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PROBLEMS & PARADIGMS

Prospects & Overviews

Inheritance and maintenance of small RNA-mediated epigenetic effects

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Abstract

Heritable traits are predominantly encoded within genomic DNA, but it is now appreciated that epigenetic information is also inherited through DNA methylation, histone modifications, and small RNAs. Several examples of transgenerational epigenetic inheritance of traits have been documented in plants and animals. These include even the inheritance of traits acquired through the soma during the life of an organism, implicating the transfer of epigenetic information via the germline to the next generation. Small RNAs appear to play a significant role in carrying epigenetic information across generations. This review focuses on how epigenetic information in the form of small RNAs is transmitted from the germline to the embryos through the gametes. We also consider how inherited epigenetic information is maintained across generations in a small RNA-dependent and independent manner. Finally, we discuss how epigenetic traits acquired from the soma can be inherited through small RNAs.

KEYWORDS

chromatin, epigenetic inheritance, epigenetics, RNA interference, small RNAs, transgenerational inheritance

INTRODUCTION

Genetic and epigenetic inheritance regulates the passing of traits from parents to offspring. Genetic inheritance controls most inherited traits and relies on the transmission of genetic information using DNA as a carrier. Epigenetic inheritance, instead, has been initially described as the study of heritable changes in gene expression that are not caused by changes in the DNA sequence.^[1] A more recent definition of epigenetic inheritance is “gene expression changes that are mutation independent and heritable in the absence of the triggering event.”^[2] According to both definitions, epigenetic inheritance is not caused by changes in the DNA sequence. Therefore, studies spanning the last two decades have focused on identifying and characterizing the molecules responsible for the epigenetic inheritance of traits. Small noncoding

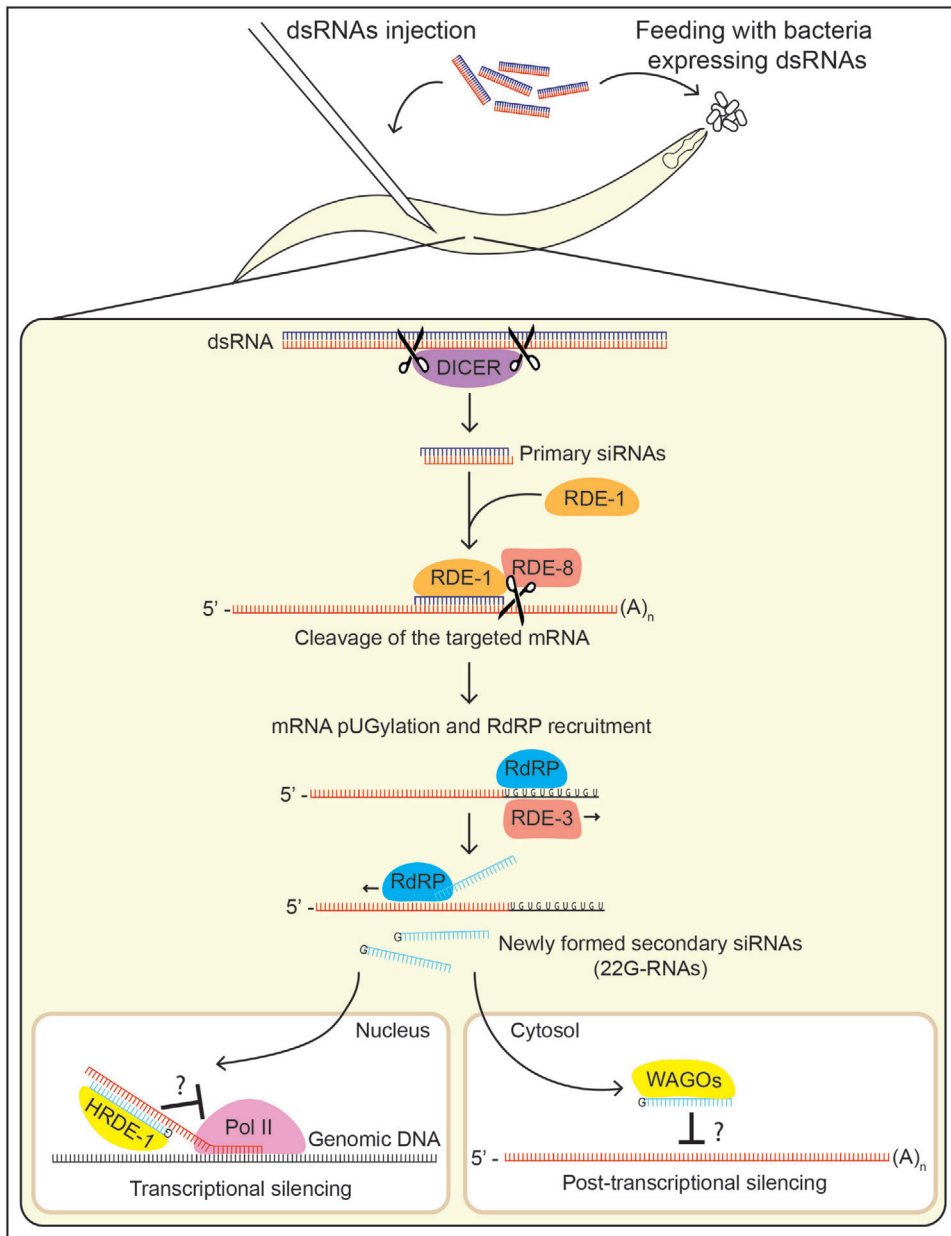
RNAs have been identified as epigenetic factors, together with histone modifications and DNA methylation.^[3–5] Pioneering studies of the RNA interference (RNAi) phenomenon in the nematode *Caenorhabditis elegans* first revealed the potential role of small RNAs in epigenetic inheritance. Worms injected with double stranded RNAs (dsRNAs) complementary to an mRNA target displayed the silencing of the gene locus even in offspring not exposed to dsRNAs^[6,7] (see Box 1). The discovery of heritable RNAi opened the road to the idea that small RNAs could be transmitted and propagated over generations to maintain the epigenetic silencing of the targeted gene. For this reason, the phenomenon of the RNAi, which is conserved among most eukaryotes,^[8] has been extensively used in the past two decades as a model system to investigate the mechanisms of RNA-mediated epigenetic inheritance effects.

