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The soft genome

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Caenorhabditis elegans (_C. elegans_) nematodes transmit small RNAs across generations, a process that enables transgenerational regulation of genes. In contrast to changes to the DNA sequence, transgenerational transmission of small RNA-mediated responses is reversible, and thus enables "soft" or "flexible" inheritance of acquired characteristics. Until very recently only introduction of foreign genetic material (viruses, transposons, transgenes) was shown to directly lead to inheritance of small RNAs. New discoveries however, demonstrate that starvation also triggers inheritance of endogenous small RNAs in _C.elegans_. Multiple generations of worms inherit starvation-responsive endogenous small RNAs, and starvation also results in heritable extension of the progeny's lifespan. In this Commentary paper we explore the intriguing possibility that large parts of the genome and many additional traits are similarly subjected to heritable small RNA-mediated regulation, and focus on the potential influence of transgenerational RNAi on the worm's physiology. While the universal relevance of this mechanism remains to be discovered, we will examine how the discoveries made in worms already challenge long held dogmas in genetics and evolution.

The Permeability of the Weismann Barrier

The classical view held following the formulation of the Modern Synthesis, which combined population genetics with the ideas of Mendel and Darwin, was that the environment couldn't directly shape the genetic makeup of an organism. The surrounding's only contribution to evolution is in determining the forces of selection.[1] An immediate implication of this conviction, which became somewhat outdated due to many developments in the study of epigenetics, is that the door is shut on the possibility of inheriting acquired traits; evolution is "blind," "clueless," and "directionless."

One of the major reasons for the assumption that the interactions with the world cannot directly affect the following generations, hypothesized by Friedrich Leopold August Weismann in the late 19th century,[2] is that the germline was thought to be segregated by a theoretically impermeable barrier.[3] Acceptance of Weismann's rule (the "Weismann Barrier") dictates that: "*genetic information cannot transfer from the soma to the germline.*"[4]

A polemic discussion over the existence and "permeability" of this barrier stirred the scientific community when the modern theory of evolution was framed. However, due to both scientific and even political reasons, eventually Weismann's principle was recognized as a fundamental truth, and became commonly known as "the second law of biology."[4]

Nevertheless, recent research has uncovered epigenetic phenomena (heritable changes that arise independently from changes in the DNA sequence), and in particular mechanisms of RNA interference (RNAi) in *Caenorhabditis elegans* nematodes, which offer an explanation to how the barrier may be breached, allowing interactions between the soma and the environment to alter heredity, in both transient and stable ways.[5] As extensive reviews have very recently elaborated on RNAi mechanisms and RNAi inheritance ,[6-11] we will only highlight some milestone findings, while our focus will be to examine the unknown: the potential of

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transgenerational silencing in challenging the current conceptual limits of heredity.

There are still many "unknowns" in the field of epigenetic inheritance; we wish to draw the reader's attention to the 4 fundamental questions that need to be answered, and that will be highlighted throughout the text:

1. Does the inheritance of a specific non-DNA agent (RNA, chromatin, other) precedes, or establishes, the inheritance of the other epigenetic marks? ("Which comes first?")
2. How, to what extent, and depending on which factors, can epigenetic information transfer between the soma and germline?
3. Which environmental conditions can induce the biogenesis and inheritance of small RNA, and what is typical to these environments?
4. How is transgenerational information maintained, or reset?

From Soma to Germline in *C. elegans*

In 1998 Fire and Mello showed that double strand RNA (dsRNA) catalyzes gene silencing in *C. elegans*, and that the silencing effect of dsRNAs which are injected to different worm somatic tissues spreads systemically, and affects non-treated progeny.[12] Soon after, it was shown that RNAi could be induced simply by feeding worms on bacteria which express dsRNA,[13] and moreover that the silencing is maintained in the next generation as well. Thus, silencing can pass from digested bacteria in the gut, to other non-gut tissues, including the germline, in clear defiance of Weismann's role.

