAs in the germ cells of the exposed parent. They called this gene heritable RNAi defective 1 [*hrde-1*: identified previously as WAGO-9; also implicated in stable transgenerational RNAi at about the same time (see Ashe et al., 2012; Bagijn et al., 2012; Luteijn et al., 2012; Shirayama et al., 2012)]. Under normal circumstances, HRDE-1 directs the nuclear RNAi pathway to trimethylate histone H3 at lysine 9 (H3K9me3) at genomic sites targeted by RNAi to promote inheritance of RNAi. In an *hrde-1* background, germline silencing is lost over ensuing generations and animals show gradually increasing defects in gamete formation and will ultimately become sterile. Buckley et al. (Buckley et al., 2012) identified the Argonaute protein HRDE-1 as the agent that directs gene silencing events in germ cell nuclei leading to multigenerational RNAi as well as promoting germ-cell lineage immortality.

A series of experiments carried out by Ashe et al. (Ashe et al., 2012) showed that the transgenerational inheritance of environmental RNAi and the piRNA silencing pathway converge on the same germline nuclear RNAi/chromatin pathway. Ashe et al. used a single copy insertion of a reporter transgene expressing a histone–GFP fusion protein in the germline of *C. elegans* to screen for mutations that were defective for transgenerational inheritance (Ashe et al., 2012). In wild-type worms, a single exposure to RNAi against the GFP transgene was heritable for at least four generations. In silenced animals, there was a significant decrease in mRNA levels for the transgene as well as in preRNA levels. A small RNA profile was made using high-throughput sequencing for P0 animals exposed to environmental RNAi, control (empty vector) RNAi and for worms in the fourth generation after RNAi exposure. In the P0 animals exposed to RNAi, Ashe et al. found abundant sense and antisense small RNAs (primary siRNAs). In contrast, in animals four generations later, they found only antisense small RNAs with the characteristics of secondary siRNAs (Ashe et al., 2012). Given the large number of progeny produced by a single hermaphrodite (>200) and the high level of these siRNAs, Ashe et al. hypothesized that the secondary siRNAs must be generated by each generation of animals. To compare genes needed for piRNA silencing and heritable environmental RNAi, they did a forward genetic screen using strains with GFP-based sensors for heritable RNAi and for piRNA silencing (Ashe et al., 2012). They found that, like environmental RNAi, the piRNAs could trigger long-term silencing that could last for at least 20 generations. After being initiated by the piRNA trigger, the signalling no longer depends on piRNA; however, it is dependent on the nuclear RNAi/chromatin pathway. By comparing the genes necessary for both types of silencing, they determined that a core set of nuclear RNAi and chromatin factors is necessary for multigenerational inheritance of both environmental RNAi and piRNA silencing. The genes they identified include the germline-specific nuclear Argonaute HRDE-1/WAGO-9, the HP1 orthologue HPL-2, and two putative histone methyltransferases, SET-25 and SET-32.

Self-recognition

The original role of RNAi was thought to be a defence against foreign (e.g. viral genes or multiple copy transgenes) DNA that inserted itself into the *C. elegans* genome and used it to replicate. For this to work, the mechanisms of RNAi then need a way to identify non-self RNAs versus self RNAs. While it is important to recognize foreign sequences, it is equally important to recognize and protect endogenous self RNAs. A number of studies have investigated how *C. elegans* achieves this and they have come to the conclusion that small RNA molecules carry the memory for identification of both self and non-self RNAs (reviewed in Albuquerque and Ketting, 2014). For recognition of non-self RNAs, the Argonaute protein PRG-1 plays a critical role. PRG-1 uses piRNA sequences (more than 30,000 annotated piRNAs have been described thus far for *C. elegans*) to identify non-self targets. When PRG-1 identifies and targets non-self RNA, it recruits RdRP, which makes small RNAs that are antisense to the original targeted sequence and thus activate the cytoplasmic dsRNA silencing machinery through WAGO-9 and activate transcriptional silencing of the gene that produced the original non-self transcript through HRDE-1 (Ashe et al., 2012; Lee et al., 2012). This PRG-1-initiated transcriptional silencing is maintained transgenerationally by HRDE-1. How do self transcripts and stable transgenes avoid being targeted by this PRG-1 system? Claycomb et al. (Claycomb et al., 2009) found that the essential Argonaute protein CSR-1 (chromosome segregation and RNAi deficient) is associated in complexes with a large number of small RNAs derived from expressed genes; however, these genes were not downregulated. This led to the hypothesis that CSR-1 might be involved in the recognition of self. Although this process is still not fully understood, a series of recent papers has shed some light on it.

