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Genetic requirements for Piwi-induced stable transgenerational gene silencing in *Caenorhabditis elegans*

Miguel Duarte Dias de Vasconcelos Almeida

Dissertação

Mestrado em Biologia Evolutiva e do Desenvolvimento

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Para todos/to all,
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Resumo

A descoberta, em 1998, da interferência por RNA (RNAi) revelou que pequenos RNAs não codificantes estão envolvidos no controlo da expressão genética. Atualmente, várias vias de RNAi são conhecidas em Eucariotas. Apesar de divergirem em muitos aspetos, todas possuem complexos regulatórios envolvendo uma proteína Argonauta e um pequeno RNA. O complexo Argonauta-pequeno RNA vai ligar-se a RNAs mensageiros (mRNAs) com sequência complementar. De seguida, a expressão do mRNA alvo será afetada, nomeadamente, o mRNA poderá ser degradado ou poderá ocorrer inibição da sua tradução. O silenciamento genético promovido pelas vias de RNAi pode também dar-se ao nível da cromatina. Nesse caso, há o recrutamento de complexos enzimáticos capazes de modificá-la. Assim, utilizando estas plataformas mecanísticas, as vias de RNAi regulam a expressão genética a vários níveis, tendo particular importância no controlo de elementos genéticos considerados egoístas ou parasíticos, como os elementos transponíveis (TEs).

As proteínas Argonautas podem ser divididas em três subfamílias: a subfamília Ago, cujos membros são expressos ubiquamente e associam-se a RNAs em cadeia dupla; a subfamília Piwi, que são expressas na linha germinal dos metazoários e associam-se a um tipo específico de pequenos RNAs de cadeia simples, os piRNAs; e a subfamília Wago, cujos componentes estão apenas presentes em Nemátodes, tal como *Caenorhabditis elegans*.

Em *C. elegans,* existem cinco classes de pequenos RNAs, divididas de acordo com a sua dimensão, alterações químicas e Argonautas às quais se associam: pequenos RNAs primários, 22G-RNAs, 21U-RNAs, 26G-RNAs e microRNAs. Os pequenos RNAs primários são RNAs de cadeia dupla que, uma vez dentro das células do nemátode, são clivados em pequenos RNAs. Posteriormente, estes irão associar-se a uma Argonauta que removerá uma das cadeias e o complexo pequeno RNA-Argonauta ligar-se-á a um mRNA alvo. Estes pequenos RNAs primários, não justificam, por si, o silenciamento genético robusto que é observado. Para um forte silenciamento, é necessário que haja amplificação do sinal. Para este efeito, são recrutadas para o mRNA alvo RNA Polimerases dependentes de RNA (RdRPs) que sintetizam pequenos RNAs secundários. Estes são denominados 22G-RNAs por exibirem um comprimento de 22 nucleótidos e uma forte tendência para guanosina na sua extremidade 5'. Por sua vez, os 22G-RNAs vão associar-se a Argonautas da classe Wago, exercendo silenciamento genético de formas que ainda não são completamente claras. Recentemente, foi descoberta em *C. elegans* uma Argonauta expressa nas células somáticas, denominada NRDE-3, que após a ligação a 22G-RNAs, migra para o núcleo e interage com pré-mRNAs com sequência complementar ao 22G-RNA que transportam.

Após o reconhecimento de um pré-mRNA complementar, a NRDE-3 recruta os fatores nucleares NRDE-1, NRDE-2 e NRDE-4 que, por sua vez, irão inibir a progressão da Polimerase de RNA II e recrutar enzimas ou complexos enzimáticos modificadores de cromatina, como as metiltransferases de histonas. Estas depositarão marcas na cromatina dos genes alvo, provocando silenciamento genético. Um exemplo concreto é a trimetilação no nono resíduo de lisina da cauda da histona H3 (H3K9me3).

Em Eucariotas, as proteínas Piwi, associadas a piRNAs, mantêm TEs silenciados, sendo fulcrais para a manutenção da linha germinal. Os 21U-RNAs de *C. elegans,* assim denominados por terem 21 nucleótidos de comprimento e uma forte propensão para uracilo na sua extremidade 5', foram recentemente identificados como piRNAs, pois associam-se às proteínas Piwi, dependendo delas para a sua síntese na linha germinal. *C. elegans* tem duas proteínas Piwi, PRG-1 e PRG-2 que são expressas na linha germinal. É de notar que PRG-1 é preponderante para a atividade dos 21U-RNAs, enquanto PRG-2 não aparenta ser tão relevante. Os 21U-RNAs são transcritos principalmente de duas vastas regiões localizadas no cromossoma IV. Curiosamente, os 21U-RNAs diferem de piRNAs de outros sistemas por serem transcritos como unidades individuais, graças a um motivo conservado, localizado a montante do 21U-RNA, que é reconhecido por fatores de transcrição da família *Forkhead*.

Após a transcrição de dado 21U-RNA, este é processado de uma forma que não é completamente clara, mas que inclui metilação a 3'. Seguidamente, os 21U-RNAs vão associar-se à PRG-1 e juntos vão ligar-se a mRNAs alvo que poderão ser transcritos de TEs ou de outros genes que codifiquem para proteínas expressas na linha germinal. Esta ligação não exige perfeita complementaridade de bases, tolerando algumas bases desemparelhadas. O complexo PRG-1- 21U-RNA conduz ao recrutamento de RdRPs o que, pela produção de 22G-RNAs e a sua associação a WAGOs, irá reforçar o silenciamento genético. Esta via silencia, de forma robusta, TEs na linha germinal. Presumivelmente, outros genes importantes para a gametogénese também poderão ser regulados pelos 21U-RNAs.

Em *C. elegans,* o silenciamento genético induzido por RNAi pode ser herdado. No entanto, o silenciamento acaba por diluir-se após um número muito curto de gerações. A base mecanística e os fatores envolvidos eram, até há pouco tempo, desconhecidos. Estudos recentes identificaram os 22G-RNAs e a marca de cromatina H3K9me3 na base de silenciamento genético hereditário. Tanto as proteínas NRDE como as WAGO foram diretamente implicadas. No entanto, a indução de silenciamento genético transgeneracional nunca foi atribuída a piRNAs.

A caracterização de um novo tipo de silenciamento genético induzido por pequenos RNAs, em particular por piRNAs, que tenha capacidade de ser mantido por um número indefinido de gerações, foi o foco do trabalho experimental apresentado nesta dissertação.

Este silenciamento foi originalmente encontrado em estirpes portadoras de transgenes em cópia única no genoma, com expressão específica na linha germinal. Estes transgenes foram silenciados espontaneamente e o silenciamento foi transmitido de forma estável e totalmente penetrante à sua descendência. Pelo papel dos 21U-RNAs no seu estabelecimento, este silenciamento genético foi denominado de silenciamento epigenético induzido por RNAi (RNAe). Uma vez estabelecido, o RNAe torna-se independente de PRG-1 e perpetua-se indefinidamente sem sinais de reversão.

Os objetivos desta dissertação almejavam a identificação de genes envolvidos na manutenção deste novo paradigma de silenciamento genético, que ocorre na linha germinal de *C. elegans*. Para tal, recorri a cruzamentos genéticos, cruzando estirpes portadoras do transgene silenciado por RNAe com estirpes mutantes para um gene candidato. Através deste sistema experimental, a reativação do transgene indica que o gene candidato está envolvido na manutenção do RNAe. Os meus resultados apoiam o envolvimento de 22G-RNAs na manutenção do RNAe, uma vez que uma mutação no gene *mut-7*, codificante para uma exonuclease putativa e que foi previamente implicado na biogénese de 22G-RNAs, anula o RNAe. As WAGOs foram também implicadas, uma vez que *wago-9* mutado reativa o transgene. Recentemente, outros demonstraram que WAGO-9 está presente no núcleo e poderá atuar na linha germinal de forma análoga a NRDE-3. A via NRDE de RNAi nuclear foi também implicada, uma vez que mutantes de *nrde-1* e *nrde-2* não apresentam RNAe. Um efeito materno foi igualmente notado, dado que a reativação do transgene foi consistentemente observada na segunda geração de homozigotia para o gene candidato. Além disso, um mutante defetivo para RNAe foi recuperado após triagens de mutantes. Este mutante apresentou sensibilidade a RNAs em cadeia dupla exógenos, indicando que o mutante não faz parte da classe RNAi defetivo (Rde). Tomados em conjunto, estes resultados apoiam um modelo em que, após o estabelecimento do RNAe, há produção de 22G-RNAs que se poderão ligar a WAGO-9 e juntos migrarão para o núcleo. No núcleo, o complexo 22G-RNA - WAGO-9 irá recrutar as proteínas NRDE1, NRDE-2 e NRDE-4, que por sua vez poderão recrutar complexos enzimáticos modificadores de cromatina, depositando H3K9me3. Para o restabelecimento de RNAe a cada geração, provavelmente ocorrerá deposição materna de 22G-RNAs nos embriões. Por sua vez, estes pequenos RNAs herdados irão provavelmente restabelecer as marcas na cromatina. Mais, os 22G-RNAs presumivelmente serão sintetizados *de novo* a cada geração. Os resultados aqui apresentados elucidam quais as vias e genes necessários para o RNAe, um novo fenómeno de regulação estável e duradoura da expressão genética. Estes estudos contribuem para a compreensão da regulação da expressão genética por pequenos RNAs. A um nível mais amplo, o RNAe poderá ter implicações na compreensão de fenómenos evolutivos e do desenvolvimento.

Palavras-Chave

Piwi, piRNA, silenciamento genético, RNAe, *Caenorhabditis elegans*

Abstract

In metazoans RNAi regulates gene expression and has great importance in the most diverse biological processes. The spreading of RNAi-induced gene silencing through multiple generations has been described. However such gene silencing events are transient, eventually disappearing. The existence of RNAi-induced epigenetic silencing (RNAe), capable of stable transmission through numerous generations, is a long-standing question in the field. In animals, Piwi proteins and piRNAs control transposons and maintain the integrity and functionality of the germline. The *C. elegans* Piwi protein, PRG-1 and its piRNAs have been recently implicated in the induction of RNAe in germline expressed single-copy transgenes. Those strains showed stable silencing with no reversion over time. The work reported here used those RNAe strains to identify genes involved in the maintenance of RNAe. Genetic crosses of RNAe strains with mutant candidate genes revealed that MUT-7, a putative exonuclease involved in 22G-RNA biogenesis, is required for RNAe. Moreover, WAGO-9, a germline nuclear Argonaute, and proteins belonging to the nuclear RNAi pathway, NRDE-1 and NRDE-2, are also required. In those crosses, RNAe was only depleted in the second homozygous generation for the candidate gene introduced, revealing a striking maternal effect. In parallel, forward genetic screens identified one hit. The hit was RNAi-sensitive, indicating that the hit does not belong to the RNAi-deficient (Rde) class. Altogether, those results support a model where 22G-RNAs, WAGOs and the NRDE pathway act together to maintain RNAe indefinitely. Reestablishment of RNAe in every generation may be explained by maternal deposition of 22G-RNAs into embryos, followed by reinforcement of chromatin marks. Furthermore, to assure perpetuation of RNAe, 22G-RNAs may be synthesized *de novo* in every generation. The results reported here shed light on the requirements of RNAe in the germline of *C. elegans*. This new paradigm may be of great importance to understand the regulation of gene expression, with implications in evolution and development.

Keywords

Piwi; piRNA; gene silencing; RNAe; *Caenorhabditis elegans*

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Abbreviations

CSR	Chromosome segregation and RNAi defective
DIC	Differential interference contrast microscopy
DRH	Dicer related helicase
Ds	Double-stranded
EGO	Enhancer of *glp-one*
EGFP	Enhanced green fluorescent protein
EMS	Ethyl methanesulfonate
Endo-siRNA	Endogenous siRNA
Exo-siRNA	Exogenous siRNA
FKH	Forkhead family of transcription factors
HENN-1	HEN-1 of nematode-1
HMT	Histone methyltransferase
HP1	Heterochromatin protein 1
HPL	HP1 like
H3K9me3	Histone H3 lysine-9 trimethylation
mRNA	Messenger RNA
MUT	Mutator
NRDE	Nuclear RNAi defective
piRNA	Piwi-interacting RNA
PRG	Piwi related gene
PTGS	Post-transcriptional gene silencing
RDE	RNAi defective
RdRP	RNA-dependent RNA Polymerase
RISC	RNA-induced silencing complex
RNAe	RNA-induced epigenetic silencing
RNAi	RNA interference
RRF	RNA-dependent RNA polymerase family
siRNA	Small interfering RNA
SMG	Suppressor with morphogenetic effect on genitalia
sRNA	Small RNA

Ss	Single-stranded
TE	Transposable element
UCM	Upstream conserved motif
UTR	Untranslated region
WAGO	Worm-specific Argonaute

I. INTRODUCTION

I. INTRODUCTION

The central dogma of molecular biology proposed by Francis Crick (Crick, 1958) states that the flow of biological information is linear from DNA to proteins. DNA is conceived as the information reservoir, which is transcribed into RNA that it is later translated into a protein. RNA was typically regarded as a mere transient intermediate between DNA and protein with no relevant regulatory roles in gene expression. The discovery of enzymatic activity of RNA (Kruger *et al*., 1982) was the first suggestion that there was much more to know about RNA. However, the scientific community had to wait until 1998 (Fire *et al*., 1998) for the discovery of what we now know to be the event of RNA-mediated regulation of gene expression by excellence: RNA interference (RNAi). This discovery changed our comprehension on RNA, revealing that it is an important regulatory molecule, key to many biological processes, rather than an inert intermediate. In 50 years, the central dogma of molecular biology, although not being incorrect, turned obsolete, not contemplating several additional layers of complexity.