In worms, dsRNA transporters (most notably the Systemic Interference Deficient gene, SID-1) and proteins that are involved in trafficking of vesicles are required for systemic transfer of small RNAs and acquisition of dsRNA from the environment (e.g. SID-2).[14] However, the details regarding the process that leads RNAi from the soma to the germline are not clear, and the factors that mediate such transfer have not been identified. Specifically, it is still not known whether SID-1, which is the best-studied gene involved in systemic RNAi, is required for soma to germline small RNA transfer or transgenerational RNAi. Until now, in worms, only silencing effects that are triggered via exogenous small RNAs where directly shown to transfer between cells. A direct demonstration of cell-to-cell transfer of endogenous small RNAs is still missing in *C. elegnas*, and it is not known whether SID-1 is involved in the process, assuming that it takes place. In different organisms, including humans, additional methods for spreading RNAi among cells have been found, and certain endogenously-produced small RNA species, for example microRNAs, transfer across tight cell-cell connections (e.g. immunological synapses), and also systemically via vesicles such as exosomes.[15,16]

Transgenerational Inheritance of RNAi

In worms, in addition to silencing genes in the non-treated offspring, RNAi against a minority of the genes tested (13/171, 7.6%),[7] and mostly against germline-experssed genes, can produce a long lasting effect. Here we define "long lasting effects" as RNAi effects which last longer than 2 generations. Our definition is based on the fact that only effects that last longer than 2 generations can be considered as truly "transgenerational" and not "intergenerational." The exposure of the third generation's germ cells, while in the mother, to the original RNA trigger can be ruled out, since the heritable agents will be diluted by a factor of millions. The Fire lab constructed a sensitive test to assess the durability of heritable RNAi, using injected RNAi that targets a temperature sensitive dominant allele of *oma-1*. In these experiments it was discovered that RNAi usually peters out after 2-3 generations, but in some cases can last for at least 7 generations.[17] Another experiment from the Plasterk group showed that RNAi against some genes was essentially stable, lasting for more than 80 generations.[7] Both eggs and sperm can transmit heritable RNAi, and importantly, RNAi inheritance occurs even in the absence of the DNA template of the target gene.[18]

How can dsRNA-induced silencing persist in worms for multiple generations in spite of a theoretically huge dilution effect? (each worm lays more than 200 eggs on average). Multigenerational RNA-based silencing responses are enabled granted to the action of complementary mechanisms. First, an RNA-dependent RNA polymerase (RdRP)-mediated amplification mechanism generates new ("secondary") small RNAs in every inheriting generation.[19,20] Second, inherited RNA molecules affect the DNA by directing chromatin modifications, which can shut down transcription, and augment the persistency of the silencing effect[7] (**Fig. 1**). Since this paper is focused on small RNA inheritance, we point the reader to comprehensive reviews that discuss Chromatin-mediated inheritance, and other transgenerational epigenetic mechanisms in other systems, most notably in fungi and plants[8,21,22] (see also **Text Box 1**).

RNA and chromatin inheritance are not mutually exclusive processes; on the contrary, small RNA, chromatin, and DNA methylation-based epigenetic phenomena have been shown to be at least partly interdependent in a variety of organisms.[22] In worms, RNAi and chromatin-based gene regulation is coordinated by nuclear argonaute proteins that carry 22G small RNAs in the germline, HRDE-1 (Heritable RNAi Deficient-1), and CSR-1 (Chromosome-Segregation and RNAi deficient-1), and NRDE (Nuclear RNAi Deficient) proteins, as detailed elsewhere.[6]

Both small RNAs and chromatin marks are detectible in the progeny of worms that are treated with RNAi. One study found that the heritable small RNAs are detectable before the chromatin marks, and thus suggested that small RNAs are the primary heritable material, while the chromatin marks are reconstructed *de novo* in every generation.[23] The question of whether heritable small RNAs precede heritable chromatin marks or vice versa is a hotly debated issue, which required more research before it can be resolved. The

current models for epigenetic inheritance which take into account feed forward interactions between RNAi factors and chromatin marks and an association between RNAi factors and nascent RNA transcripts (an interface which enables the recruitment of RNA-binding proteins that carry "guiding" small RNAs to the DNA, where interaction with chromatin-modifiers and modification of the chromatin can take place) rely heavily on insights from other organisms, especially fission yeast.[24] However, regardless of the exact mechanism, and "who came first," it is clear that both of these processes act together, and that both are important for epigenetic inheritance in worms as well.