Seth et al. (Seth et al., 2013) carried out a series of experiments to investigate self-recognition *in C. elegans*. Additionally, a number of studies have linked RNAi-related silencing to epigenetic silencing through alterations of chromatin marks, suggesting that the mechanisms of RNAi and chromatin modification support and interact with one another. Because the propagation of chromatin marks occurs in *cis*, and RNAi can propagate in *trans*, this allows for the coordinated regulation of whole gene families on sister chromatids. Shirayama et al. (Shirayama et al., 2012) called PRG-1 transgenerational epigenetic silencing 'RNA-induced epigenetic silencing' (RNAe), although Billi et al. (Billi et al., 2013) called it exo-RNAi. Although the majority of small RNA pathways studied to date are involved in silencing genes, there are a few examples in which small RNAs appear to activate genes. Shirayama et al. (Shirayama et al., 2012) discovered a transactivating signal that counteracted RNAe when they crossed worms with an RNAe transgene and homologously active transgenes. Because this process involved the epigenetically transmitted, RNA-induced transactivation of a silent allele, Seth et al. (Seth et al., 2013) called this phenomenon RNA-induced epigenetic gene activation (RNAa). Seth et al. (Seth et al., 2013) showed that CSR-1 is required for RNAa. They found that a transgene with RNAa activity showed an accumulation of CSR-1 small RNAs. To test whether transactivation required CSR-1 activity, they crossed *oma-1*::GFP (RNAa) to GFP::*cdk-1*(RNAe). The FI offspring were exposed to either *csr-1* RNAi or a control RNAi. OMA-1::GFP is normally expressed through the cytoplasm of oocytes. Accumulation of GFP::CDK-1 in the nucleus was used to measure transactivation. When the F1 worms were exposed to a control RNAi, all of the F1 showed transactivation of GFP::*cdk-1* (RNAe). In contrast, when F1 worms were exposed to *csr-1* RNAi, none of the worms showed transactivation of GFP::CDK-1. This type of transactivation did not occur in a *csr-1* mutant background.

The findings of Seth et al. (Seth et al., 2013) about the role of CSR-1 in the activation of genes are correlational, not causal; in a series of elegant experiments, Wedeles et al. (Wedeles et al., 2013) provided direct mechanistic evidence that CSR-1 actively turns on genes. In these experiments, they (Wedeles et al., 2013) used a tethering system to force CSR-1 to interact with a transcript from a transgene encoding GFP, leading directly to its expression. The binding of CSR-1 to this transgene also protected it from silencing by another normally fully penetrant silenced GFP transgene that is not bound to CSR-1. This protection of the silenced GFP transgene occurred over several generations. These experiments led to another very interesting phenomenon: over generations, the CSR-1 transcript could work in *trans* to activate a previously silenced GFP transgene that did not have the tethering site that makes it associate with CSR-1. Taken together, these data suggested that there is an accumulation of 'something' over generations that establishes activation (Wedeles et al., 2013). One hypothesis is that the 'something' is small RNAs bound to CSR-1; however, this has yet to be proven.

In a study of gene expression during spermatogenesis, Conine et al. (Conine et al., 2013) reported that CSR-1 interacts with two additional Argonaute proteins, ALG-3 and ALG-4, to activate expression of genes required for spermiogenesis. During spermiogenesis, ALG3/4 amplify expression of small RNAs that represent an epigenetic memory of male-specific gene expression. This memory is transmitted to offspring by CSR-1, which is highly expressed in mature sperm. Interestingly, Conine et al. also observed that males carried a large number of CSR-1 small RNAs targeting mRNAs that are important for oogenesis. Thus, sperm contain CSR-1 small RNA complexes that together represent a memory for paternal gene expression that is transmitted transgenerationally. Taken together, these experiments on CSR-1 suggest that patterns of gene expression from both maternal and paternal genomes are represented by CSR-1 small RNA complexes in developing zygotes, and that CSR-1 is an excellent candidate for recognition of self in the *C. elegans* genome.

Cecere et al. (Cecere et al., 2014) carried out genome-wide profiling of newly transcribed RNAs and found that sense oriented RNA polymerase II transcription was increased by the CSR-1 pathway. In a CSR-1 mutant background, they found a global increase in transcription of antisense RNAs and transcription of normally silent regions of chromatin. Based on these results, they (Cecere et al., 2014) hypothesized that the CSR-1 pathway also plays an important role in maintaining the directionality of active transcription, which enhances the distinction between transcriptionally active and silent regions of the genome.