In addition to heritable RNAi responses, the inheritance of complex traits, such as fertility, immune responses to pathogen infection, different types of stresses, and learned behavior, are controlled by

Abbreviations: dsRNAs, double-stranded RNAs; EV, extracellular vesicle; PTM, post-translational modification; RdRP, RNA-dependent RNA polymerase; RNAi, RNA interference; TE, transposable element; WAGO, worm-specific Argonaute protein

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Box 1. RNA interference in the nematode *C. elegans*

The phenomenon of RNA interference (RNAi) is induced in nematodes upon the ingestion of double stranded RNAs (dsRNAs) from bacteria or by direct injection of dsRNAs into the body of the worms. The endoribonuclease enzyme Dicer cleaves the dsRNAs inside *C. elegans* cells to release small interfering RNAs (siRNAs).^[10,11] The loading of siRNAs into the conserved family of Argonaute proteins allows the targeting of a complementary mRNA and induces its repression. In *C. elegans*, the Argonaute protein RDE-1 loads the siRNAs derived from dsRNAs but does not directly trigger the silencing of the complementary targets.^[12,13] RDE-1 recruits an endoribonuclease, RDE-8, which cleaves the mRNA target.^[14] Subsequently, the enzyme RDE-3 adds stretches of uridine (U) and guanosine (G) ribonucleotides to the 3' termini of the cleaved mRNA target, resulting in a pUGylated mRNA.^[15,16] Next, an RNA-dependent RNA polymerase (RdRP) recognizes pUGylated mRNA fragments and generates small single-stranded RNAs of 22 nucleotides in size, called 22G-RNAs, which are all antisense to the pUGylated mRNA target.^[15] The RdRP synthesis of 22G-RNAs serves to amplify the silencing signal initiated by siRNAs derived from dsRNAs.^[17,18] In *C. elegans*, there is an extended clade of Argonaute proteins known as Worm-specific Argonautes (WAGOs). The 22G-RNAs produced by the RdRP are loaded into WAGOs, which are the effector of the silencing responses.^[17,18] The WAGOs loaded with 22G-RNAs repress the expression of the mRNA target at the post-transcriptional and the transcriptional level. The transcriptional silencing is usually associated with the deposition of histone post-translational modifications (PTMs) (reviewed in Ref. [19]). Once initiated, the silencing can persist for multiple generations without a dsRNA trigger.^[6]

heritable small RNAs (reviewed in Ref. [5]). The capacity of small RNAs to be delivered to the embryo by the gametes and their ability to propagate and maintain the epigenetic responses across generations is an essential aspect of the epigenetic inheritance processes controlled by small RNAs. The delivery of small RNAs from the germline to the embryo employs different mechanisms, which ultimately maintain the heritable small RNA responses across multiple generations. Mechanisms regulating the soma to germline transfer of small RNAs also participate in the inheritance of somatic responses to different stimuli across generations.^[5,9]

This review focuses on the different strategies used by eukaryotic organisms to deliver small RNAs from the germline to the progeny. We discuss active mechanisms that participate in maintaining the heritable information transmitted by the small RNAs delivered to the embryos. We also highlight small RNA-independent mechanisms for maintaining heritable epigenetic signals in the offspring and how somatic small RNAs can trigger heritable epigenetic responses.