Until very recently it was not clear whether transgenerational transmission of RNAi enables inheritance of physiologically relevant acquired traits, or whether it is triggered only by administration of exogenous dsRNA. However, the discovery of dedicated argonaute proteins that specifically affect inherited RNAi, and not RNAi *per se* (HRDE-1,[25]), and traits that are affected by heritable small RNAs (see below), suggests that *C.elegans* nematodes breach the Weismann barrier in order to complement their genome in an adaptive fashion; Indeed, as described below, heritable small RNAs enable progeny to remember their ancestors' reactions to environmental challenges.

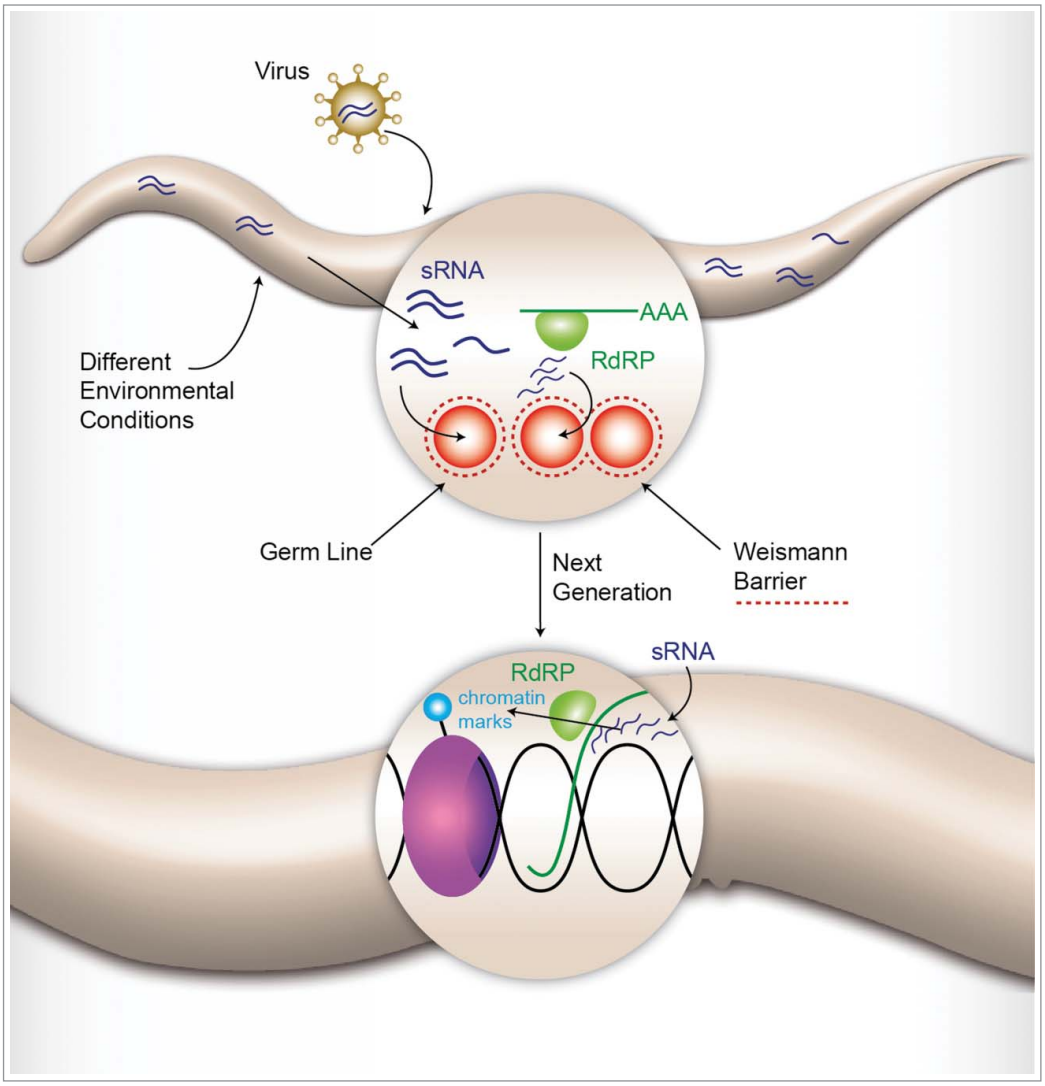

Figure 1. RNAi acts systemically in *C. elegans* nematodes. Moreover, the RNAi response is amplified via the action of RdRPs, and involves also chromatin remodeling. Transfer of RNAi to the germline produces a heritable response that is re-initiated in the next generations.

Inheritance of Adaptive Immunity via Transgenerational RNAi

Inherited RNAi was recently linked to several immunological functions.[26] Worms are remarkably resistant to viruses because anti-viral small RNAs (viRNAs) destroy viruses with great efficiency.[27] Acquired viRNAs, which are amplified by RdRPs, are heritable for multiple generations, and serve as "Inherited vaccines," to provide the progeny with innate anti-viral protection.[20,26,28,29] In addition, heritable small RNAs protect the genome from other intruding elements, such as transposons, by permitting only the expression of endogenous genes. The logic for distinguishing "self" from "foreign" is based on transgenerational memory: genes that should be expressed in the germline are "marked" with heritable endogenous small RNAs that are carried over by the nuclear argonaute CSR-1, and correspond to the previous generations' germline-expressed genes.[30,31] If a gene is transcribed in the germline without such heritable "RNA licensing"[32,33] the RNAi system recognizes it as "foreign" (as a parasitic mobile element) and silences it by producing heritable PIWI-interacting small RNAs (piRNAs) which shut off the gene's expression at the chromatin level.[25,32,34,35] Experimental elimination of these heritable endogenous small RNAs resulted in a "mortal germline" and infertility phenotypes.[25,31] Thus, different small RNA species protect the genome by acting as

heritable "Guest" and "Black" lists, which restrict expression in the germline.[26]

Starvation-Induced Small RNA Inheritance

Can *C. elegans* use heritable small RNAs to regulate different endogenous signaling cascades, and thus to propagate long lasting adaptive physiological responses to challenges, or is the use of RNA inheritance restricted for immunological functions?