Longevity

A great deal of research has uncovered a number of important genetic pathways that mediate longevity in *C. elegans*. An interesting factor in these studies is that the majority of them were carried out in the presence of the drug 5-fluorodeoxyuridine (FUdR), which inhibits the proliferation of germline stem cells and blocks egg production in adults (this makes the experiments much easier to do as it means worms do not have to be moved daily). However, as Greer et al. (Greer et al., 2010) point out, this masks any influence that reproduction might have on longevity. In a pair of papers, Greer et al. (Greer et al., 2010; Greer et al., 2011) investigated effects of RNAi of methyltransferases, proteins containing the SET enzymatic domain of methyltransferases, or orthologues of regulators of histone methylation, on longevity in fertile worms. Their results pointed to the ASH-2 trithorax complex as a regulator of *C. elegans* lifespan. The ASH-2 complex trimethylates histone H3 at lysine 4 (H3K4) and is composed of three members: ASH-2, WDR-5 and the H3K4 methyltransferase SET-2. Mutations or knock-downs of any of these three genes extended worm lifespan. In contrast, mutating or knocking down the H3K4 demethylase RBR-2 greatly decreased lifespan. To investigate where in the animal these genes were required, Greer et al. (Greer et al., 2010) repeated the RNAi experiments in *rrf-3* animals in which RNAi works in the germline, but not in somatic cells, as well as tissue-specific rescue of mutations in the genes using a *pie-1* promoter that is expressed only in the germline. Both of these sets of experiments indicated that ASH-2, WDR-5, SET-2 and RBR-2 function in the germline was sufficient to alter lifespan. Interestingly, the lifespan effects of the ASH-2 complex and RBR-2 did not appear to involve significant alterations in fertility or fecundity; for the lifespan alteration, ASH-2 and RBR-2 required the continuous production of mature eggs. Through these experiments, Greer et al. (Greer et al., 2010) demonstrated that genes that make up an H3K4me3 methyltransferase complex play a key role in regulating lifespan in *C. elegans* and that the ASH-2 complex and RBR-2 regulate lifespan by controlling trimethylation of H3K4.

In their second series of experiments, Greer et al. (Greer et al., 2011) asked whether the lifespan effects of perturbing the activity of the ASH-2 complex in a parental generation of *C. elegans* would persist and regulate lifespan for more than a single generation. They found that decreasing levels of WDR-5 decreased H3K4me3 levels and extended the lifespan of worms. To test whether there were transgenerational epigenetic effects of the decrease in WDR-5 in the parental animals, Greer et al. crossed wild-type males (P0=+/+) with *wdr-5* hermaphrodites (P0=*wdr-5*/*wdr-5*) to generate an F1

generation of heterozygotes (+/*wdr-5*). These heterozygotes were then self-crossed to create an F2 generation consisting of +/+, +/*wdr-5* and *wdr-5*/*wdr-5* offspring. These animals were genotyped and each group was self-crossed to create an F3 generation of +/+, +/*wdr-5* and *wdr-5*/*wdr-5* and then an F4 and F5 generation. A control wild-type male and hermaphrodite cross was done at the same time to compare lifespans across the generations. Interestingly, a comparison of lifespan for genetically wild-type F3 descendants from P0 *wdr-5* parents (*wdr-5*/*wdr-5*) still showed a 20% extension of lifespan when compared with descendants from pure wild-type parents. Interestingly, this effect lasted through only four generations; by the F5 generation, wild-type descendants from *wdr-5*/*wdr-5* mutant parents no longer showed extended lifespans. They repeated these experiments with another member of the ASH-2 complex, the H3K4me3 methyltransferase enzyme SET-2 that, together with ASH-2 and WDR-5, functions to regulate H3K4me3 levels and longevity in *C. elegans*. The results were strikingly similar to what was observed in WRD-5 animals in that genetically wild-type descendants from *set-2*/*set-2* parents showed extended lifespan in F3 and F4 generations, but not in the F5 generation. To test whether the effect was linked to the fact that the crosses had been done with mutant hermaphrodites and wild-type males, the experiment was repeated with P0 consisting of wild-type hermaphrodites and mutant male SET-2 worms with the same results. Thus, the transgenerational effect on longevity was not linked to a particular gender in the parental generation. The final member of the complex, ASH-2, plays a key role in the conversion of H3K4 dimethylation (H3K4me2) to H3K4 trimethylation (H3K4me3). RNAi of ASH-2 in wild-type worms decreased global H3K4me3 levels during development and extended longevity. Greer et al. (Greer et al., 2011) knocked down ASH-2 with RNAi in P0 and investigated whether this would affect lifespan of later generations. They showed that the RNAi was effective in lowering ASH-2 mRNA and protein levels in P0 worms, and that these levels were normal in subsequent generations. This indicated that the ASH-2 RNAi itself was not inherited. Even though ASH-2 levels were normal in F1, F2 and F3 worms, they all showed extended lifespan compared with unexposed wild-type worms. By F4, worms showed normal wild-type lifespans. Taken together, these experiments show that disruption of the components of the H3K4me3 methyltransferase complex (ASH-2, WDR-5 and SET-2) in P0 alters the lifespan of subsequent generations. This supports the hypothesis that the transgenerational inheritance of longevity is the result of epigenetic changes that can persist over three to four subsequent generations.