POSSIBLE MECHANISMS OF SMALL RNA TRANSMISSION TO THE NEXT GENERATION

Various examples of inherited acquired traits are controlled by heritable small RNAs (reviewed in Ref. [5]). The transfer of small RNA from parent to offspring requires small RNAs to be packaged in gametes and directly transmitted to the embryo. Gametes can directly acquire these small RNAs from germline and somatic tissue. How small RNAs are packaged in the gametes is also very important as unbound small RNAs are rapidly depleted upon loss of AGO proteins.^[10,11] In addition, many cellular factors and nucleases can also contribute to degrading small RNAs.^[12–17] Therefore, organisms might have evolved mechanisms to protect functional small RNAs to ensure their delivery via gametes to the next generation and avoid their degradation and loss. In the current section, we discuss recent advances in the field to elucidate different modes of small RNA transfer employed by animals.

Delivery of small RNAs protected in phase-separated germ granules

In most animals, the germline is characterized by the presence of phase-separated condensates known as germ granules or nuage in *Drosophila*, sponge, balbiani, and chromatoid bodies in mature gametes (reviewed in Ref. [18–20]). Germ granules are composed of RNA and proteins. Structural proteins within germ granules include Vasa and other helicases of the DEAD-box RNA helicase family and are conserved across species.^[21–26] Another conserved group of germ granule residents includes proteins involved in small RNA pathways such as DICER, Argonaute family proteins along with small RNAs.^[27–35]

In mammals, the germ granules dissolve in mature oocytes and are re-established de novo in primordial germ cells.^[18,36] On the other hand, in the worm, fruit fly, frog, and fish, germ granules are present throughout the development of gametes and are maternally deliv-

ered to the embryo.^[18] Therefore, the inheritance of germ granules might serve to transmit small RNAs from the germline to the embryos. The role of germ granules in small RNA inheritance has been extensively studied in the nematode *C. elegans*. Germ granules are localized peri-nuclear in the developing germline and detach from the nuclear envelope in mature oocytes.^[37,38] Upon fertilization, germ granules polarize to the cytoplasmic region destined to form germline precursor cells. After the 16-cell stage, the germ granules regain their perinuclear localization^[38] coincidentally with the zygotic genome activation of the germline precursor cell. The transfer of germ granules to the embryo also facilitates the transfer of the small RNA pool to the embryo.^[34,39–41] Embryos having mutations in germ granule components exclusively involved in the formation of zygotic germ granules fail to transmit heritable small RNA silencing responses, suggesting that the germ granules in embryos are responsible for small RNA heritability.^[40] Since translation and RNA degradation does not occur in germ granules,^[42,43] they might constitute a protective environment to prevent small RNA degradation and ensure their maternal delivery to embryos.

C. elegans germ granules also harbor the enzymes involved in small RNA amplification, such as the RNA-dependent RNA polymerases (RdRP) along with Argonautes in addition to conserved proteins like Vasa.^[18,25] They also contain proteins involved in the mRNA pUGylation, another heritable RNA intermediate molecule capable of triggering transgenerational silencing^[44,45] (see Box 1). These enzymatic activities and the Argonaute proteins are spatially separated and occupy distinct compartments in the germ granules.^[34] *C. elegans* germ granules have three distinct compartments: the P granules, the Z granules, and the mutator foci.^[34] Most *C. elegans* Argonaute proteins and RNA binding proteins including the PGL family of proteins and GLH proteins (VASA homologs) localize to the P granule. P granule components, including GLH-1, and the nuclear Argonaute HRDE-1, which promotes transcriptional silencing, are required for the transgenerational inheritance of RNA silencing responses.^[39,46–48] Due to the proximity of germ granules to the nuclear pores and the loading of RdRP-dependent small RNAs exclusively from exonic regions by the nuclear Argonaute HRDE-1, it is plausible that the nuclear Argonaute can shuttle between the nucleus and germ-granule to load small RNAs. The Z granule contains two factors, ZNFX-1, and the Argonaute WAGO-4, which are essential for the inheritance of post-transcriptional silencing effects in the embryo, complementing the nuclear function Argonaute HRDE-1, which transmit heritable transcriptional silencing.^[34,49] The mutator foci contain proteins involved in small RNA synthesis, including the RdRP and factors involved in RNA pUGylation, an RNA substrate that recruits RdRP to generate small RNAs essential for heritable RNAi.^[44,45,50] Therefore, besides being a vehicle for maternal small RNA inheritance in the zygote, germ granules can also be necessary for reinitiating the de novo synthesis of small RNAs in embryos (Figure 1).