The discovery of RNA interference and regulatory RNAs

Although antisense RNA was shown to trigger gene silencing (Fire *et al*., 1991; Guo and Kemphues, 1995; Izant and Weintraub, 1984; Rosenberg *et al*., 1985), silencing could not be solely explained by antisense RNA binding to messenger RNA (mRNA). Finally, in 1998, Fire and colleagues (Fire *et al*., 1998) showed that double-stranded RNA (dsRNA) is the trigger for post-transcriptional gene silencing (PTGS) in the nematode *Caenorhabditis elegans*. Moreover, they demonstrated that just a low amount of dsRNA molecules per cell can give rise to sequence-specific gene silencing.

This breakthrough preluded the swift confirmation of the existence of RNAi in plants (Waterhouse *et al*., 1998), fungi (Volpe *et al*., 2002) and other metazoans: in the fruitfly (Kennerdell and Carthew, 1998), in the zebrafish (Wargelius *et al*., 1999) and in mammalian cells (Elbashir *et al*., 2001). These studies supported the idea that RNAi is a widespread, evolutionarily conserved mechanism for the regulation of gene expression.

From then on, our understanding of several species of non-coding RNAs as gene expression regulatory molecules has increased tremendously. Non-coding RNAs revealed to be extremely prolific in terms of quantity, diversity, function and interactions (Ghildiyal and Zamore, 2009; Ketting, 2011). This dissertation will focus on non-coding small RNAs (sRNAs) involved in RNAi reactions.

The Argonaute proteins

A key commonality of the RNAi pathways that lead to gene silencing is the participation of an Argonaute protein, which binds sRNA molecules. In eukaryotes, the Argonaute protein family is subdivided into three subfamilies, categorized according to sequence homology: the Ago subfamily, the Piwi subfamily, and the worm-specific Wago subfamily (**Figure 1A, B**) (Hutvagner and Simard, 2008). Amongst eukaryotes the Argonaute proteins are conserved, but their number varies considerably, ranging from one in the fission yeast to twenty-seven in *C. elegans* (Ender and Meister, 2010; Hutvagner and Simard, 2008).

The Ago proteins are ubiquitously expressed and associate with dsRNAs, both microRNAs and small interfering RNAs (siRNAs). Piwi subfamily proteins are mainly expressed in the germline and bind to single-stranded (ss) Piwi-interacting RNAs (piRNAs), whereas the Wago subfamily proteins associate with secondary siRNAs in *C. elegans* (Ender and Meister, 2010; Hutvagner and Simard, 2008). All Argonaute proteins possess four protein domains (**Figure 1C**): a N domain, a PAZ domain, a MID domain and a PIWI domain. Crystallographic studies increased our understanding on the contributions of each of those domains to Argonaute function in gene silencing. The PAZ domain was shown to form a specific binding pocket for the 3'-end of ssRNAs (Ender and Meister, 2010; Hutvagner and Simard, 2008). The MID domain was reported to be a binding pocket for the 5'-phosphate of the small RNA with which the Argonaute binds (Ender and Meister, 2010; Hutvagner and Simard, 2008). In turn, the structure of the PIWI domain adopts a RNase H fold, which was consequently thought to be responsible for the catalytic activity of the Argonaute-sRNA complexes (Song *et al*., 2004). Specifically, a conserved motif of three aminoacids is responsible for cleavage activity, the so-called catalytic triad (Song *et al*., 2004). This catalytic activity is important for both the extraction of one strand from the RNA duplex and sequence-specific cleavage of target transcripts (Hutvagner and Simard, 2008). Nonetheless, the cleavage activity of Argonaute proteins is not merely explained by the presence of a PIWI domain. Some Argonautes, despite having the canonical residues in the catalytic site, don't cleave. This indicates that other factors or sRNAs may be needed (Hutvagner and Simard, 2008). Lastly, a function for the Argonaute N domain has recently been found in the unwinding of the small RNA duplexes during RNA-induced silencing complex (RISC) assembly (Kwak and Tomari, 2012).

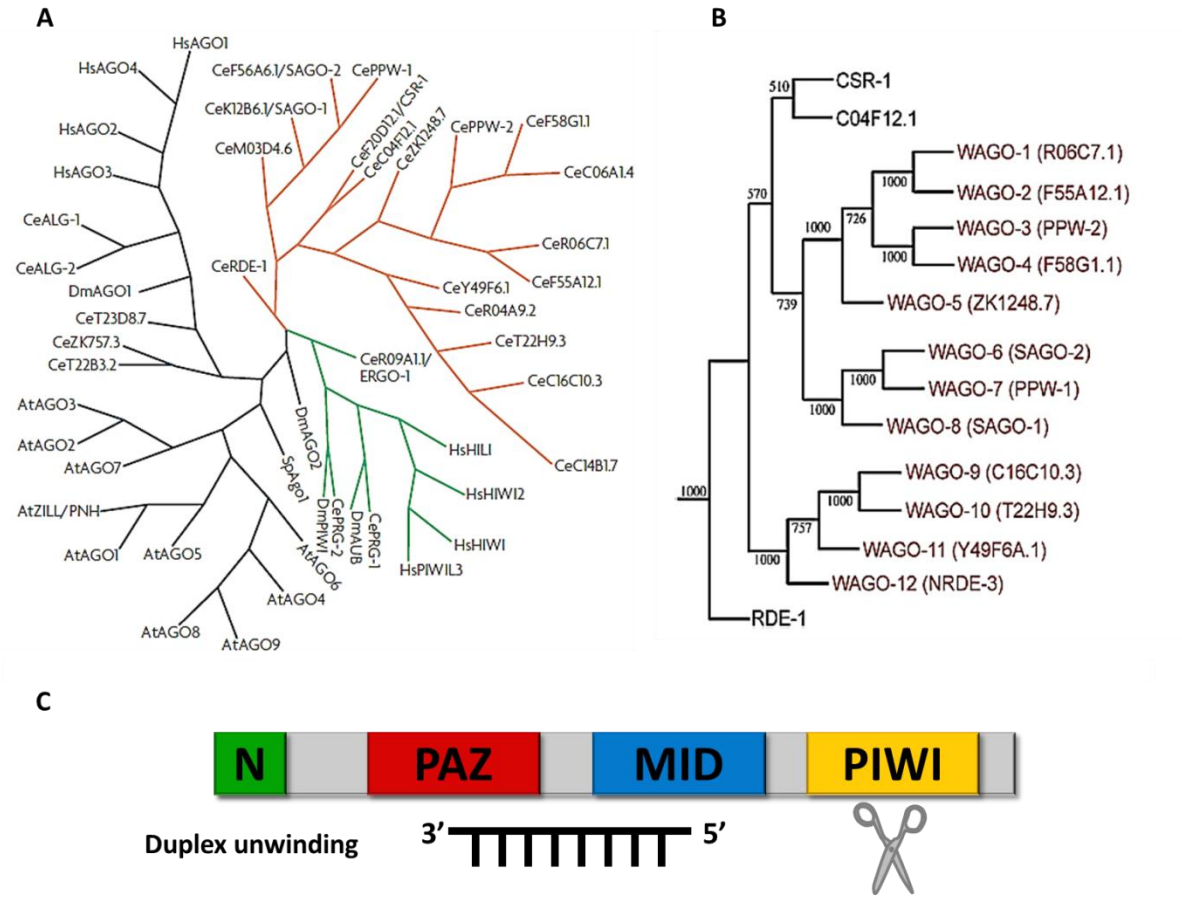

Figure 1. Overview of the Argonaute family proteins. (**A**) Representative elements of the three Argonaute subfamilies in various organisms are shown. In black there is the Ago subfamily, in green the Piwi subfamily and orange the worm-specific Wago family. After Hutvagner and Simard, 2008. Sp, *Schizosaccharomyces pombe*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; At, *Arabidopsis thaliana*. (**B**) Phylogenetic tree focusing on the Wago protein subfamily. Adapted from Gu *et al*, 2009. (**C**) Schematics of the four Argonaute protein domains. The N domain is involved in duplex unwinding, while the PAZ and MID bind respectively the 3' and 5'-ends of the sRNA. Moreover, Argonaute catalytic activity is mediated by the PIWI domain.

Small interfering RNAs

siRNAs may arise either from exogenous or endogenous sources (**Figure 2**). In exogenous siRNA (exo-siRNA) pathways, an exogenous long dsRNA is cleaved into ds-siRNAs by Dicer, a RNase III endonuclease with specificity for dsRNAs (Ghildiyal and Zamore, 2009; Ketting, 2011). The cleaved products are approximately 21 nucleotides long and bear small 3'-overhangs. For target regulation, the siRNAs must be loaded into Argonaute proteins, forming the RISC (Ghildiyal and Zamore, 2009; Ketting, 2011). Then, together with other auxiliary factors, the RISC will cleave the target mRNA, thereby silencing the transcript.

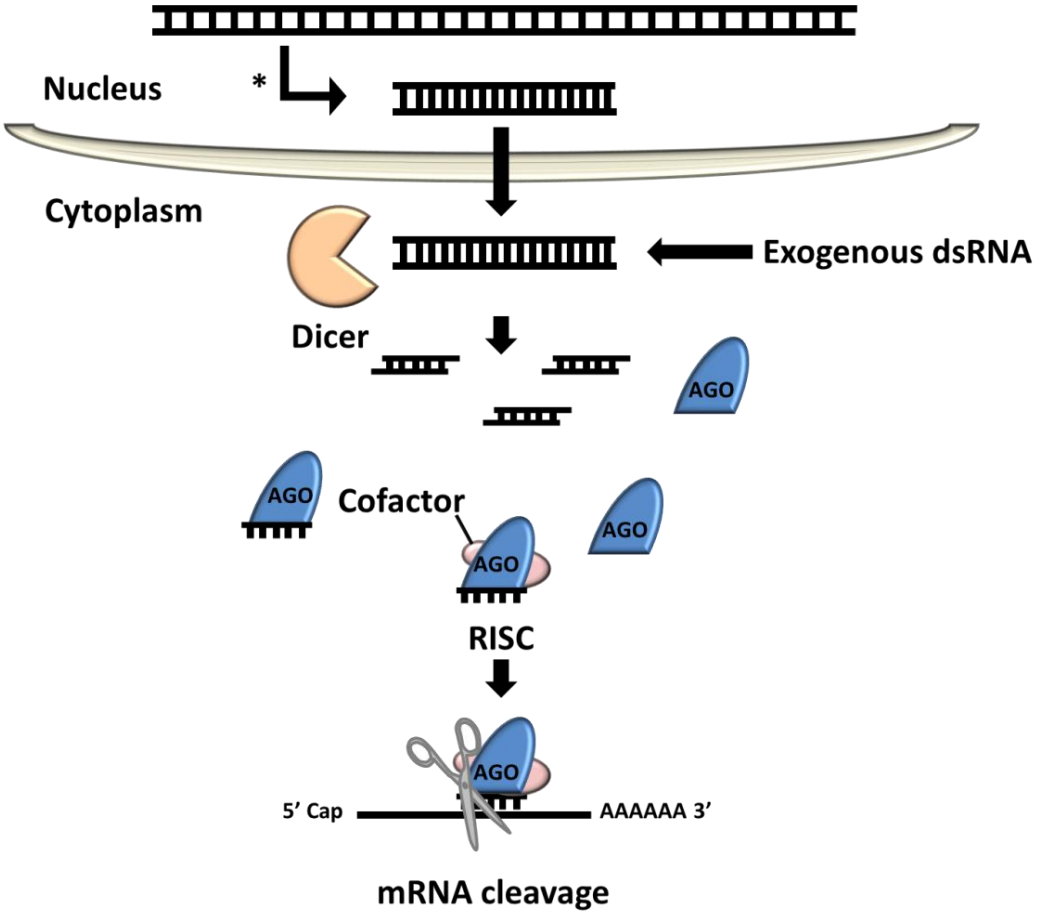

Figure 2. Representative siRNA pathways. A typical siRNA pathway is depicted. dsRNA can derive from exogenous sources or (*) it can be synthesized from endogenous *loci* in a variety of ways: from structured loci, bidirectional or convergent transcription, etc (Ghildiyal and Zamore, 2009). The dsRNA is subsequently processed by Dicer into small dsRNAs. Those will bind to an Argonaute which will slice out one of the strands. The Argonaute bound with a siRNA and its auxiliary factors comprises the mature RISC. The latter will cleave target mRNAs with sequence complementarity to the siRNA. AGO, Argonaute protein; RISC, RNA-induced silencing complex.