Greer et al. (Greer et al., 2010) showed that while the ASH-2 complex increases lifespan in P0 through activity in the germline, the H3K4me3 demethylase RBR-2 is necessary for this lifespan extension. Greer et al. (Greer et al., 2011) asked whether RBR-2 was also necessary for the transgenerational increase in lifespan after disruption of members of the ASH-2 complex. They exposed genotypically wild-type F3 descendants of P0 *wdr-5*/*wdr-5* worms to RBR-2 RNAi and found that the lifespan extension was no longer observed. They also found normal lifespans in F3 wild-type descendants from P0 *set-2;rbr-2* parents had wild-type lifespans. Taken together, these results suggest that the transgenerational inheritance of lifespan that results from alterations in the ASH-2 complex members is dependent on the H3K4me3 demethylase RBR-2. Interestingly, other experiments indicated that, by itself, RBR-2 does not have transgenerational epigenetic effects. As found for the within-lifetime lifespan effects of ASH-2 complex disruption (Greer et al., 2010), the transgeneration epigenetic lifespan-extending effects of the complex also required an intact and functioning germline.

After uncovering transgenerational epigenetic effects of H3K4me3 modifiers on lifespan, Greer et al. (Greer et al., 2011) tested whether chromatin modifiers of other marks (*set-9*, *set-15* and *utx-1*) or genes from other lifespan pathways including insulin signalling (*age-1* and *dod-23*), mitochondria (*cco-1* and *cyc-1*) and stress resistance (*asm-3*) also showed transgenerational effects. In contrast with their observations with the ASH-2 complex, knock-down of any of these genes showed effects only on P0 and no effects of subsequent generations. From this, Greer et al. (Greer et al., 2011) concluded that that transgenerational extension of lifespan is specific to H3K4me3 chromatin modifiers. In terms of mechanism, the lifespan effects did not appear to be mediated by a heritable global decrease in H3K4me3 levels as global H3K4me3 levels were not decreased in F3 and F4 generation genetically wild-type descendants from *wdr-5* and *set-2* parents. To test whether the lifespan effects might be the result of heritable altered marks on specific genes, Greer et al. (Greer et al., 2011) did a genome-wide comparison of gene expression in F4 and F5 wild-type descendants from *wdr-5* mutant and wild-type ancestors, and pure *wdr-5* mutant descendants. Using microarray data, they found 759 genes that were differentially regulated in *wdr-5* pure mutants compared with wild-type worms. When expression of these genes was compared in the F4 and F5 generations of wild-type descendants from *wdr-5* mutant worms, a significant subset of WDR-5-regulated genes were still differentially regulated in the F4 but not in the F5 generation. Analysis of the types of genes that showed altered expression included longevity genes known to be expressed in the germline as well as enrichment for several types of metabolic pathways.

Greer and colleagues' study (Greer et al., 2011) is the first to report a transgenerational epigenetic effect on longevity. Although the traditional view holds that histone marks and DNA methylation are erased between generations, this may not always be the case. The data from these studies are consistent with the hypothesis that H3K4me3 at specific locations may be maintained and passed on through several generations. An interesting question not answered by these studies is why four generations? There does not appear to be a gradual diminution of the effect across F1–F4; the degree of increase in lifespan is comparable across these generations. Why then is the effect suddenly lost at five generations?