In *Drosophila*, the Argonautes Aubergine (Aub) and AGO3 involved in the amplification of PIWI-interacting RNAs (piRNA) (reviewed in Ref. [51, 52]) are maternally inherited to silence transposons.^[53–55] Aub and Ago3 localize in phase-separated germ granules known as nuage.^[32,51,52,56] Contents of nuage are transmitted to oocytes from

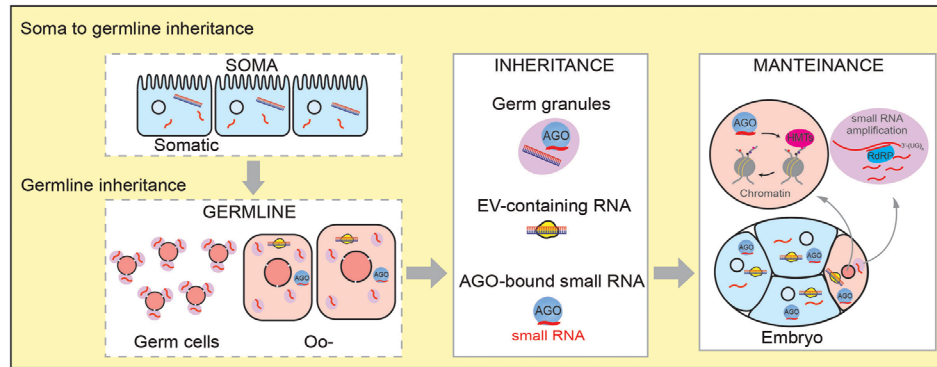


FIGURE 1 Schematic of small RNA-mediated epigenetic inheritance based on *C. elegans* studies but applicable to other organisms as well. Gametes containing phase-separated germ granules, EVs, and Argonaute (AGO) loaded with small RNAs can transmit to the progeny germline derived RNA molecules in the form of small RNAs, dsRNAs, or other RNA silencing intermediate molecules, such as pUGylated mRNAs (see Box 1). These RNA molecules can serve to propagate germline traits or even somatic traits. In the case of somatic trait transmission, an exchange of RNA molecules between somatic and germ cells must occur. Alternatively, somatic cells might directly or indirectly influence the production of small RNAs in the germline. The inherited RNA molecules in the developing embryo can act in somatic blastomeres to trigger somatic gene expression changes that ultimately lead to the transmission of somatic phenotype. In parallel, the inherited RNA molecules must be maintained in germline blastomere and develop germline to be propagated across multiple generations. Two possible maintenance mechanisms are the amplification and de novo production of small RNAs through RdRP enzymes like in *C. elegans* (see Box 1) or other small RNA amplification mechanisms such as the piRNA ping-pong cycle in *Drosophila*

nurse cells by yet unknown mechanisms, leading to the formation of pole plasm in oocytes.^[57] This transport of nuage content to germplasm of oocyte most likely facilitates the transfer of piRNA and Argonaute proteins from mother to embryo.

In zebrafish, PIWI protein known as ZIWI is maternally provided and localizes to nuage^[58] and loss of ZIWI leads to germ cell apoptosis.^[58] Additionally, about 180 miRNAs belonging to 109 different families were found to be maternally inherited in the embryo. However, their role is still uncharacterized,^[59] and it is also unknown how they are delivered or if nuage plays any role in their inheritance.

These examples from different species highlight the functional conservation of the phase-separated germ granule components and their involvement in delivering functional small RNAs from the germline to the embryo. Therefore, one possible function of germ granules is to create a proper environment to preserve functional small RNAs for their inheritance (Figure 1). Moreover, multiple Argonaute proteins reside in germ granules. Thus, the granule environment might confer the proper balance and loading of small RNAs into the different Argonautes, essential for their functions.

Delivery of AGO-bound small RNAs to the embryo

In mice, both P-bodies and germ granules are known to dissolve in mature oocytes and are re-established de novo in primordial germ cells two days post specification.^[18,36,60,61] This brings in an important question of how the small RNAs are transmitted from the germline to the embryo. Argonaute proteins that localize to germ granules in the germline are also maternally inherited despite the dissolution of germ granules in mature oocytes, raising the possibility that small RNAs could be inherited in an AGO-bound state to facilitate their

delivery to the embryo.^[62] The maternally provided Argonaute AGO2 is essential for early embryogenesis in maternal to zygotic mRNA degradation.^[63,64] Furthermore, AGO2 protein localizes with P-bodies marker DCP1A, which is identical to peri-nuclear granules^[64] that are assembled de novo in the embryo.^[18] Therefore, small RNAs may be inherited from germline to embryo in an AGO-bound state and then eventually segregate again to the P-bodies in the embryo.