The endogenous siRNAs (endo-siRNAs) were first discovered in plants (Hamilton *et al*., 2002) and *C. elegans* (Ambros *et al*., 2003). In *C. elegans,* the production of endo-siRNAs differs extensively from the biogenesis pathway reported above for exo-siRNAs. It relies on RNA-dependent RNA Polymerases (RdRPs) without the participation of Dicer (**Figure 3**). The RdRPs synthesize short RNA molecules that directly bind an Argonaute protein (Aoki *et al*., 2007; Pak and Fire, 2007; Sijen *et al*., 2007). The discovery of endo-siRNAs in flies (Aravin *et al*., 2003; Czech *et al*., 2008; Ghildiyal *et al*., 2008) and mammals (Watanabe *et al*., 2008; Yang and Kazazian, 2006) followed, showing that they are widespread amongst eukaryotes. However, their biogenesis is mechanistically different from the endo-siRNAs of worms and plants, since flies and mammals do not possess RdRPs. For example, they can be produced by convergent transcription or structured loci (Ghildiyal and Zamore, 2009). However, the downstream steps seem to be identical to exo-siRNA

pathways (**Figure 2**). Genetic studies attributed a role for endo-siRNAs in the silencing of transposable elements (TEs) (Chung *et al*., 2008; Ghildiyal *et al*., 2008). Another class of sRNAs was proven to be crucial for TE control of these in the metazoan germline. Those are the piRNAs.

piRNAs and the transposon menace

TEs are mobile DNA elements that are present in most eukaryotic genomes, often comprising a large percentage of the genome content (Malone and Hannon, 2009; Slotkin and Martienssen, 2007). They are highly mutagenic, since they can insert within protein-coding genes, leading to changes in gene expression (Slotkin and Martienssen, 2007). TEs are considered “selfish” or “parasitic”, because there is a negative correlation between the copy number of a certain TE in a genome and the fitness of the host. However, TEs are thought to contribute greatly to evolution by introducing raw genetic variation, shaping genomic and transcriptomic landscapes (Malone and Hannon, 2009; Slotkin and Martienssen, 2007). To avoid detrimental effects to the fitness, organisms must control the mobility of TEs. The germline must be especially resistant to TEs to assure that the next generation will not have TE-derived defects. In the germline, TEs are silenced mainly by epigenetic mechanisms, where RNAi plays an essential role (Malone and Hannon, 2009; Slotkin and Martienssen, 2007). It should be noted that, for the purpose of this dissertation, by epigenetics it is meant: *the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states* (Bird, 2007).

In *Drosophila*, an abundant class of endogenous sRNAs was named as piRNAs after the discovery of their association with Piwi proteins in the germline (Brennecke *et al*., 2007; Gunawardane *et al*., 2007; Saito *et al*., 2006; Vagin *et al*., 2006). This association of Piwi proteins with piRNAs was also observed in mammals (Aravin *et al*., 2006; Girard *et al*., 2006; Grivna *et al*., 2006; Lau *et al*., 2006) and fish (Houwing *et al*., 2007). Piwi proteins and piRNAs are thought to be present in almost all metazoans, since they can be found in phyla, like Cnidaria and Porifera, that diverged before the appearance of Bilaterians (Grimson *et al*., 2008).

The major functions of piRNA pathways in animals is to silence TEs in the germline, allowing the correct development and the maintenance of germline integrity (Siomi *et al*., 2011). piRNAs are also believed to regulate other targets, namely non-repetitive protein-coding genes (Siomi *et al*., 2011). For example, pachytene piRNAs (which expression starts in the pachytene stage of meiosis) in mice do not seem to directly target TEs (Aravin *et al*., 2006; Girard *et al*., 2006) and their function remains unknown. On the other hand, the

mechanism of TE silencing by piRNAs and Piwi proteins reached a reasonable level of understanding in flies. The prevailing model of piRNA biogenesis states that there are two piRNA biogenesis pathways conserved in animals: a primary processing pathway and the so-called ping-pong amplification loop (Brennecke *et al*., 2007; Gunawardane *et al*., 2007; Lau *et al*., 2006; Robine *et al*., 2009). Succinctly, the first provides an initial pool of piRNAs that target TEs, while the latter amplifies the number of sequences targeting TEs. For robust silencing, a concerted action of both pathways is required (Siomi *et al*., 2011).

The *C. elegans* RNAi pathways

Worms can respond to exogenous dsRNA (Fire *et al*., 1998) and have several endogenous sRNA species (Ketting, 2011). Early, forward genetic screens revealed factors involved in RNAi (Grishok *et al*., 2000; Ketting *et al*., 1999; Tabara *et al*., 1999b). RDE-1, an Argonaute, and MUT-7, a putative exonuclease, were amongst them. Also, it was noted that, similarly to other animals, RNAi in *C. elegans* silences TEs in the germline (Ketting *et al*., 1999; Tabara *et al*., 1999b).

For effective exo-siRNA-induced gene silencing, an amplification step is necessary (Sijen *et al*., 2001). It is now known that RdRPs are recruited to mRNAs that are bound with primary siRNAs, and then synthesize secondary siRNAs. This synthesis occurs in an unprimed manner, preferentially in the 5'-to-3' direction, antisense to the mRNA (**Figure 3**) (Pak and Fire, 2007; Sijen *et al*., 2007). The secondary siRNAs exhibit 5'-triphosphates, while Dicer cleavage products usually display 5'-monophosphates, suggesting that Dicer is not involved. Genetic analysis revealed that the RdRPs EGO-1 (Smardon *et al*., 2000) and RRF-1 (Sijen *et al*., 2001), as well as the helicase DRH-3 (Aoki *et al*., 2007; Duchaine *et al*., 2006) are necessary for secondary siRNA biogenesis in the germline. Upon their production, secondary siRNAs associate with WAGO proteins (**Figure 1B; Figure 3**) (Yigit *et al*., 2006). This amplification step allows few primary siRNA molecules to yield robust gene silencing via secondary siRNAs.

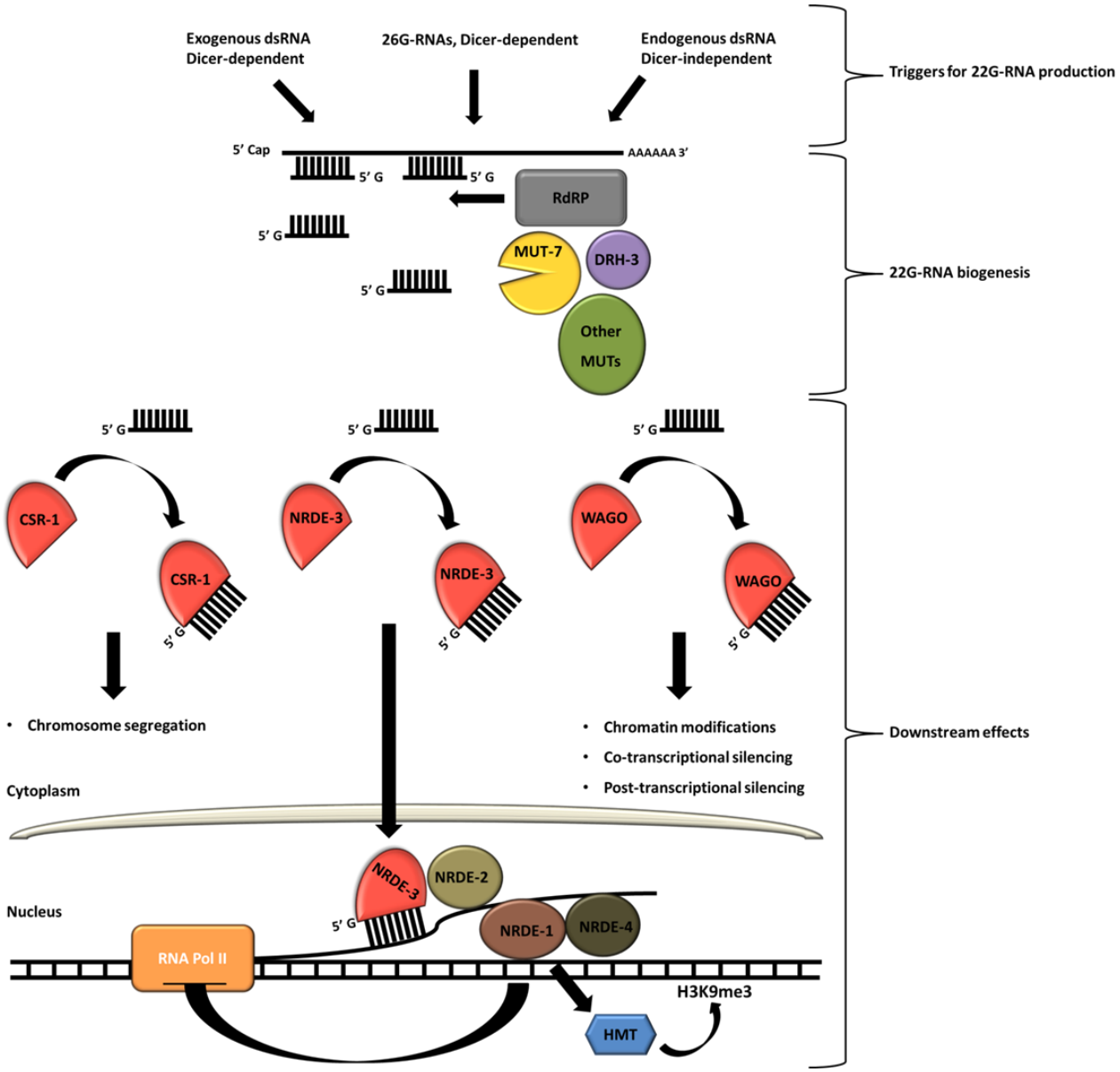

Figure 3. The *C. elegans* 22G-RNA and NRDE pathways. 22G-RNA biogenesis can be triggered in various ways. After their biogenesis they can associate with different Wago subfamily members. 22G-RNAs associated with CSR-1 are involved in chromosome segregation while 22G-RNAs associated with NRDE-3 go to the nucleus of somatic cells to induce, in concerted action with other NRDE proteins, deposition of H3K9me3. In the germline, other WAGOs may have an analogous role, inducing chromatin modifications. In addition, 22G-RNAs associated with WAGOs may induce silencing both co-transcriptionally and post-transcriptionally. RdRP, RNA-dependent RNA Polymerase; HMT, histone methyltransferase; RNA Pol II, RNA Polymerase II.

Sequencing and comparative genomics approaches allowed the identification of microRNAs and two classes of germline-specific endo-siRNAs in *C. elegans* (Ambros *et al*., 2003; Ruby *et al*., 2006). Both classes have a strong bias for a 5'-guanosine. One class is the 26G-RNAs, which have a length of 26 nucleotides and bear 5'-monophosphates. The other class has a length of 22 nucleotides and 5'-triphosphates. Those are identical to secondary-siRNAs and were named 22G-RNAs. They are strongly expressed in the germline

and are maternally deposited into embryos (Gu *et al*., 2009). Nowadays, several factors involved in their biogenesis are known, including MUT-7 and other Mutator proteins (Mutators are genes that, when defective, are characterized by high mutagenic rates attributable to TE mobility), DRH-3 and the RdRPs EGO-1 and RRF-1 (Gu *et al*., 2009; Yigit *et al*., 2006). 22G-RNAs can be functionally divided into two main separate subpopulations: one that associates with WAGO proteins (Gu *et al*., 2009; Yigit *et al*., 2006) and other that associates with CSR-1, another germline-specific Argonaute that is important for chromosome segregation (Claycomb *et al*., 2009; van Wolfswinkel *et al*., 2009). The prevailing model suggests that 22G-RNAs and the WAGOs are involved in a surveillance system in the germline, maintaining TEs, aberrant transcripts and other genes silenced (Gu *et al*., 2009). This may be achieved by mechanisms that are not likely to involve cleavage, since WAGOs do not possess the catalytic motif (Yigit *et al*., 2006). On the other hand, CSR-1-associated 22G-RNAs target thousands of germline-expressed protein-coding genes to assure that chromosome segregation occurs in a secure manner (Claycomb *et al*., 2009; van Wolfswinkel *et al*., 2009).

26G-RNAs have Dicer-dependent biogenesis and are required for gametogenesis (Ketting, 2011). Interestingly, 26G-RNAs have been implicated in synthesis of 22G-RNAs revealing the complexity of *C. elegans* RNAi pathways. Indeed, the exo- and endo-siRNA pathways interact extensively, sharing factors, like the 22G-RNAs and the WAGOs (Duchaine *et al*., 2006; Gu *et al*., 2009; Lee *et al*., 2006; Yigit *et al*., 2006).

The piRNAs of *C. elegans* and downstream pathways

Two Piwi-like proteins were identified in *C. elegans* by homology and were named PRG-1 and PRG-2 (**Figure 1A**) (Cox *et al*., 1998). Large-scale sequencing efforts unveiled a class of sRNAs with 21 nucleotides-long bearing a strong bias for uridine-monophosphate in the 5'-end (Ruby *et al*., 2006). Soon after, 21U-RNAs were identified as the *C. elegans* piRNAs given their association with PRG-1 (Batista *et al*., 2008; Das *et al*., 2008; Wang and Reinke, 2008). Similarly to piRNAs in other organisms, 21U-RNAs have, as previously mentioned, a 5'-uridine monophosphate and their processing is Dicer-independent (Batista *et al*., 2008; Das *et al*., 2008; Ruby *et al*., 2006). Also, 21U-RNAs show methylation on the 2'-hydroxyl group at the 3'-end. This methylation is deposited by the conserved methyltransferase HENN-1 (**Figure 4**) (Billi *et al*., 2012; Kamminga *et al*., 2012; Montgomery *et al*., 2012). There are nearly 16,000 unique annotated 21U-RNA sequences, transcribed mainly from two broad regions on chromosome IV. Unlike flies and vertebrates, 21U-RNAs are located in-between protein-coding genes and there is no strand bias (Batista *et al*., 2008;

Das *et al*., 2008; Ruby *et al*., 2006). Another striking difference to other systems is the existence of an upstream conserved motif (UCM) adjacent to all the 21U-RNAs, suggesting that 21U-RNAs are transcribed as individual units. The UCM is conserved between *C. elegans*, *C. briggsae and C. remanei*, while the sequences of the 21U-RNAs *per se* are not (Batista *et al*., 2008; Das *et al*., 2008; de Wit *et al*., 2009; Ruby *et al*., 2006). Most recently, definitive proof of the individual transcription of 21U-RNAs has been found (Cecere *et al*., 2012). Indeed, Forkhead (FKH) family transcription factors can bind to the UCM and induce expression of 21U-RNAs. In addition, 21U-RNA precursor transcripts are capped and longer than 21 nucleotides (**Figure 4**) (Cecere *et al*., 2012).