Olfactory imprinting

Lorenz (Lorenz, 1935) first described how early experience during a critical period could permanently alter behaviour in individual animals; he called this process imprinting. Remy and Hobert (Remy and Hobert, 2005) described a form of olfactory imprinting in *C. elegans*. They found that worms exposed to the volatile odorant benzaldehyde during the first 24 h after they hatched significantly increased chemotaxis towards benzaldehyde as adults when compared with worms not exposed to the compound early in development. This olfactory imprinting was dependent on expression of *sra-11*, an orphan G-protein-coupled seven-transmembrane receptor that is a member of a large family of chemoreceptor-encoding genes. Cell-specific rescues showed that olfactory imprinting was dependent on expression of *sra-11* in the AIY pair of interneurons. Remy (Remy, 2010) showed that this olfactory imprinting could be passed transgenerationally. If a single generation was exposed to the imprinting paradigm, then their offspring (F1) also showed a preference for benzaldehyde, but the next generation (F2) did not. In contrast, if worms were exposed to the benzaldehyde imprinting paradigm for four generations, then it

was stably inherited through at least 40 generations. These data suggest that somewhere between one and four generations of exposures there is a switch between a transitory, reversible effect and a stable, inherited effect. To date, no mechanism has been identified to underlie this behavioural epigenetic effect, but studies of other transgenerational epigenetic effects in *C. elegans* offer a number of great candidate genes to begin the investigations.

Summary

Together, these papers reveal the complexity and variability of the transgenerational epigenetic phenomenon in *C. elegans*. Many new molecules and mechanisms have been uncovered and the rich details of numerous processes have been revealed. Despite these discoveries, there are still many unanswered questions. In some cases, epigenetic silencing lasts for three to four generations, for some, dozens of generations. Why these differences? What determines whether a gene will be subject to transgenerational epigenetic silencing, and for how long? Why do independently created, genetically identical transgenic lines behave differently? What other mechanisms are yet to be uncovered? This review has only covered a subset of the research on mechanisms of transgenerational epigenetics that has been done using *C. elegans*; more detailed reviews can be found elsewhere (see Bagjin et al., 2012; Benayoun and Brunet, 2012; Billi et al., 2013; Brasset and Chambeyron, 2013; Kelly, 2014; Feng and Guang, 2013; Lim and Brunet, 2013; Vella and Slack, 2005; Wenzel et al., 2011). This is a rich field of study, and it is clear that *C. elegans* offers unique opportunities to rapidly uncover the molecular machinery regulating this amazing process.

It is, however, important to note that most of the studies reported here focused on transgenerational inheritance under highly controlled laboratory conditions using transgenes generated by the researchers. The studies show the ways that transgenerational inheritance can occur in the laboratory. We still do not know when it occurs in nature. What types of experiences are epigenetically encoded, altering gene expression for future generations? In one of the first reports of epigenetic inheritance, offspring of pregnant rats exposed to pesticides showed epigenetic effects for at least the next four generations (Anway et al., 2005). With its short life cycle, *C. elegans* will be a great model system in which to explore the real world triggers of epigenetic inheritance. The understanding of the mechanisms of epigenetic inheritance determined by studies like those described here can then be used to attempt to alter or mitigate the effects of these natural triggers.