Similarly, the *C. elegans* Argonaute CSR-1 loads small RNAs antisense to most germline genes,^[27,65] which are maternally delivered to the embryo in an AGO-bound state in the cytosol of the embryo via the oocyte.^[62] The maternally inherited CSR-1 and its associated small RNAs facilitate the degradation of maternal mRNAs in somatic blastomeres by CSR-1 slicer activity.^[62] Another *C. elegans* Argonaute WAGO-4, essential for heritable RNAi,^[66] is maternally inherited in the 1-cell embryo before segregating with the germline blastomere inside the germ granules. Interestingly, WAGO-4 binds small RNAs antisense to targets which largely overlaps with CSR-1 and is essential for the inheritance of small RNAs.^[66] These examples suggest that in the absence of continuous germ granules or even in the organisms with functional granules, small RNAs can be inherited directly from the germline to the embryo in an AGO-bound state. This mode of inheritance can function alongside the germ granules and requires further investigations (Figure 1).

TRANSFER OF SMALL RNAs OF SOMATIC ORIGIN FROM GERMLINE TO EMBRYO

The separation of soma from germline, proposed by Weissmann,^[67] implicates that only germ cells contribute to heritable traits. Nonetheless, it is becoming increasingly clear that heritable epigenetic traits

transmitted from parental germline to the embryo originate in response to somatic cell stimuli. This soma to germline transmission of epigenetic information can be mediated by either dsRNA^[6,68] or endogenous small RNAs.^[9,69] Classic examples of these processes include dsRNA transfer from *C. elegans* intestine to the germline during RNAi induced upon feeding with bacteria-expressing dsRNAs. In *Drosophila*, somatic nurse cells control the transfer of nuage components to oocytes germ plasm^[57] and in mouse, epididymosomes originating from epididymis control the small RNA cargo of sperm.^[69,70] These dsRNA or small RNAs transferred from the soma to the germline are further transmitted to the embryo (Figure 1).

Transfer of dsRNA via dsRNA transporters and channels

In *C. elegans*, dsRNA feeding or somatic injection of dsRNA results in heritable RNAi. The incorporation of dsRNA delivered by bacteria feeding in intestinal cells requires the dsRNA transporter SID-2. This membrane protein is responsible for dsRNA uptake in intestinal cells via endocytosis. Subsequently, the dsRNA channel protein SID-1 is required for releasing dsRNA from the vesicles in the germline.^[71-74] The dsRNAs imported in the germline are then transmitted to the embryo, requiring the dsRNA channel protein SID-1 to release dsRNA from vesicles transmitted from germline to embryo.^[75,76] In *C. elegans*, dsRNAs injected in the body cavity of the worm connecting the intestine to the germline colocalize with yolk-containing vesicles.^[77] These vesicles originate from the intestine and might also transfer dsRNAs directly to the oocytes. Even though the presence of dsRNA in yolk vesicles remains controversial,^[71] the receptor for uptake of yolk vesicles is involved in heritable RNAi in the absence of SID-1.^[71,77] Thus, the yolk-containing vesicles can be used as an alternative route for soma to germline dsRNA transfer and small RNA inheritance, which is an interesting proposition suggesting a coupling between the inheritance of traits via dsRNA transfer with maternal delivery of nutrition to the embryo.

In mammals, the membrane expression of the SID-1 homolog, SIDT1, enhances the uptake of siRNA, resulting in increased gene silencing in cell lines.^[78] In mouse, SIDT1 is expressed in the stomach on the apical surface of gastric mucous cells (pit cells) localized to the plasma membrane, where it appears to be involved in miRNA uptake.^[79] SID-2 homolog, SIDT2, is essential for dsRNA uptake upon herpes simplex virus 1 and encephalomyocarditis virus in the cytosol, resulting in the activation of the innate immune system.^[80] SIDT1 has been shown to participate in this process, even though it is not essential.^[81]

Although *Drosophila* lacks any known homolog of SID-1, *Drosophila* S2 cells can internalize the dsRNA by Scavenger Receptor-mediated endocytosis, albeit with lower efficiency.^[82] Scavenger receptor is conserved across species and is essential for the uptake of small RNAs associated with high-density lipoprotein.^[83] Despite the role of SIDT1, SIDT2, and Scavenger receptors in dsRNA uptake, it is unknown if they

can transfer dsRNA from soma to germline in mammals, and it remains an intriguing possibility to explore further.

Transfer of small RNAs via extracellular vesicles

The presence of small RNAs, including miRNAs, in extracellular vesicles (EVs), has been extensively studied in different organisms, including humans (reviewed in Ref. [84, 85]). Here we focus exclusively on the transfer of vesicles from soma to the germline, or from germline to embryo, in the process of trait inheritance in animals. A recent study in *C. elegans* demonstrated the involvement of virus-like particles (VLPs) encoded by the retrotransposon *Cer-1* in small RNA-mediated learned pathogen avoidance and transgenerational inheritance and the horizontal transfer of the avoidance phenotype on exposure to *Pseudomonas aeruginosa* PA-14.^[86] Thus, VLPs might also constitute another way of transferring small RNA-mediated epigenetic information. In addition to dsRNA, *C. elegans* vesicles might also transfer small RNAs and other RNAi intermediate molecules such as pUGylated RNAs from the soma to the germline.