PRG-1 and PRG-2 proteins are 91% identical and is likely that they were formed after a recent duplication event (Cox *et al*., 1998; Das *et al*., 2008). Interestingly, 21U-RNAs show different interactions to PRG-1 and PRG-2. Indeed, an abundant piRNA, 21UR-1, is depleted both in *prg-1; prg-2* double mutants and *prg-1* mutants (Batista *et al*., 2008; Das *et al*., 2008; Wang and Reinke, 2008). Conversely, 21UR-1 is present in *prg-2* mutants (Batista *et al*., 2008; Das *et al*., 2008). Although a certain level of redundancy might exist, it seems that PRG-2 functionally diverged from PRG-1. The requirement for Piwi proteins is specific of 21U-RNAs, since *prg-1* and *prg-2* mutants do not downregulate sRNAs from other RNAi pathways (Batista *et al*., 2008; Das *et al*., 2008). Additionally, in conditional mutants with an absent germline, PRG-1, PRG-2 and 21U-RNAs are absent, suggesting that their expression is restricted to the germline. Localization studies supported these data (Batista *et al*., 2008; Wang and Reinke, 2008).

Several fertility problems were identified and quantified in *prg-1* mutants such as an inferior number of germ-cells, reduced brood size and temperature-sensitive sterility (Batista *et al*., 2008; Cox *et al*., 1998; Das *et al*., 2008; Wang and Reinke, 2008; Yigit *et al*., 2006). These fertility defects were associated with spermatogenesis and oogenesis (Batista *et al*., 2008; Das *et al*., 2008; Wang and Reinke, 2008). Also, *unc-130* mutants, a germline-enriched FKH transcription factor strongly involved in 21U-RNAs expression, shows a similar fertility phenotype as *prg-1* mutants (Cecere *et al*., 2012). This further supports the idea that 21U-RNAs and factors associated to their biogenesis may have other, still unidentified, roles in gametogenesis. Altogether these data suggest that both Piwi proteins, but predominantly PRG-1, are necessary for proper gametogenesis.

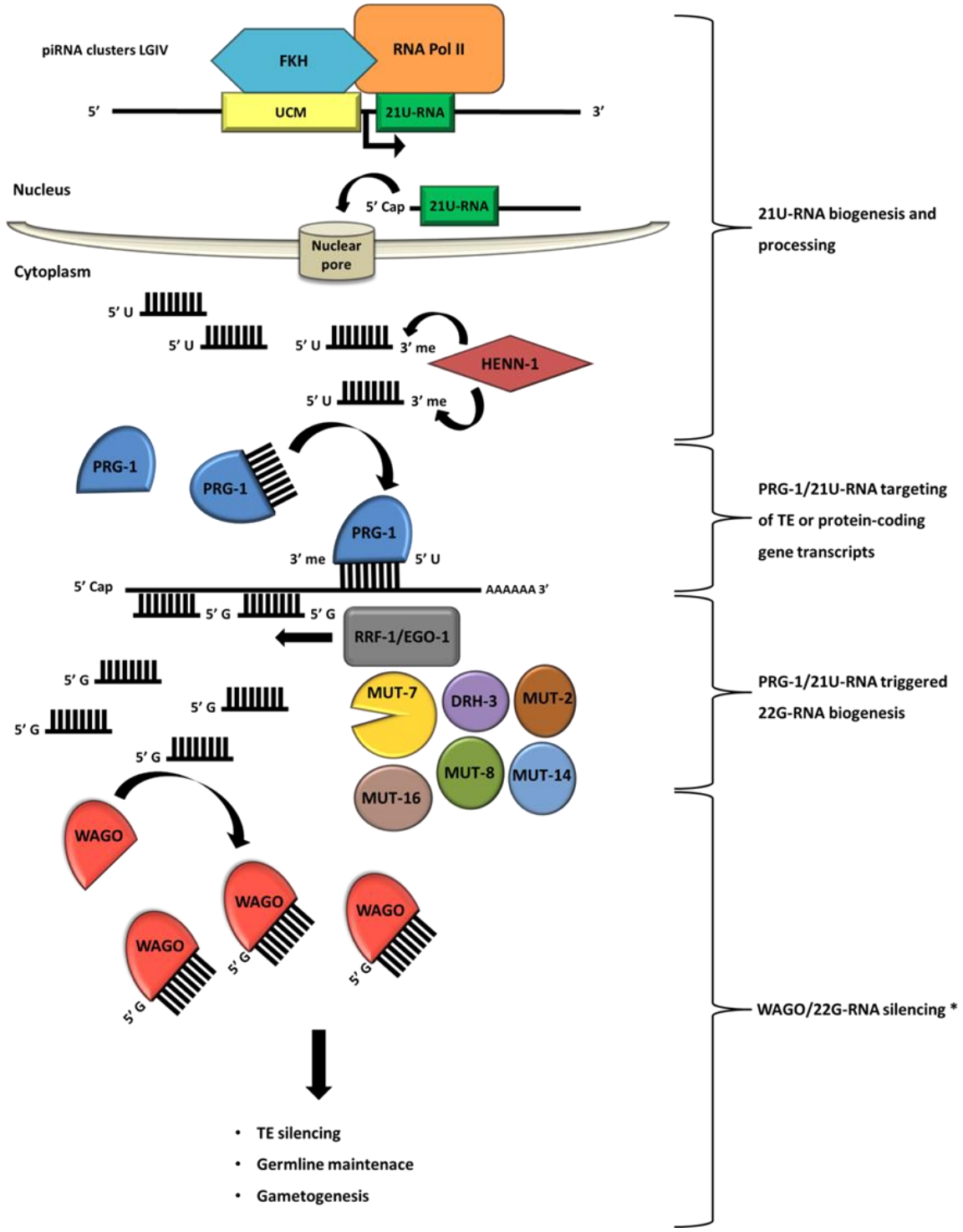

Figure 4. The 21U-RNA pathway in *C. elegans*. 21U-RNAs are transcribed in the germline after the recognition of the UCM by FKH proteins. The precursor transcript is capped and longer than 21 nucleotides. After export to the cytoplasm, mature 21U-RNAs are further processed and will associate with PRG-1. In turn, the complex PRG-1-21U-RNA will target TE transcripts and other protein-coding genes. After binding to a complementary sequence, RdRPs are recruited and start synthesizing 22G-RNAs. 21U-RNAs also bind to sequences that only have partial complementarity. Other factors are involved in their biogenesis. Afterwards, the 22G-RNAs will associate with WAGOs and will provoke TE silencing, germline maintenance and proper gametogenesis. * The silencing may be in the form of chromatin modifications, co-transcriptional or PTGS. FKH, Forkhead family of transcription factors; RNA Pol II, RNA Polymerase II; UCM, upstream conserved motif.

Not surprisingly, like piRNAs and Piwi in other animals, 21U-RNAs and PRG-1 are involved in TE silencing. Only two out of the 16,000 21U-RNAs found were perfectly complementary to the sequences of one TE, termed Tc3, and Piwi mutants showed increased levels of Tc3 transposase mRNA (Das *et al*., 2008). Recent work has showed that 21U-RNAs actually bind to targets with mismatches (Bagijn *et al*., 2012; Lee *et al*., 2012). In this way, assuming up to three mismatches, the possible number of targets increases to 700,000. Moreover, 21U-RNA activity was associated with silencing of numerous TEs and protein-coding genes in the germline, besides Tc3 (Bagijn *et al*., 2012; Lee *et al*., 2012).

Although no evidence for the existence of a ping-pong amplification loop was found in *C. elegans* (Bagijn *et al*., 2012; Das *et al*., 2008), there would certainly be some form of amplification of the 21U-RNA pathway, to achieve effective silencing. Upon Tc3 upregulation in *prg-1* mutants, a loss of endo-siRNAs targeting Tc3 was observed. The presence of those endo-siRNAs is dependent on MUT-7 (Das *et al*., 2008). It is now known that there is an endo-siRNA pathway downstream of the 21U-RNA pathway (Bagijn *et al*., 2012; Das *et al*., 2008). This downstream pathway comprises the amplification mechanism to the 21U-RNAs. A battery of RNAi-related genes required for this process have been identified and the endo-siRNAs implicated in this process are the 22G-RNAs (Bagijn *et al*., 2012). The prevailing model for the combined action between the 21U-RNA and the 22G-RNA pathways is depicted in **Figure 4**.

Transgenerational epigenetic inheritance in *C. elegans*

Previously, RNAi-induced effects spreading through a finite number of generations have been observed in *C. elegans* (Alcazar *et al*., 2008; Grishok *et al*., 2000). For multigenerational RNAi-induced gene silencing, it is required that some silencing signal must be transferred, via the germline, to the progeny. Other than RNA, chromatin modifications pose as an appealing hypothesis. RNAi screens in worms revealed that several factors with possible roles in chromatin binding and remodeling were implicated in RNAi (Dudley *et al*., 2002; Kim *et al*., 2005; Robert *et al*., 2005; Vastenhouw *et al*., 2006). Also, Vastenhouw and colleagues found a RNAi-induced, not fully penetrant gene silencing event that was transmitted indefinitely, for several generations, in the absence of the trigger dsRNA (Vastenhouw *et al*., 2006). Chromatin factors were implicated, which led the authors to propose that the silencing was occurring at the transcriptional level (Vastenhouw *et al*., 2006). Also, transgenerational epigenetic inheritance of longevity (Greer *et al*., 2011) and antiviral immunity (Rechavi *et al*., 2011) were observed in *C. elegans*. Virus-derived siRNAs

(Rechavi *et al*., 2011) and defective elements of a chromatin modifier complex (Greer *et al*., 2011) were reported to underlie those phenomena.

Recently, Guang and colleagues described NRDE-3, a worm-specific Argonaute protein (**Figure 1B**) that is involved in nuclear RNAi in the soma, in *C. elegans* (Guang *et al*., 2008). After RNAi, NRDE-3 is loaded with a 22G-RNA and shuttled to the nucleus (**Figure 3**). The conserved protein NRDE-2 is required for the association of NRDE-1 with pre-mRNAs (Burkhart *et al*., 2011; Guang *et al*., 2010). Also, NRDE-2 and NRDE-4 are required for the association of NRDE-1 to chromatin (Burkhart *et al*., 2011). Together, the NRDE proteins elicit co-transcriptional silencing of pre-mRNAs by reducing RNA Polymerase II occupancy (Burkhart *et al*., 2011; Guang *et al*., 2010; Guang *et al*., 2008). Moreover, NRDE-1 probably interacts with histone methyltransferases to deposit histone H3 lysine-9 trimethylation (H3K9me3) reinforcing silencing of sites targeted by RNAi (**Figure 3**) (Burkhart *et al*., 2011). Thus, the NRDE pathway acts downstream of 22G-RNAs, linking co-transcriptional silencing with H3K9me3, a chromatin modification typically associated with repressive chromatin states (Kouzarides, 2007).

Two recent studies reported a transgenerational RNAi-induced gene silencing, where NRDE-induced H3K9me3 deposition was observed in genes targeted by dsRNA (Burton *et al*., 2011; Gu *et al*., 2012). siRNAs against the gene targeted by RNAi were also observed across generations. Nevertheless, this silencing was brief. siRNAs were depleted and H3K9me3 reverted to background levels after 2-3 generations (Burton *et al*., 2011; Gu *et al*., 2012). Altogether, these observations in *C. elegans,* linking RNAi and chromatin modifications, provide a cornerstone for the occurrence of inheritable RNAi-induced gene silencing events that can be transferred across generations. However, inheritable silencing events, whereby sRNAs and chromatin modifications can induce robust gene silencing that surpasses generational boundaries indefinitely, have not been reported so far.