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Competing interests

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References

Ambros, V. (2001). microRNAs: tiny regulators with great potential. *Cell* **107**, 823-826.
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.
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 Genet.* **7**, e1001391.
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., Pintacuda, G., Sakaguchi, A., Sarkies, P., Ahmed, S. and Miska, E. A. (2012). piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* **150**, 88-99.
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.
Benayoun, B. A. and Brunet, A. (2012). Epigenetic memory of longevity in *Caenorhabditis elegans*. *Worm* **1**, 76-80.
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*. *Curr. Biol.* **14**, 1639-1643.
Bender, L. B., Suh, J., Carroll, C. R., Fong, Y., Fingerman, I. M., Briggs, S. D., Cao, R., Zhang, Y., Reinke, V. and Strome, S. (2006). MES-4: an autosome-associated histone methyltransferase that participates in silencing the X chromosomes in the *C. elegans* germ line. *Development* **133**, 3907-3917.
Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-366.
Billi, A. C., Fischer, S. C. and Kim, J. K. (2013). Endogenous RNAi pathways in *C. elegans* (May 7, 2014). In *WormBook* (ed. The *C. elegans* Research Community, WormBook). Available at: http://dx.doi.org/10.1895/wormbook.1.170.1.
Brasset, E. and Chambeyron, S. (2013). Epigenetics and transgenerational inheritance. *Genome Biol.* **14**, 306.
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.
Burton, N. O., Burkhart, K. B. and Kennedy, S. (2011). Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **108**, 19683-19688.
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. *Nat. Struct. Mol. Biol.* **21**, 358-365.
Claycomb, J. M. (2012). *Caenorhabditis elegans* small RNA pathways make their mark on chromatin. *DNA Cell Biol.* **31 Suppl. 1**, S17-S33.
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.
Conine, C. C., Moresco, J. J., Gu, W., Shirayama, M., Conte, D., Jr, Yates, J. R., III 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.
Cui, M. and Han, M. (2007). Roles of chromatin factors in *C. elegans* development. In *WormBook* (ed. The *C. elegans* Research Community, WormBook). Available at: http://dx.doi.org/10.1895/wormbook.1.139.1.
de Albuquerque, B. F. M. and Ketting, R. F. (2013). Is this mine? Small RNAs help to decide. *Dev. Cell* **27**, 599-601.
Feng, X. and Guang, S. (2013). Small RNAs, RNAi and the inheritance of gene silencing in *Caenorhabditis elegans*. *J. Genet. Genomics* **40**, 153-160.
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, S. E. (2010). Small RNA-mediated gene silencing pathways in *C. elegans*. *Int. J. Biochem. Cell Biol.* **42**, 1306-1315.
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.
Greer, E. L., Maures, T. J., Hauswirth, A. G., Green, E. M., Leeman, D. S., Maro, G. S., Han, S., Banko, M. R., Gozani, O. and Brunet, A. (2010). Members of the H3K4 trimethylation complex regulate lifespan in a germline-dependent manner in *C. elegans*. *Nature* **466**, 383-387.
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.
Grishok, A. (2005). RNAi mechanisms in *Caenorhabditis elegans*. *FEBS Lett.* **579**, 5932-5939.
Grishok, A., Tabara, H. and Mello, C. C. (2000). Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494-2497.
Guang, S., Bochner, A. F., Pavelec, D. M., Burkhart, K. B., Harding, S., Lachowiec, J. and Kennedy, S. (2008). An Argonaute transports siRNAs from the cytoplasm to the nucleus. *Science* **321**, 537-541.
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**, 6-23.
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, H. C., Gu, W., Shirayama, M., Youngman, E., Conte, D., Jr and Mello, C. C. (2012). *C. elegans* piRNAs mediate the genome-wide surveillance of germline transcripts. *Cell* **150**, 78-87.
Li, T. and Kelly, W. G. (2011). A role for Set1/MLL-related components in epigenetic regulation of the *Caenorhabditis elegans* germ line. *PLoS Genet.* **7**, e1001349.

Lim, J. P. and Brunet, A. (2013). Bridging the transgenerational gap with epigenetic memory. *Trends Genet*. **29**, 176-186.
Lorenz, K. (1935). Der kumpan in der umwelt des vogels, der artgenosse als auslosendes moment sozialer verhal-tungsweisen. *J. Orn. Lpz*. **83**, 137-213; 289-413.
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*. *EMBO J*. **31**, 3422-3430.
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 Genet*. **6**, e1001091.
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.
Remy, J. J. (2010). Stable inheritance of an acquired behavior in *Caenorhabditis elegans*. *Curr. Biol*. **20**, R877-R878.
Remy, J. J. and Hobert, O. (2005). An interneuronal chemoreceptor required for olfactory imprinting in *C. elegans*. *Science* **309**, 787-790.
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. *Dev. Cell* **27**, 656-663.
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.
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.
Vanfleteren, J. R., Van Bun, S. M. and Van Beeumen, J. J. (1989). The histones of *Caenorhabditis elegans*: no evidence of stage-specific isoforms. An overview. *FEBS Lett*. **257**, 233-237.
Vastenhouw, N. L., Brunschwig, K., Okihara, K. L., Müller, F., Tijsterman, M. and Plasterk, R. H. (2006). Gene expression: long-term gene silencing by RNAi. *Nature* **442**, 882.
Vella, M. C. and Slack, F. J. (2005). *C. elegans* microRNAs. In *WormBook* (ed. The *C. elegans* Research Community, WormBook). Available at: http://dx.doi.org/doi/10.1895/wormbook.1.26.1.
Wedeles, C. J., Wu, M. Z. and Claycomb, J. M. (2013). Protection of germline gene expression by the *C. elegans* Argonaute CSR-1. *Dev. Cell* **27**, 664-671.
Wenzel, D., Palladino, F. and Jedrusik-Bode, M. (2011). Epigenetics in *C. elegans*: facts and challenges. *Genesis* **49**, 647-661.
Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.