In mammals, the vesicular transport of small RNAs has been extensively documented. During sperm maturation and their transit from the epididymis, there is a massive transfer of proteins and RNA, including small RNA from EVs present in the epididymal fluid known as epididymosomes (reviewed in Ref. [87]). Considering that sperm are transcriptionally silent, the role of epididymosomes becomes critical in establishing the sperm epigenome and in the transfer of paternal epigenetic traits to the progeny, including small RNAs.^[88,89] Sperm show high-level plasticity of small RNAs during their maturation with two waves of piRNA production in testes followed by loss of piRNAs and gain of another class of small RNAs known as tRFs (tRNA fragments derived by tRNA cleavage).^[69] The delivery of small RNAs, including miRNAs, via epididymosomes has been shown to be essential for the mammalian sperm epigenome and subsequent fitness of the embryo under specific conditions.^[69,70,90-93] In fact, the paternal diet and stress has been shown to regulate levels and composition of sRNAs, including miRNAs and tRFs in the sperm, and impact offspring phenotype^[94-97] (also reviewed in Ref. [98]).

In *Drosophila*, accessory gland cells secrete vesicles under the control of bone morphogenetic protein (BMP) signaling, which fuse with sperm after mating. These exosomes play a role in preventing remating by the female for the reproductive advantage of the male.^[99] However, the composition of these vesicles is not known. Moreover, whether these vesicles of somatic origin can deliver epigenetic information to sperm, like in mouse, remains to be determined.

The transport of small RNAs via EVs might provide a stable environment to avoid degradation and control the transfer of small RNAs to the gametes in a temporal manner. However, it is not clear how these transported small RNAs are released from the vesicles and whether they are directly released in the gametes or the embryo. Further studies are required to understand the release of the small RNAs from the vesicles, which may also involve a role for RNA transporters, as seen in the case of dsRNA release in the embryos.^[75,76]

HOW ARE INHERITED SMALL RNAs MAINTAINED ACROSS GENERATIONS?

At the beginning of embryogenesis, animal embryos are transcriptionally silent and are loaded with maternal components, including maternal mRNAs, proteins, and small RNAs. Maternal components are generally degraded with the activation of the zygotic genome^[100] in a process known as the maternal to zygotic transition.^[100] To exert heritable functions, carriers of epigenetic information, such as small RNAs, must propagate their information beyond embryogenesis and possibly until adulthood. Consequently, without a propagation mechanism, maternally inherited small RNAs would be initially diluted and finally degraded during embryonic and postembryonic development, and their effect would be limited to early embryogenesis. *C. elegans* RNAi, for example, can last for more than 80 generations after the exposure to the initial dsRNA trigger if silenced worms are actively selected.^[101] Thus, the RNA silencing effect could be propagated almost indefinitely. This is not limited to the phenomenon of exogenous RNAi. Transgenes targeted by endogenous small RNAs are also silenced transgenerationally.^[102] In addition, changes in the levels of endogenous small RNAs can occur stochastically and can also be inherited for a few generations, although they generally show lower stability compared with transgenes' silencing.^[103]

This section discusses several mechanisms that have evolved to, directly or indirectly, propagate the silencing effect mediated by inherited small RNAs. These involve the amplification of small RNAs and the crosstalk with other epigenetic factors, such as the histone post-translational modification (PTMs).

Active mechanisms in the propagation and maintenance of heritable small RNA responses

Yeast, plant, and nematodes rely on the activity of RdRP enzymes for the amplification of small RNAs. RdRPs, catalyze the synthesis of an RNA molecule using an RNA as a template. They are very ancient enzymes that are essential for the replication of all RNA virus genomes. Most RdRPs are specific to viruses.^[104] However, RdRPs are also present in eukaryotes but not in vertebrates,^[105] and they are evolutionarily related to viral RdRPs.^[106]

RdRPs are involved in the production of secondary small RNAs and participate in several small RNA pathways in a variety of organisms including yeasts, plants, and nematodes (reviewed in Ref. [107–109]).