The silence of the sensor

A recently developed experimental system has been described in which a single-copy transgene can be silenced by a specific abundant 21U-RNA - 21UR-1 (Bagijn *et al*., 2012). This is achieved by the presence of a fully complementary 21UR-1 binding-site in the transgene 3'-UTR. *egfp* is also included in the transgene, thereby providing a tractable system that can be used as a sensor for piRNA activity in *C. elegans* (**Figure 5A**). For simplicity, this sensor will henceforth be addressed as 21Usensor. EGFP is not observed in germline nuclei in worms carrying the 21Usensor – in a wild-type background -, demonstrative of a fully functional piRNA pathway (**Figure 5B, C**). On the other hand, when

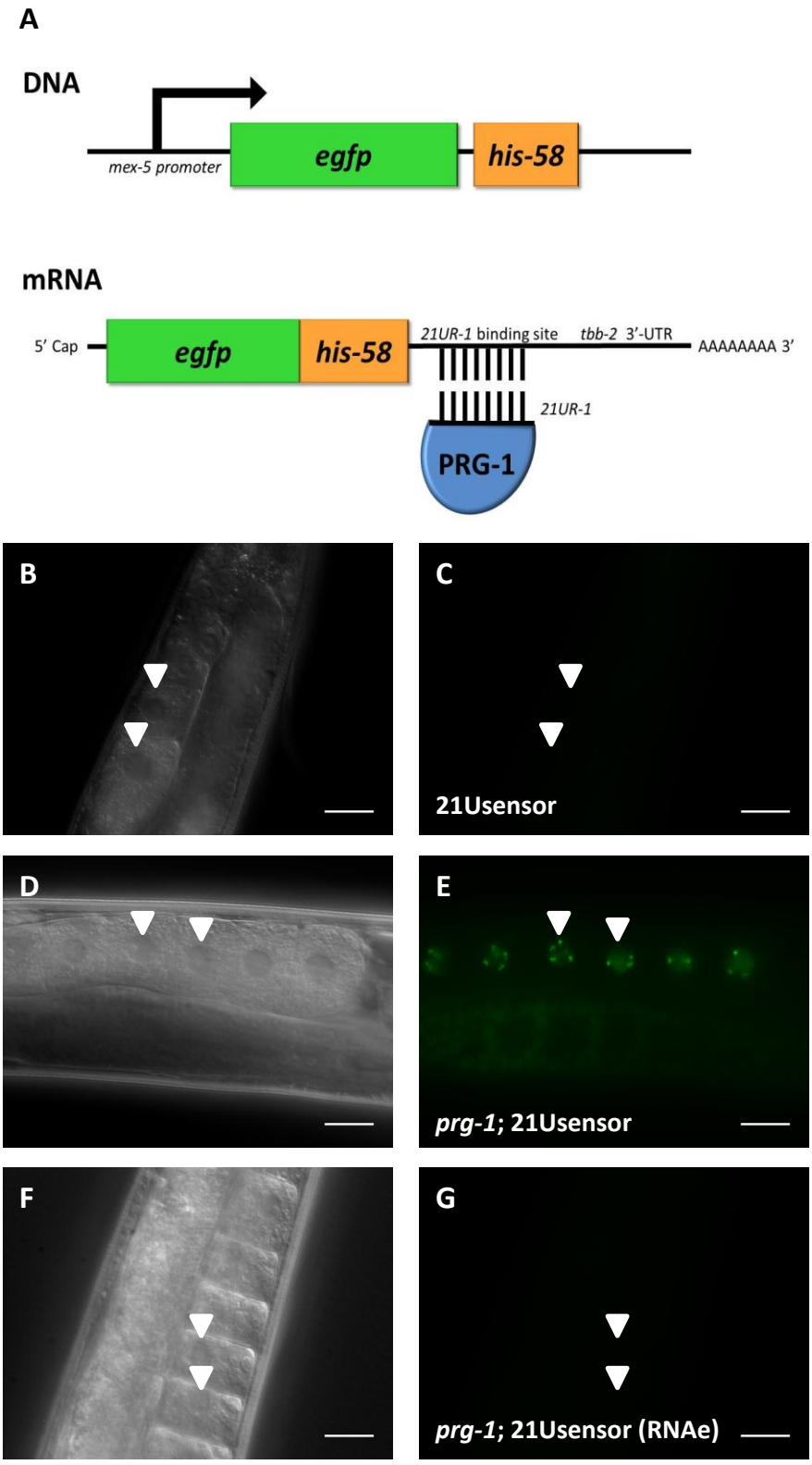

Figure 5. RNAe strains do not show transgene expression even in the absence of *prg-1*. (**A**) Schematics of the single-copy transgene, designated as 21Usensor, used in this study. It consists of *egfp* fused to an homolog of histone H2B (*his58*) controlled by a germline-specific promoter. The mRNA has a binding site for a single abundant 21U-RNA. See Materials and Methods for further details. (**B**) and (**C**) show worms carrying the 21Usensor in a wild-type background. The piRNA pathway is fully functional and, as such, the transgene is silenced. In (**D**) and (**E**), worms carrying the sensor and a *prg-1* mutation show EGFP expression, since the 21U-RNA pathway is defective. Strains where RNAe was found show silencing of the transgene even when *prg-1* mutations are present, as can be seen in (**F**) and (**G**). Images (**B**), (**D**), (**F**) were acquired with differential interference contrast (DIC), whilst images (**C**), (**E**) and (**G**) were acquired with fluorescence microscopy. Arrowheads pinpoint germline nuclei. Scale bars represent 25 µm.

a certain component of the piRNA pathway is impaired, the 21Usensor is expressed. This can be observed in *prg-1* mutants (**Figure 5D, E**).

In mutants for the methyltransferase *henn-1*, EGFP is expressed in lower levels, indicating that the 21U-RNA pathway is not completely abrogated (Kamminga *et al*., 2012). In these mutants, 21U-RNAs can still be detected, so the low levels of EGFP expression might be explained by the lack of stability of sRNAs without the methylation in the 3'-end (Li *et al*., 2005). Most curiously, *henn-1*; 21Usensor strains had the tendency to show silencing of the transgene every generation, in a considerable number of individuals (Luteijn *et al*., 2012). Once worms showing such silencing were singled and allowed to reproduce by selfing, the transgene remained silenced in all progeny and propagated through several generations, without signs of reactivation (Luteijn *et al*., 2012). By removing the *henn-1* mutation and crossing-in a *prg-1* mutation, the sensor remained silenced (**Figure 5F, G**). A *prg-1* mutation should reactivate the sensor, because the 21U-RNA pathway is not functional (compare **Figure 5D, E** with **F, G**). Similarly, RNAi against *prg-1* should also reactivate the 21Usensor, but that does not occur (Luteijn *et al*., 2012). Those observations suggest that there must be signals, other than 21U-RNAs, that are able to perpetuate the silencing even in the absence of the initial trigger. For simplicity, this extremely stable form of dominant gene silencing will be designated throughout this dissertation as RNA-induced epigenetic silencing (RNAe). Strains with transgenes silenced by RNAe will be addressed as 21Usensor (RNAe).

Objectives

The purpose of the work presented here was to find the pathways involved and the factors required for the maintenance of RNAe across generations. To do this, *prg-1*; 21Usensor (RNAe) worms were used and crossed with worm strains carrying mutations in candidate genes, aiming to observe reactivation of the 21Usensor, *i.e.* EGFP expression in germline nuclei. Several factors that were previously shown to be involved in, or related to, RNAi pathways were tested, as well as several worm-specific Argonautes. Another approach was to mutagenize worms with ethyl methanesulfonate (EMS) and screen for mutants that reactivated the 21Usensor.

II. MATERIALS AND METHODS

II. MATERIALS AND METHODS

Worm strains

Nematode populations were cultured according to standard procedures (Brenner, 1974) in solid nematode growth medium (NGM) with a lawn of OP50 *E. coli* strain. All the strains used in this study, including the 21Usensor strain, have the Bristol N2 strain genetic background. The strains produced in this work are derived from *prg-1 (pk2298)*; 21Usensor (RNAe) worms, thus having the N2 genetic background. Consult **Table A** in the appendix for a detailed list of the strains used in this study as well as its sources. Also, a detailed list of the strains produced during this study is presented in **Table B** in the appendix.

21Usensor

The 21Usensor was produced and provided by the Eric Miska laboratory in Cambridge and it has already been described (Bagijn *et al*., 2012). A depiction of the *in vivo* piRNA sensor used in this study can be seen in **Figure 5A**. The sensor is comprised by the coding sequence of *egfp* fused to the coding sequence of histone H2B (*his-58* in *C. elegans*) and their transcription is controlled by the promoter of a germline-specific gene, *mex-5.* Furthermore, the mRNA has the 3'-UTR of the *C. elegans* homolog of β-tubulin (also named *tbb-2*), which is not known to be regulated by any small RNA pathways. Finally, the component which allows the semi-quantitative analysis of piRNA activity is a sequence complementary to one abundant endogenous piRNA, the 21UR-1. This target-site is just upstream of the *tbb-2* 3'-UTR. The histone H2B will allow EGFP localization to the germline nuclei (Bagijn *et al*., 2012). To genotype the worms regarding the presence of the 21Usensor, PCR with two sets of primers was done (**Table C** in the appendix). One pair of primers targeted sequences inside the 21Usensor, while the other pair binds to the regions flanking the 21Usensor. The latter could only amplify successfully when the 21Usensor is not inserted in the genome. Strains homozygous for the 21Usensor are designated throughout this dissertation and in the appendix as "21Usensor".

Mutagenesis screen

Mutagenesis was done on *prg-1*; 21Usensor (RNAe) worms to find mutants showing reactivation of the 21Usensor. For this, worms were rinsed off their plates and washed

several times with M9. Next, the worms were bleached to achieve synchronization of the individuals for the following generation. After bleaching, eggs were thoroughly washed with M9 and left to hatch overnight. The following day L1 worms were plated on fresh medium. When those worms reached the adult stage, they were washed off the plates with M9 and incubated for 4 hours in 0,24 $mg.\mu L^{-1}$ EMS. After incubation and subsequent washes, healthy looking young adults were picked to new plates and incubated at room temperature. Between 150 and 200 F1 worms were individualized and scored for EGFP as adults under a Zeiss M2Bio epifluorescence microscope. Following two individual mutagenesis experiments, after which no hit was found, the screen was extended to the F3 generation. In this set-up, F2 worms instead of F1 worms were individualized and both the F2 and F3 progenies were scored for EGFP. Once found, the hits were isolated to fresh medium and cultured for future analysis.

RNAi assay

The RNAi experiment was conducted as previously reported (Kamath *et al*., 2003). The strategy used was to deliver the dsRNA to *C. elegans* by feeding them with *E. coli* strains, which contain a plasmid that is responsible for the production of dsRNA against the gene of interest. The dsRNA is produced by base-pairing after bidirectional transcription from two flanking T7 promoters. Furthermore, the vector has a resistance marker to Ampicillin. *E. coli* strains were inoculated in LB with 50 $mg.\mu L^{-1}$ Ampicillin and incubated at 37^{o}C overnight. The promoters are inducible with Isopropyl β-D-1-thiogalactopyranoside (IPTG), therefore 0,2 $mg.\mu L^{-1}$ IPTG was added 3 hours before plating. The medium in the Petri dishes also contains Ampicillin and IPTG in the same concentrations. dsRNA targeting *pos-1* was chosen for this experiment, because it is expressed in the maternal germline and is required for proper embryonic development (Tabara *et al*., 1999a). As a result, the eggs laid by worms that fed *pos-1* dsRNA do not hatch. 21Usensor and *prg-1*; 21Usensor (RNAe) strains were used as positive controls, while RNAi-resistant *mut-7* mutants were used as a negative control (Ketting *et al*., 1999). 10 young L4 worms were picked per strain to 6 cm Petri dishes with *E. coli* expressing dsRNA targeting *pos-1*. Other 10 young L4s per strain were placed in plates seeded with *E. coli* carrying an empty vector (EV), *i.e.* with no dsRNA expression. The next day, each of the adults was individualized into 3 cm plates seeded with the same *E. coli* they were in previously. After 24 hours of laying eggs, adults were removed and the total amount of eggs laid was counted. The next day, for the EV progenies, the dead eggs were counted. For *pos-1* progenies, the hatched L1s were counted, except for *mut-7* worms, where the dead eggs were totaled instead. Results are presented as survival percentages

mean ± standard error of the mean. Statistical significance between *pos-1* and EV treatments was determined by student's T-test, using Microsoft Office Excel 2010 ™. *p-value* < 0.05 was considered as statistically significant.

Genetic crosses

To increase the number of males of a certain worm strain, L4 hermaphrodites were heat-shocked for 6 hours at 32 ^{o}C, or 1 hour at 37 ^{o}C. This method increases the probability of chromosome non-disjunction events in meiosis, leading to the X0 karyotype in some embryos and the consequent development of males in the next generation. Crosses were set-up with approximately a 2:1 sex-ratio (males/females), according to male availability. 6 cm Petri dishes with just 20 µL of *E. coli* were used for the crosses to increase the chance of mating. F1 plates with approximately 50% of males were selected and L4s picked to lay eggs for the next generation. After 24 hours of laying eggs, the worms were lysed and an allele present in the strain from which males were used was genotyped, to confirm that the next generation was indeed cross-progeny. The allele used for genotyping depended on the males used in each cross. Next, F2 adults were scored for EGFP expression on a Zeiss M2Bio microscope, lysed and genotyped by conventional PCR for the 21Usensor and the mutant allele introduced. Moreover, Sanger sequencing was used to genotype *prg-1 (pk2298)*. F2 worms homozygous for the 21Usensor and the mutant allele introduced were selected to proceed to the F3. Scoring for EGFP expression and genotyping was repeated on the F3. For a complete list of the primers used in this study for genotyping, consult **Table C** in the appendix. Chromatograms from sequencing were analyzed using FinchTV version 1.4.0 software developed by the Geospiza Research team (http://www.geospiza.com). The *smg-2* and *smg-5* alleles were not genotyped by PCR. Instead, homozygosity was assessed by the characteristic protruded vulva phenotype (Hodgkin *et al*., 1989).