As mentioned above, until very recently RNA inheritance was shown to ensue only as a response to the introduction of foreign genetic elements (viruses, transposons, transgenes), which supply the substrate from which small RNAs could be produced. Natural environmental conditions that could trigger the production of endogenous small RNAs are poorly characterized. We were inspired to examine the potential of starvation to induce the production of small RNAs, and to lead to epigenetic effects, since transgenerational effects in response to different diets were shown to occur in diverse animal models, and importantly also in humans, see.[36-38] We therefore tested whether heritable small RNAs arise in *C. elegans* following L1 starvation, and whether a heritable physiological effect can be detected.[39] We hypothesized that L1 arrest would dramatically change the worm's endogenous small RNA pools, and thus the levels of small RNAs that could potentially be inherited, because more genes change their expression during L1 starvation than throughout the entire course of larval development.[40] In line with our hypothesis, we observed significant changes in the levels of primary endogenous small RNAs (26Gs) and secondary small RNAs (22G) in adults that experienced severe starvation as L1s (6 d without food). In addition, we found 31 genes that were putative targets of both 26Gs and 22Gs, which were differentially expressed following starvation.[39]

Crucially, we were able to detect a large and highly statistically significant degree of similarity between the small RNA pools of adult animals that experienced starvation as L1s, and the small RNA pools of their F3 progeny that grew *Ad libitum* for 3 consecutive generations. We focused on the inheritance of 22G Small RNAs as this small RNA species is amplified by RDRPs, and is known to be the cargo of the argonatues that carry the heritable RNAi signal, HRDE-1 and CSR-1.[39] The similarity between the small RNA pools of the fed F3 worms and their ancestors that experienced starvation as L1s stemmed primarily from the similarity between the pools of their 22G small RNAs. We examined clusters of 22G small RNAs that align in the antisense orientation to particular genes (Small RNAs that Target specific Genes, or "STGs"). Similar changes in 22G expression was observed for 26.3% of the STGs that were upregulated in the P0 generation following starvation (152/578, p-value < 1.399e-71), 52% of the downregulated STGs (311/597, p-value < 1.256e-292), and 91% of the 22Gs that overlap with 26G STGs (p-value < 5.629e-29, fold-enrichment=26.1).[39]