C. elegans RdRPs play essential roles in amplifying the RNAi responses and generating antisense small RNAs loaded into Argonaute effector proteins (see Box 1). These RdRP-dependent small RNAs are not only produced during RNAi responses but are also triggered by other endogenous small RNAs, such as the piRNAs.^[46,110] The RdRP-dependent small RNAs triggered by piRNAs are propagated across generations even in the absence of piRNAs in *C. elegans*.^[46,102,111,112] In a process similar to the RNAi. During RNAi, primary small RNAs trigger the production of secondary small RNAs that are maternally inherited. Secondary small RNAs can also trigger the de novo produc-

tion of the tertiary small RNAs.^[111] Therefore, the RdRPs can initiate a new cycle of small RNA amplification at each generation, starting from the maternally inherited pool of small RNAs. The amplification of the small RNA pool by the RdRPs plays an essential role in maintaining the silencing signal in the absence of the initial trigger. Interestingly, this transgenerational amplification process depends on the nuclear RNAi pathway^[47,111] which is also involved in the deposition of histone PTMs at the targeted locus.^[113,114] Thus, the chromatin silencing and the nuclear RNAi pathway might be linked to the zygotic production of small RNAs starting from the inherited small RNA pool. However, it is still unclear how the nuclear Argonaute and the chromatin silencing influence the production of heritable small RNAs. Finally, small RNA production across generations can also be triggered by inherited pUGylated mRNAs (see Box 1).^[44,45] The presence of pUGylated mRNAs is sufficient to trigger transgenerational silencing responses, and pUGylated mRNAs are inherited in embryos upon the initial dsRNA-mediated silencing in the parental germline (Figure 1).^[44,45] In addition, the de novo production of pUGylated mRNAs starting from the initial pool of maternally inherited small RNAs might also be required to maintain the transgenerational silencing responses.

In the fission yeast *Schizosaccharomyces pombe*, the RNAi pathway is responsible for the epigenetic maintenance of the constitutive heterochromatic state of pericentromeric repeats.^[109] The working model, reviewed extensively in,^[109] suggests that pericentromeric repeats are transcribed into long noncoding RNAs processed into primary small RNAs and loaded onto AGO-1 within the RNA-induced transcriptional silencing complex (RITS). The RITS binds by base complementarity to nascent noncoding centromeric transcripts and triggers the recruitment of the RdRP. This, in turn, converts the noncoding nascent RNA into a dsRNA that is processed by Dicer to produce new small RNAs that are in turn loaded in the RITS complex. Furthermore, the RITS interacts with a complex containing H3K9 methyltransferase, which triggers the deposition of the heterochromatic H3K9me2/3 histone marks.^[109] A similar feedback loop between small RNAs and H3K9me2/3 participates in maintaining the epigenetic silencing of euchromatic genes over several meiotic and mitotic divisions.^[109] Therefore, the propagation of the epigenetic silencing of euchromatic genes might also depend on a similar RdRP-dependent amplification mechanism.

RdRPs are not conserved in vertebrates,^[105] suggesting that animals lacking RdRPs might have evolved other small RNA amplification strategies. For instance, the amplification of the piRNA-dependent silencing of transposable elements (TEs) in the *Drosophila* germline does not rely on an RdRP-dependent amplification mechanism. Instead, most piRNAs are generated from piRNA clusters, composed mainly of defective transposons, and located in heterochromatic regions. These heterochromatic genomic clusters generate primary piRNAs complementary to sense transcripts of functional TEs located in euchromatic regions. Primary piRNAs loaded into PIWI Argonautes mediate the cleavage of active TEs, producing sense secondary piRNAs that are loaded on other PIWI proteins that cleave TEs from heterochromatic transcripts. Every cleavage generates a new piRNA in

the opposite orientation with this strategy. This process constitutes a piRNA amplification machinery, not relying on RdRPs, named the ping-pong cycle.^[115,116]

Maternally inherited piRNAs are essential to control TEs at each generation in *Drosophila*. Indeed, progeny from crosses between wild-caught males and laboratory-strain females are sterile. In contrast, progeny from crosses between laboratory-strain males and wild-caught females are fertile despite having the same genotype. This phenomenon, known as hybrid dysgenesis, is caused by the mobilization of specific classes of TEs, called I-elements, and it depends on maternally inherited piRNAs and their amplification through the ping-pong cycle.^[53,55,117]

The presence of an active small RNA amplification machinery allowed some organisms to rely on a constant amplification of small RNAs to maintain RNAi-mediated silencing over generations. However, besides the RdRPs in *C. elegans* and the piRNA ping-pong cycle in flies, other active mechanisms to propagate small RNA-mediated epigenetic silencing over generations remain to be determined.