Complementation crosses were set with preferentially the same sex-ratio mentioned above (2 males per 1 female) according to male availability. Germline EGFP expression was scored in F1 worms from plates with approximately 50% males, indicative of cross-progeny.

Image acquisition and processing

Images were acquired with a Leica DM6000 epifluorescence microscope equipped with EGFP fluorescence filters and with differential interference contrast microscopy (DIC). The same exposure times and illumination were applied to all the pictures taken. Worms were sedated directly on the slide, with 0.01 M sodium azide (NaN_3). Acquiring and

processing was done using Leica LAS software. Also, ImageJ version 1.45s, NIH was used to process the fluorescence and DIC pictures (http://imagej.nih.gov/ij).

III. RESULTS

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Requirements for RNAi-related factors in the maintenance of RNAe

Several factors previously implicated in RNAi pathways were tested for involvement in RNAe. This was done by crossing *prg-1*; 21Usensor (RNAe) worms (**Figure 5F, G** and **Figure 6A**) with strains carrying mutations in genes of interest. **Table 1** shows which genes were tested and which did reactivate the sensor.

Table 1. RNAi-related factors involved in the maintenance of RNAe.

Genotype	Gene function	Germline EGFP
mut-7 (pk204)[1] *mut-7 (pk204); prg-1 (pk2298)*[1]	3'-to-5' exonuclease; 22G biogenesis	++[2]
nrde-1 (gg088) *nrde-1 (gg088); prg-1 (pk2298)*	Nuclear RNAi	+[3] ++[3]
nrde-2 (gg091); prg-1 (pk2298)	Nuclear RNAi	++[2]
hpl-2 (ok916) *hpl-2 (ok916); prg-1 (pk2298)*	HP1 homolog	-
smg-2 (e2008) *smg-2 (e2008); prg-1 (pk2298)*	Component of the NMD pathway; 22G biogenesis	-
smg-5 (r860)	Component of the NMD pathway; 22G biogenesis	-

The alleles used are between parentheses. NMD, nonsense-mediated decay.

[1]Both reciprocal crosses were done and showed the same result.

[2]EGFP expression detected in the second homozygous generation.

[3]EGFP expression detected in the F3. The F2 was not scored for EGFP.

MUT-7, a putative 3'-to-5' exonuclease was shown to be required for TE silencing (Ketting *et al*., 1999) and 22G-RNA biogenesis (Bagijn *et al*., 2012; Das *et al*., 2008; Gu *et al*., 2009). As expected, MUT-7 is required for the maintenance of RNAe (**Figure 6B and C**). NRDE-1 and NRDE-2, factors involved in nuclear RNAi (Burkhart *et al*., 2011; Guang *et al*., 2010), are also required for RNAe (**Figure 6D-F**). HPL-2, is one of the *C. elegans* Heterochromatic protein 1 (HP1) homologs that was shown to be required for RNAi in the soma (Grishok *et al*., 2005), silencing of repetitive sequences in the germline (Robert *et al*., 2005) and germline development (Couteau *et al*., 2002). Unexpectedly, *hpl-2* mutants did not revoke RNAe. Also, *smg-2* and *smg-5*, two genes involved in the nonsense-mediated decay pathway that were shown to be involved in RNAi (Domeier *et al*., 2000; Gu *et al*., 2009), namely in the synthesis of 22G-RNAs, did not reactivate the sensor.

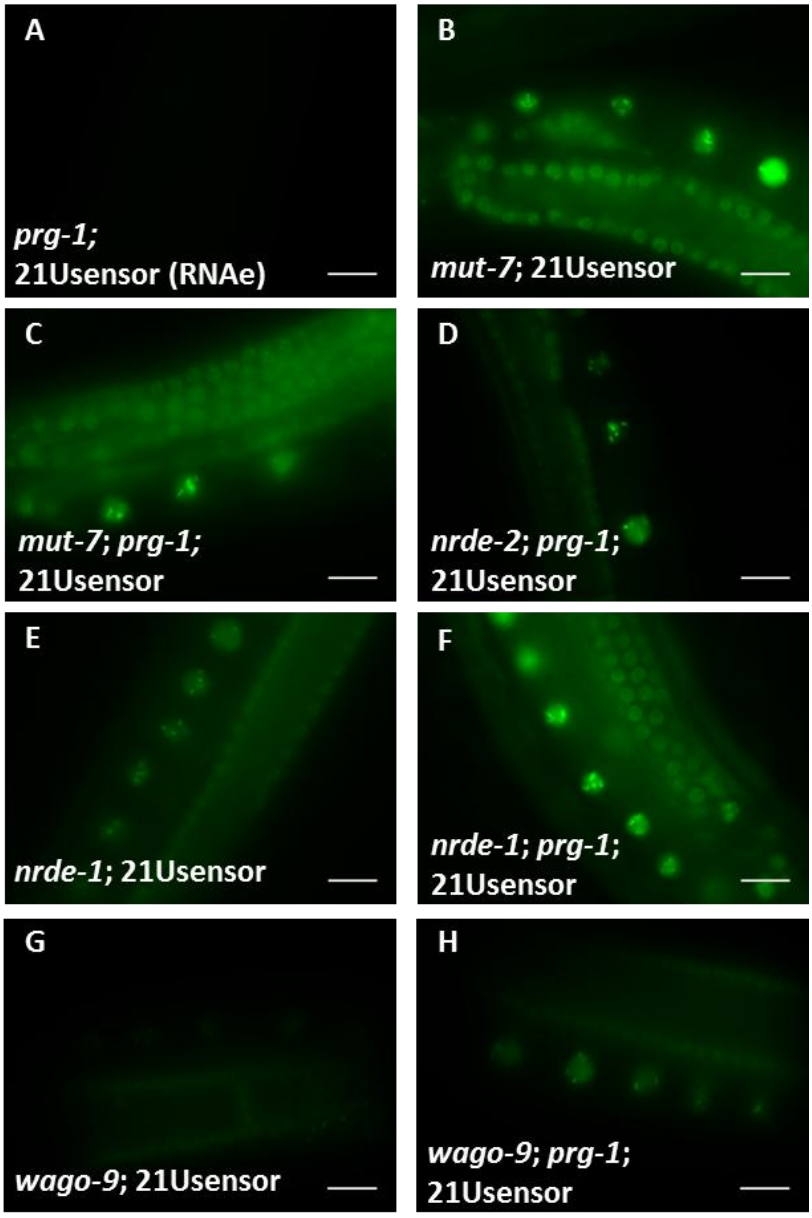

Figure 6. Different degrees of 21Usensor reactivation after RNAe loss. (**A**) *prg-1*; 21Usensor (RNAe) strain showing that the 21Usensor is silenced in the germline. (**B-F**) Representative fluorescence images of adult worms from strains that lost RNAe, showing reactivation of the 21Usensor. In those cases, reactivation was observed after crossing-in mutant alleles into the *prg-1*; 21Usensor (RNAe) background. Each of the pairs (**B**) and (**C**), (**E**) and (**F**), (**G**) and (**H**) was obtained from the same cross. In those cases, an enhanced expression can be seen in the worms that also bear the *prg-1* mutation. Scale bars represent 25 µm.

Some interesting observations were done regarding the way RNAe is lost. Indeed, EGFP expression was only detected in the F3 progeny of F2 homozygous mutants for *mut-7* or *nrde-2*. All the F2s were still positive for RNAe, even the *mut*-7 or *nrde-2* homozygous mutants carrying at least one copy of the 21Usensor (one copy of the 21Usensor is enough to detect EGFP expression). Also, the reactivation was independent of the *prg-1* mutation. F3 worms that showed EGFP expression, upon the loss of RNAe, were homozygous for *mut-7 or nrde-2* but their *prg-1* genotype varied. The F2 parents of F3 worms that reactivated the 21Usensor also had variable *prg-1* genotypes. The EGFP expression was not scored in the

F2s of the *nrde-1* cross, but it seems likely that it followed the same pattern of reactivation of *mut-7* and *nrde-2*, therefore being silenced by RNAe in the F2.

The first cross set (*mut-7* x *prg-1*; 21Usensor (RNAe)) used *mut-7* males. Early studies with *mut-7* identified a parental effect in RNAi resistance and TE activation (Ketting *et al*., 1999). When a mutant *mut-7* allele was transmitted maternally, TEs were derepressed and RNAi resistance was enabled immediately in the first homozygous generation. If the mutant allele was transmitted by the male, the phenotypic effect would only appear in the second or third homozygous generation. Thus, to see if such effect happens in RNAe, the reciprocal cross was done. This cross also showed a loss of RNAe in the F3, the second generation homozygous for the mutant allele, indicating that such parental effect is not occurring in RNAe (**Table 1**).

Worm-specific Argonaute requirements for the maintenance of RNAe

It is very likely that Argonautes would be involved in RNAe. As such, we sought to identify, by genetic crosses, which WAGOs were involved in this process. The Argonautes with high aminoacid similarity to NRDE-3, which are also thought to have nuclear functions, were chosen to test if they are required for the maintenance of RNAe (NRDE-3 clade in **Figure 1B**). Previously, these WAGOs were shown to act together with 22G-RNAs exerting gene silencing (**Figure 3 and Figure 4**) (Gu *et al*., 2009; Guang *et al*., 2008; Yigit *et al*., 2006). From the *wago* mutant alleles tested, only *wago-9* reactivated the 21Usensor, thereby depleting RNAe (**Table 2** and **Figure 6G, H**). Similarly to what was observed for *mut-7* and *nrde-2*, EGFP expression was only observed in the F3, the second homozygous generation for *wago-9.* Again, *prg-1* did not influence the reactivation. Furthermore, loss of functional NRDE-3 did not deplete RNAe (**Table 2**). This was expected because NRDE-3 was shown to be expressed in somatic nuclei and not in the germline (Guang *et al*., 2008).

Table 2. Requirements for Worm-specific Argonautes in RNAe.

Genotype	Gene function	Germline EGFP
wago-9 (tm1200)	Germline nuclear WAGO	+/-[1]
wago-9 (tm1200); prg-1 (pk2298)		+[1]
wago-10 (tm1332)	Nuclear WAGO	-
wago-10 (tm1332); prg-1 (pk2298)		
wago-11 (tm1127); prg-1 (pk2298)	Nuclear WAGO	-
C04F12.1 (tm1637)	WAGO	-
wago-10 (tm1332); wago-11 (tm1127); prg-1 (pk2298)	Nuclear WAGOs	-
nrde-3 (gg066)	Somatic nuclear WAGO	-
nrde-3 (gg066); prg-1 (pk2298)		

The alleles used are between parentheses.

[1]EGFP expression detected in the second homozygous generation.

The 21Usensor is reactivated in different degrees after RNAe loss

In all crosses wherein the 21Usensor showed reactivation, this reactivation *per se* was independent of the *prg-1* genotype. However, the 21Usensor was reactivated in different degrees, *i.e.* different levels of EGFP expression were observed, depending on the presence or absence of functional PRG-1. This relationship is illustrated in **Figure 6B-H** and is conceptually organized in the **Tables 1 and 2** according to a relative hierarchy of expression levels (+/- < + < ++). The *mut-7* strains comprised an exception in the sense that both *mut-7*; 21Usensor and *mut-7*; *prg-1*; 21Usensor showed, approximately, the same levels of EGFP (++, **Figure 6B, C**). *nrde-1*;*prg-1*;21U, *nrde-2*; *prg-1*;21Usensor and both *mut-7* strains showed the strongest expression (++, **Figure 6B-D, F**). *nrde-1*; 21Usensor worms showed a slightly inferior level of expression (+, **Figure 6E**). Unfortunately, no *nrde-2*; 21Usensor worms with functional PRG-1 could be isolated and consequently no such strain was founded and used for comparison with **Figure 6D**. Given that NRDE-1 and NRDE-2 belong to the same nuclear pathway, it seems likely that EGFP expression levels would have been similar between *nrde-1*; 21Usensor (+, **Figure 6E**) and *nrde-2*; 21Usensor, comparable to the relation of *nrde-1*; *prg-1*; 21Usensor (++, **Figure 6F**) to *nrde-2*; *prg-1*; 21Usensor (++, **Figure 6D**). Finally, *wago-9* strains showed lower EGFP expression levels (**Figures 6G, H**). In *wago-9*; *prg-1*; 21Usensor (**Figure 6H**), the expression levels resemble those of *nrde-1*; 21Usensor (+, **Figure 6E**), while *wago-9*; 21Usensor show the lowest EGFP expression levels (+/-, **Figure 6G**). Altogether, these results suggest that, after depletion of RNAe, different factors may contribute differently to the 21U-RNA and downstream pathways.