When we examined the putative targets of the heritable 22G STGs, we detected a strong enrichment for genes that are involved in nutrition-related functions. Transgenerational regulation of these genes could in theory prepare the progeny for additional hungers. Examination of mRNA levels of genes that were suspected to be targets of heritable 22Gs suggested that in the F3 generation, germline-expressed genes that were previously shown to bind CSR-1 (in immunoprecipitation experiments), increase their expression, while HRDE-1-targets appear to lower their expression.[39]

It is not yet clear whether starvation initiates small RNA production in the soma or in the germline. However, production of the starvation-responsive small RNAs and regulation of their putative mRNA targets was abrogated in *rde-4* mutants (a dsRNA-binding protein, which acts upstream in endo-siRNA production). The heritable changes in the levels of both the starvation-induced small RNAs and their mRNA targets was abolished in *hrde-1* mutants.[39]

Interestingly, another group recently showed that exposing worms to brief periods of starvation is enough to reset the accumulative transgenerational sterility that results when *prg-1* mutants are cultivated in 25 degrees (PRG-1 is required for biosynthesis of piRNAs, another small RNA species).[41]

In addition to the small RNA inheritance that ensues following starvation, we observed that the lifespan of animals that derive from starved great-grandparents is longer than the lifespan of animals whose great-grandparents were continuously fed.[39] Previous experiments have already linked longevity with epigenetic inheritance. Greer et al. have shown that inheritance following incomplete reprogramming of chromatin states (in wild type descendants of mutants for ASH-2, WDR-5 or SET-2, which compose the histone H3 lysine 4 trimethylation complex) affects the lifespan of the progeny.[42] More research is required in order to understand whether the regulation of genes by inherited small RNAs is causing the heritable increased longevity that is observed upon starvation, and following perturbation of transgenerational chromatin reprogramming.

It is important to understand which other environmental conditions are capable of initiating transgenerational responses, and L1 starvation could be an important case study to learn from. How does starvation trigger the production of specific endo-siRNAs? One intuitive possibility is that during L1 arrest cognate mRNAs, which are transcribed in response to starvation, serve as templates for the synthesis of specific endo-siRNAs.[39] For example, bidirectional transcription could produce dsRNA that would then be processed into small RNAs.[39] In theory, every environmental condition that would elicit the production of small RNAs that are capable of reaching the germline, or that would affect the pool of already germline-expressed small RNAs, could result in transgenerational effects.

Combining the "Soft" and "Hard" Genomes

Here we suggest that heritable RNA molecules give rise to a rapidly-evolving epigenome, a "Soft RNA Genome" which together with other epigenetic marks, communicates with the stable, and slowly-

evolving "Hard" DNA genome, regulates it, and gets regulated by it. The RdRPs that replicate the "Soft" RNA genome have low replication fidelity (error every $\sim 10^4$ bases), and since RNA pools fluctuate in response to changing environmental signals, the "Soft genome" should increase the interspecies variability on which selection can act. In addition, the "Soft" RNA genome, as the name suggests, is flexible. Since the "Soft" RNA genome represents the reactions of the previous generations to the surroundings, it can either linger transiently, if the environment changes, or establish a stable regulation, when the response is selected due to its relevancy and adaptive nature. Maintenance of the epigenetic effects that are initiated by inherited RNA responses could occur in 2 phases. First, transient epigenetic inheritance could be achieved via RdRP-mediated amplification and feed-forward interactions with chromatin marks.[24] Later, if the environmental pressure remains relevant for many generations, the response could be assimilated via mutations or deletions, which accumulate if a gene stays off, by natural selection.[43,44] If indeed certain heritable epigenetic responses yield long lasting effects that are "fixed" in the genome, then this type of mechanism could have influenced the rate of the evolutionary process. This is a wild hypothesis that demands serious experimental examination.