Maintenance of heritable silencing by small RNAs and histone PTMs

Histone PTMs also control epigenetic heritable responses. Indeed, even if histone PTMs undergo a global erasure in gametes and embryos, some loci retain histone PTMs, and these are important for epigenetic inheritance (reviewed in Ref. [4]). Therefore, histone PTMs constitute one of the vehicles of epigenetic information transmitted across generations. Furthermore, active and repressive histone modifications are transgenerationally inherited in several organisms, including mammals, nematodes, and flies (reviewed in Ref. [3, 118]). In plants, yeasts, and animals, small RNAs can initiate the deposition of histone PTMs. For example, in *Arabidopsis thaliana*, small RNAs direct the deposition of H3K9me2 in embryos, and this process is essential for epigenetic reprogramming.^[119] During *C. elegans* RNAi, the nuclear Argonaute protein HRDE-1 promotes the deposition of the repressive marks H3K9me3,^[114] H3K27me3,^[113] and H3K23me3.^[120] These histone PTMs are also crucial for the transgenerational inheritance of the RNA silencing responses. Worms lacking SET-25, the histone methyltransferase responsible for the deposition of H3K9me3, fail to inherit the RNAi silencing.^[121] However, the lack of the main methyltransferases responsible for H3K9 dimethylation seems to differently impact the transgenerational inheritance of RNAi depending on the target gene.^[122] Therefore, small RNAs could initiate silencing maintained by repressive histone PTMs in a small RNA-independent manner. In this scenario, the transcriptional and post-transcriptional gene silencing triggered by small RNAs in the parental generation can lead to the deposition of heritable histone PTMs. The transmission of small RNA-mediated histone PTMs in animals lacking an active small RNA amplification machinery might be sufficient to maintain the silencing over generations in the absence of small RNAs. Alternatively, maternally inherited small RNAs might trigger the deposition of new histone PTMs in the embryo after epi-

genetic reprogramming and ultimately maintain the silencing in the progeny.

THE TRANSMISSION OF ACQUIRED TRAITS BY GERMLINE INHERITED SMALL RNAs

The gametes are the cells responsible for propagating genetic and epigenetic information to the zygote. Given that the gametes are produced in the germline, this tissue must be responsible for heritable genetic and epigenetic information. Also, epigenetic information acquired from somatic tissue might be transmitted to the zygote by the gametes. Despite the striking observation that somatically acquired traits, such as responses to different stresses or pathogens, are heritable, it is still challenging to have a precise molecular understanding of these processes. As discussed in previous sections, epigenetic factors such as small RNAs can move across separate cellular compartments and between different tissues. Thus, somatic small RNAs transmitted from the soma to the germline can ultimately be inherited in the embryo. However, inherited small RNAs should signal back to the soma to propagate the somatic phenotype across generations. Moreover, the simultaneous propagation of small RNAs in the germline might be required to maintain the inherited somatic phenotype.

For instance, the epigenetic inheritance of neuronal traits, such as the transmission of the learned avoidance of a pathogenic bacterium in *C. elegans*, is controlled by a germline small RNA pathway.^[86,123,124] A bacteria encoded small RNA is processed in the intestine and can produce gene expression changes in the neuron.^[124] The germline piRNA pathway controls the inheritance of this phenotype for up to four generations.^[124] Another study in *C. elegans* showed that neuronal small RNAs could potentially change the abundance of small RNAs in the germline.^[125] Interestingly, some of these small RNAs with altered expression are inherited for up to three generations and can influence gene expression changes that ultimately influence chemotaxis behavior.^[125]

Although the molecular mechanisms of inheritance of acquired traits in the somatic tissue are still unclear, these observations suggest that animals have evolved such types of inheritance that constitute a selective advantage, especially in challenging environments, such as pathogen exposure.

FUTURE DIRECTIONS

It is now widely accepted that small RNAs are among the inherited maternal components and that, together with germ granules, are crucial for the first phases of embryogenesis and the process of epigenetic inheritance. However, details on the mechanism and mode of small RNA inheritance and their maintenance in the offspring are only beginning to be understood.

There are many unresolved questions in the field. How animals lacking active amplification machinery can maintain small RNAs across multiple generations is still unclear. Future work should focus on under-

standing if histone PTMs might work in a small RNA-independent manner in these organisms to overcome the absence of small RNA amplification pathways. Ultimately, the involvement of small RNAs in the inheritance of acquired traits in *C. elegans* suggests that small RNAs might act as a carrier of information from different tissues to enable the transgenerational transmission of such traits. The identification of specific mechanisms of small RNA delivery from the soma to the embryo, including EVs, supports this hypothesis. Identifying the molecular mechanisms that allow the small RNA-dependent communications between the soma and the germline will be a key aspect to begin to appreciate the complex molecular exchange of information acquired during our lifetime between different tissues and organs, which is ultimately transferred to the next generation. Finally, the investigation of these molecular mechanisms in ecologically relevant species possibly in their natural context might contribute to shedding some light on the evolution of epigenetic inheritance mechanisms beyond model organisms.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

PQ, MS, and GC conceived the review topic and wrote the manuscript. LB prepared the figures with the contribution of all the authors.

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