RNAi-sensitive hit found in a mutagenesis screen

To find other factors involved in RNAe, *prg-1*; 21Usensor (RNAe) worms were mutagenized with EMS and scored for EGFP expression in the F2. After two independent experiments, after which no hit was found, the screens were further extended into the F3 generation. Then, both the F2 and F3 generations were scored for EGFP. Consistent with the results reported above for the genetic crosses about 21Usensor reactivation in the F3, as soon as the screen was extended, worms depleted of RNAe were found. Overall, six independent mutagenesis experiments retrieved eight RNAe-depleted mutants from over 660 progenies screened. From experiment III, six hits were picked and the other two hits were isolated from experiments IV and V. Lamentably, three of the hits were not fertile. The five remaining hits are all derived from the III experiment and show stable EGFP expression in the germline. They were designated as EMSIII S1; EMSIII S3; EMSIII S4; EMSIII 261 and EMSIII 274. It should be noted that the hits also bear the *prg-1* mutation and the 21Usensor, because the worms that were mutagenized were *prg-1*; 21Usensor (RNAe). Next, complementation crosses between the hits were done. For this, males and females from different hits were put together to mate and the F1 generation was scored for EGFP expression. All the crosses between the hits did not show complementation, since EGFP expression was also observed in the F1. These crosses indicate that the hits either carry different mutations in the same gene, or the different hits are indeed the same mutation (**Table 3**). The latter hypothesis is more likely, because the hits were recovered from the same mutagenesis experiment, and five hits with different mutations in the same gene would be most unlikely. The hits were subsequently frozen for posterior analyses and the following experiments were done only in EMSIII S3; *prg-1*; 21Usensor. For simplicity it will be addressed from now on as hit S3; *prg-1*; 21Usensor.

Table 3. Complementation crosses.

Cross	F1's Germline EGFP
Hit S1; *prg-1*; 21Usensor x Hit S3; *prg-1*; 21Usensor	+
Hit S4; *prg-1*; 21Usensor x Hit S3; *prg-1*; 21Usensor	+
Hit 274; *prg-1*; 21Usensor x Hit S3; *prg-1*; 21Usensor	+
Hit 261; *prg-1*; 21Usensor x Hit 274; *prg-1*; 21Usensor	+
Hit 261; *prg-1*; 21Usensor x Hit S4; *prg-1*; 21Usensor	+
Hit S1; *prg-1*; 21Usensor x Hit 274; *prg-1*; 21Usensor	+
Hit 261; *prg-1*; 21Usensor x Hit S3; *prg-1*; 21Usensor	+
All hits x 21Usensor	-
Hit S3; *prg-1*; 21Usensor x *mut-7 (pk204)*	-
Hit S3; *prg-1*; 21Usensor x *wago-9 (tm1200)*	-
Hit S3; *prg-1*; 21Usensor x *nrde-2 (gg091)*	-
Hit S3; *prg-1*; 21Usensor x *nrde-4 (gg129)*	-

The alleles used are between parentheses.

More complementation crosses were done to exclude possible candidates. An overview of such crosses is shown in **Table 3**. Since RNAe does not seem to follow conventional Mendelian inheritance, as a control for the complementation crosses, the hits were crossed with 21Usensor worms – with wild-type background – and EGFP was scored in the F1. As expected, complementation was observed (**Table 3**), validating further complementation crosses. The complementation crosses with selected genes indicate that, in principle, the hit is not mutated in *mut-7*, *wago-9*, *nrde-2* or *nrde-4* (**Table 3**). Next, to address the sensitivity of the hit to RNAi, an experiment using *pos-1* dsRNA was done. RNAi against *pos-1* arrests embryogenesis and as a consequence the eggs don't hatch (Ketting *et al*., 1999). This experiment would indicate in which RNAi pathway the mutant allele belongs. **Figure 7** shows clearly that hit S3; *prg-1*; 21Usensor worms are RNAi-sensitive. Ten separate hit S3; *prg-1*; 21Usensor worms fed on *pos-1* RNAi laid a total of 807 eggs and remarkably, not even one egg hatched. This suggests that the hit does not belong to the RNAi defective (Rde) class, since worms carrying the hit can respond to dsRNA. Further experiments should be done to elucidate if the hit is hypersensitive to dsRNA. Also, Northern Blots to specific sRNA species and chromatin immunoprecipitation would be of great interest to this matter.

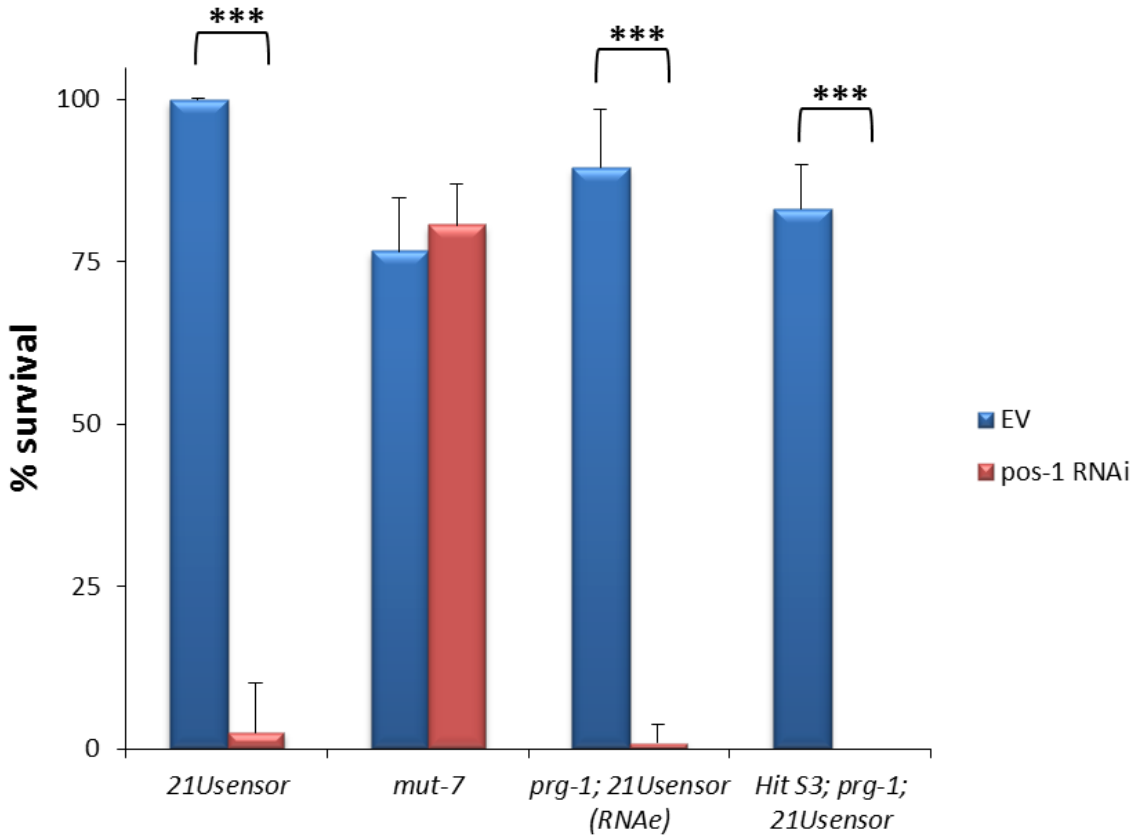

Figure 7. Hit S3; *prg-1*; 21Usensor worms are sensitive to RNAi. Experiment to assess RNAi resistance of hit S3; *prg-1*; 21Usensor worms. 21Usensor and *prg-1*; 21Usensor (RNAe) were used as positive controls, while *mut-7* mutants are RNAi-resistant and as such were used as a negative control. Blue bars represent survival percentage of the eggs laid by worms fed on *E. coli* with an empty vector (EV), while the red bars indicate the survival of eggs laid by worms that fed on *pos-1* RNAi. The progenies of 10 worms fed on EV and 10 worms fed on *pos-1* RNAi were counted for each strain. Error bars represent the standard error of the mean. *** *p-value* < 0,001.

IV. DISCUSSION

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Here I describe part of the genetic requirements for the maintenance of a new germline gene silencing phenomenon in *C. elegans*, termed RNAe. Once established, it is absolutely dominant and can be propagated indefinitely for a great number of generations without any observable signs of reversion (Luteijn *et al*., 2012). During the course of this work, others also reported this kind of dominant and heritable multigenerational gene silencing (Ashe *et al*., 2012; Shirayama *et al*., 2012). Similar reporter transgenesis-based experimental set-ups were used in those studies. RNAe was proven to be initiated by two elements: 1) exogenous dsRNA (Ashe *et al*., 2012) and 2) PRG-1 (Ashe *et al*., 2012; Lee *et al*., 2012; Luteijn *et al*., 2012; Shirayama *et al*., 2012). Wild-type PRG-1 was found to trigger the initiation of RNAe, since *prg-1* mutants did not initiate silencing. On the other hand, once RNAe is established, it becomes independent of *prg-1*, as can be seen in **Figure 5D-G** (Ashe *et al*., 2012; Luteijn *et al*., 2012; Shirayama *et al*., 2012).

Maintenance of RNAe requires 22G-RNAs, WAGOs and NRDE proteins

The results presented in this dissertation strongly indicate that 22G-RNAs, WAGOs and the NRDE pathway components are involved in the maintenance of RNAe. Explicitly, MUT-7, WAGO-9, NRDE-1 and NRDE-2 are implicated (**Table 1** and **Table 2**). Others have also sought for genetic factors involved in the maintenance of RNAe and their results are similar to the results described here (Ashe *et al*., 2012; Shirayama *et al*., 2012). Moreover, a mutagenesis screen designed to find factors involved in RNAi inheritance identified *wago-9* (Buckley *et al*., 2012). The authors renamed *wago-9* as *hrde-1* for *heritable RNAi defective 1*. WAGO-9 is expressed in germline nuclei (Buckley *et al*., 2012; Shirayama *et al*., 2012). It is thought to have a function paralleled to NRDE-3 in the soma: associating with 22G-RNAs and engaging the NRDE pathway, thereby promoting gene silencing (Ashe *et al*., 2012; Bagijn *et al*., 2012; Buckley *et al*., 2012; Luteijn *et al*., 2012; Shirayama *et al*., 2012).

Curiously, the non-requirement for HPL-2 was divergent from parallel studies which demonstrated a requirement for HPL-2 in RNAe (Ashe *et al*., 2012; Shirayama *et al*., 2012). However, Shirayama and colleagues noted that transgenes reactivated in *hpl-2* mutants showed low EGFP expression levels, in the germline (Shirayama *et al*., 2012). On the other hand, they note that *hpl-2*; *hpl-1* double mutants show higher EGFP levels. Indeed, those two HP1 homologs are known to act redundantly in the germline, although *hpl-2* activity seems to be preponderant (Couteau *et al*., 2002). Two different *hpl-2* deletion alleles were used to test the requirement for *hpl-2* in RNAe: in the present study *hpl-2 (ok916)* was used while

Shirayama *et al*. and Ashe *et al*. used *hpl-2(tm1489)*. *hpl-2(tm1489)* deletes the upstream region of the gene, probably deleting essential *cis*-regulatory elements. On the other hand, *hpl-2(ok916)* deletes the C-terminal portion of the protein (the features of both alleles can be found on http://www.wormbase.org). The different outcomes on the requirement of *hpl-2* for RNAe can be due to allelic differences. While in *hpl-2(tm1489)* there may not even be an *hpl-2* transcript, in *hpl-2(ok916)* there may be a transcript and a truncated form of the HPL-2 protein. Assuming that the truncated protein can fold properly, it could still have an hypomorphic effect that would be partially rescued by redundancy with HPL-1. This hypothesis is further supported by the predicted existence, in *hpl-2(ok916)*, of a N-terminal Chromo-Domain that is not included in the deletion.

Mutations in the *smg* genes did not reactivate the 21Usensor (RNAe). Probably they are only required for the biogenesis of a specific subclass of endogenous, genomic-encoded 22G-RNAs (Gu *et al*., 2009). This observation highlights the existence of shared and particular factors for each subpopulation of 22G-RNAs.

The genetic requirements assessed here (**Table 1**, **Table 2**, **Figure 6**) shed some light on the mechanistic basis of the maintenance of RNAe and a working model is proposed (**Figure 8**). This model depicts the events that maintain RNAe in *C. elegans* germ-cells, highlighting an extensive interplay between cytoplasmic and nuclear reactions. In the cytoplasm, a RdRP is recruited to the transcript of the gene/transgene initiating formation of 22G-RNAs. MUT-7 is presumably involved in a processing step of 22G-RNAs. WAGO-9 is thought to shuttle the 22G-RNAs to the nucleus and may subsequently act similarly to NRDE-3 in somatic nuclei, recruiting NRDE-1 and NRDE-2 to nascent transcripts (Burkhart *et al*., 2011; Guang *et al*., 2010; Guang *et al*., 2008). In turn, the NRDE proteins engage chromatin remodelling factors that reinforce H3K9me3. It is hypothesized that RNA Polymerase II elongation may also be disturbed. In agreement with this hypothesis is the RNAe requisite for SET and MES proteins (Ashe *et al*., 2012; Shirayama *et al*., 2012). Those proteins deposit repressive chromatin marks. In addition, NRDE-4, the other known protein involved in the NRDE pathway, was also implicated in the maintenance of RNAe (Ashe *et al*., 2012).