Transgenerational RNAi Inheritance Across the Animal Kingdom

Multiple transgenerational epigenetic effects have been convincingly demonstrated in unicellular organisms and plants (see **Box.1**, and [45]), however, unicellular organisms have no germline, and in plants the germline is not segregated. Although the question was not fully addressed in many phyla, most species in the animal kingdom appear to specify their germline by epigenesis without or with very limited deposition of germline-specifying cytoplasmic determinants. In *C. elegans*, the parental soma communicates with the germline, and the germline is segregated by preformation, which makes it essentially continuous and immortal.[46] Thus, if somatic small RNAs get to the nematode's germline, their signaling could propagate for multiple generations. Relevantly, and in line with the hypothesis that epigenetic information accelerates evolution, a recent study showed that protein-coding genes evolve faster in animals that segregate their germline using preformation, in comparison to animals that specify their germline by epigenesis.[47]

It is possible that in the animal kingdom multigenerational RNAi inheritance is limited to animals with a short generation time, or animals that cannot migrate efficiently, as in these organisms the ancestors' environment is more likely to resemble that of the progeny. Alternatively, the RNA-mediated epigenetic effects found in worms might only be the tip of the iceberg, and may have been primarily discovered due to the fact that *C. elegans* is genetically tractable, and has a very short life span that allows fast analysis of traits across many generations. While these are still early days, recent studies have shown that inherited RNA may enable transgenerational memorization of stress also in mammals.[48]

While mammalian genomes do not encode for the canonical RdRPs that are found in nematodes, plants and fungi, it has been recently shown that other polymerases which have RdRP capabilities amplify DICER-substrates.[49] There is even functional evidence for the existence of a (yet to be identified) human RdRP: hepatitis delta virus, a human-infecting RNA virus that lacks an RdRP, relies exclusively on host RdRP activity for replication of its RNA genome.[50] In addition to perpetuating heritable RNA signaling by RNA amplification, it is also possible that in other organisms an RNA response results in the recruitment of the DNA methylation machinery in the germline, which consolidates the RNA-induced silencing and establishes heritable and stable DNA methylation patterns.[22] While it is well established that certain DNA methylation patterns are heritable,[51] the epigenetic effects that were recorded in *C. elegans* nematodes are not mediated by this mechanism, since worms do not seem to have any cytosine methylation.[52]

Box 1. Epigenetic effects in different organisms.

RNAi mediated inheritance across the tree of life

Apart from in *C. elegans*, non-coding RNAs enable non-Mendelian genetics and inheritance of acquired traits across the tree of life.

In many species of bacteria and arcea incorporation of sliced phages into loci that are termed Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), enables RNA-mediated transgenerational immunity. CRISPR-derived RNAs guide defense proteins back to the phage's sequence, and the phage is degraded.[54]

In different ciliates heritable RNA determines which DNA sequences will be maintained or discarded in the next generations, instructs the cell on how to perform massive DNA-rearrangements, and determines gene copy number.[55]

In plants, RNAi is systemic, and small RNAs that are transferred from somatic cells to the germline, together with DNA methylation, establish transgenerational regulation.[51] In plants "Epimutations" can be stable, and require RdRPs for their propagation.[56] In flies and fish PIWI-associated RNAs (piRNAs) can establish multigenerational RNAi.[57,58]

While some evidences exist for RNA inheritance in mammals, the underlying mechanisms are still poorly understood[59]

Concluding Remarks

It is generally accepted that the first molecule of life, RNA, gave up the throne when DNA came on stage and took over. However, recent findings suggest that discussions regarding "The RNA World"[53] should not be carried out in a nostalgic tone since it is very much alive: Even in our current "DNA World," viruses are not the sole organisms to benefit from the advantages of fast-evolving and flexible RNA genomes. "Soft" RNA-based genomes might be integral to the genetic landscape of higher animals as well, co-existing side-by-side in cooperation with their "hard" and "reliable" DNA-encoded younger brothers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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