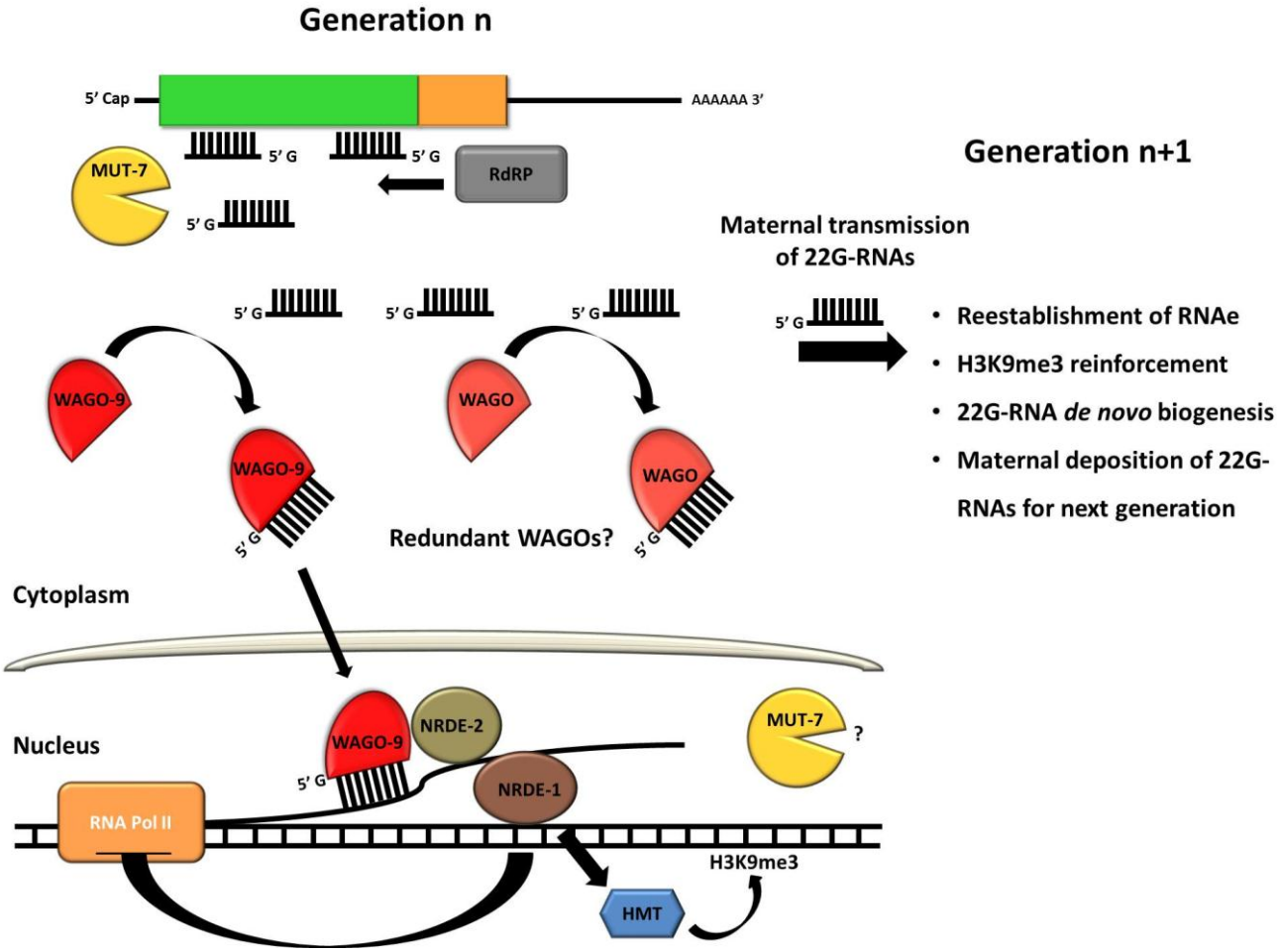

Figure 8. A model for the maintenance of RNAe in the germline of *C. elegans*. Transcripts of sequences under RNAe will lead to 22G-RNA synthesis by a RdRP. MUT-7 is involved in processing the 22G-RNAs, which are subsequently loaded into WAGO-9 that will go to the nucleus. There, it will recruit NRDE factors to prompt H3K9me3 deposition. Other WAGOs may also act redundantly together with WAGO-9. It should be noted that PRG-1 is required for complete RNAe. The 22G-RNAs are transmitted by the mother and will, in turn, re-create RNAe in the next generation by H3K9me3 reinforcement. 22G-RNAs are probably synthesized *de novo* and once again transmitted to the progeny. HMT, histone methyltransferase; RdRP, RNA-dependent RNA Polymerase; RNA Pol II, RNA Polymerase II.

Transmission and reestablishment of RNAe in every generation

The molecular interplay described above only portrays events happening in a single generation. However, there must be maintenance of the silencing in the next generations. This implies the existence of, at least, one signal that should be able to be propagated through the developing germ-cells without any kind of dilution effect (by dilution effect it is meant the partition in meiosis of a diffusible silencing signal to every daughter cell). This may be achieved by the segregation of 22G-RNAs through the mother (**Figure 8**). Accordingly, these sRNA species were previously reported to be maternally deposited in the embryos (Gu *et al*., 2009). Those 22G-RNAs would reinitiate RNAe in every generation, thereby

reinforcing H3K9me3. This is supported by the loss of 21Usensor-targeting 22G-RNAs and H3K9me3 in *nrde-1* and *wago-9* mutant backgrounds concomitant to the loss of RNAe (Luteijn *et al*., 2012). Also, transmission of sRNA species to the next generation was shown to prelude deposition of H3K9me3 (Burton *et al*., 2011; Gu *et al*., 2012). In those studies RNAi-induced H3K9me3 eventually disappears after 2-3 generations. However, in the present study, we show that RNAe lasts for an indefinite number of generations. This means that in every single generation the signal – 22G-RNAs – must be made *de novo* in order to avoid a "dilution" effect after oogenesis.

No differential reactivation of the 21Usensor was observed in two reciprocal crosses of *mut-7* mutant alleles (**Table 1**). This was unexpected given previous observations of differential phenotypic effects of *mut-7* depending on the sex segregating the mutant allele (Ketting *et al*., 1999). Even though no such parental effect was found, the consistent reactivation of the 21Usensor in the F3 generation (**Table 1 and Table 2**) is demonstrative of a striking maternal effect inherent to this system. For *nrde-1*, EGFP expression in the first homozygous generation was not addressed, but it seems likely that it followed the same pattern as *mut-7*, *wago-9* and *nrde-2.* Upon the impairment of RNAe, there is the cessation of *de novo* 22G-RNA biogenesis and deposition of repressive chromatin marks (Luteijn *et al*., 2012). According to this reasoning, after crossing-in a mutant allele for a component of RNAe, the heterozygous F1 still shows RNAe due to the inherited 22G-RNAs. Those are antisense to the 21Usensor and will reinforce H3K9me3 in the 21Usensor. Also, due to heterozygoty, there would still be *de novo* synthesis of 22G-RNAs and their transmission to the next generation. In the homozygous mutant F2, the inherited 22G-RNAs would restart RNAe. On the other hand, there would be no *de novo* generation of 22G-RNAs, thereby disabling the reestablishment of RNAe for the next generation. The recurrent reactivation of the 21Usensor in the second homozygous generation supports the model whereby maternally inherited and synthesized *de novo* 22G-RNAs assure maintenance and transmission of silencing across generations.

Diverse RNAi pathways may act redundantly in RNAe

Indications of functional redundancy have been found in the *C. elegans* RNAi pathways. Specifically, 22G-RNA biogenesis seems to depend on redundant activities of two RdRPs, EGO-1 and RRF-1 (Gu *et al*., 2009). Also, the WAGOs seem to act, together with the 22G-RNAs, in an extremely redundant way. Interestingly, the WAGOs must somehow contribute to 22G-RNA biogenesis, because in multiple WAGO mutants 22G-RNAs seem to be depleted (Gu *et al*., 2009; Yigit *et al*., 2006). There are several observations that point out for extensive crosstalk between the cytoplasm and the nucleus, during RNAe. WAGO-1 was

previously reported to locate to the cytoplasm adjacent to the nucleus, in germ-cells (Gu *et al*., 2009). Shirayama and colleagues showed that WAGO-1 is required for the maintenance of RNAe in a specific transgene (Shirayama *et al*., 2012) together with WAGO-9 (**Table 2**, **Figure 6G, H**) (Ashe *et al*., 2012; Shirayama *et al*., 2012). So, both nuclear and cytoplasmic Argonautes seem to be involved in RNAe. Furthermore, after RNAe depletion, *wago-9* shows very weak EGFP levels (**Figure 6**). This shows that WAGO-9 is probably not the only WAGO performing the shuttling of 22G-RNAs from cytoplasm to the nucleus. It also should be noted that MUT-7 was previously found in nuclear extracts and as such, nuclear functions are not ruled out (Tops *et al*., 2005). Given the profound involvement of MUT-7 in 22G-RNA biogenesis, it would be interesting to see if 22G-RNA biogenesis occurs also in, or in close association with, the nucleus.

Altogether, those observations indicate that redundancy may be observed mainly at two levels during RNAe: 1) between cytoplasmic and nuclear RNAi pathways and 2) between concurrent WAGOs within the same RNAi pathway. Thus, it seems that redundancy is a recurrent theme in RNAi pathways in worms.

RNAe and the immortal germline

The germline can be considered as an immortal cell lineage for its potential of being transmitted indefinitely from generation to generation. Forward genetic screens identified mortal germline mutants (Ahmed and Hodgkin, 2000). Those mutants can reproduce for several generations but eventually become sterile. This is thought to occur due to defective germ-cell development. Several defects will accumulate over generations, until an evolutionary dead-end is reached – sterility (Ahmed and Hodgkin, 2000; Buckley *et al*., 2012). Genes described in this dissertation as being involved in RNAe also share a mortal germline phenotype. *nrde-1*, *nrde-2* and *wago-9* mutants have been reported to become sterile after 4-5 generations, showing a mortal germline phenotype (Buckley *et al*., 2012). Taking into account the shared factors between RNAe and germline immortality, it is plausible to argue that RNAe may be important for the maintenance of germline immortality. If true, this link establishes a role for RNAe components with great evolutionary and developmental importance.

Evolutionary implications of RNAe

Given the importance of the germline, it is believed that genome and transcriptome-wide surveillance mechanisms must be very active in order to maintain germline integrity.

piRNAs and RNAe seem to play a critical role. Others proposed that 21U-RNAs and PRG-1 provide a platform for transcriptome-wide surveillance in the germline that will scan for foreign sequences, binding them in a mismatch-tolerating way (Bagijn *et al*., 2012; Lee *et al*., 2012). Consequently, the WAGO and NRDE pathways will stably silence such foreign sequences, thus providing a molecular memory of such sequences (Ashe *et al*., 2012; Lee *et al*., 2012; Luteijn *et al*., 2012; Shirayama *et al*., 2012). Another pathway is proposed to act in parallel, providing a molecular memory of the self, recognizing and protecting endogenous sequences. CSR-1 and its associated 22G-RNAs were proposed to exert this anti-silencing effect to RNAe (Lee *et al*., 2012; Shirayama *et al*., 2012). Worm populations with single-copy transgenes show phenotypic heterogeneity, *i.e.* some worms show transgene silencing, while others do not (Ashe *et al*., 2012; Luteijn *et al*., 2012; Shirayama *et al*., 2012). So, RNAe appears to be established in a partly stochastic way. One of two alternative outcomes, either being silenced or expressed, may arise after the resolution of a balance between the self- and nonself-recognizing pathways. In this scenario, the transgene would be silenced if the PRG-1 effect, binding to foreign sequences like *egfp*, would surpass the effect of CSR-1, binding to self-sequences like the endogenous sequences in the transgene. Conversely, active transgenes will arise after superiority of CSR-1 over PRG-1. (Lee *et al*., 2012; Shirayama *et al*., 2012).

RNAe provides a mechanistic basis for the control of foreign sequences, as TEs and viruses. Foreign nucleic acids can have potentially harmful consequences for the organisms, so their stable and permanent silencing is important. It should be noted that the stochasticity inherent to the initiation RNAe may have adaptive value. It reflects that the system is not completely refractory to foreign sequences, *i.e.* the licensing of foreign sequences could be beneficial under some specific circumstances, *e.g.* stress. In this sense, testing initiation of RNAe under different stress conditions could be informative. Much like plants, RNAi has been implicated in viral defense in *C. elegans* (Lu *et al*., 2005; Rechavi *et al*., 2011; Schott *et al*., 2005; Wilkins *et al*., 2005) and in one of those cases, a transgenerational inheritance of sRNAs was observed (Rechavi *et al*., 2011). RNAe, especially the PRG-1 pathway targeting foreign sequences, may provide the mechanistic basis for the defense against viruses. Also, it should be noted that RNAe is similar in some extent to paramutation, a phenomenon where some alleles can be heritably silenced after interactions with other alleles or spontaneously (Erhard and Hollick, 2011). Similarly to RNAe, RNA is thought to be subjacent to paramutation. It will be interesting to check if Piwi proteins are responsible for paramutation and viral defense.

Concluding remarks

The recent findings on RNAe in *C. elegans*, including the results described in this dissertation, establish a germline surveillance function for Piwi proteins and piRNAs, which yields an extremely stable gene silencing. This form of gene silencing is singular in many biological aspects. It has implications in the most diverse subjects, such as evolutionary biology, development, immunity and molecular biology. By taking advantage of the *C. elegans* genetic toolkit, further developments can be expected in our understanding of RNAe. Some areas of focus for future research could include the search for more factors involved in RNAe and the overlap between RNAe and germline immortality. Also, further confirmation and elaboration of the self/nonself memory model, as well as its consequences on the long-term and immunity will be valuable. The evolutionary conservation of RNAe should also be addressed. Mammal pachytene piRNAs appear as good candidates for transcriptome-wide surveillance in the germline, given their great numbers and lack of obvious targets (Aravin *et al*., 2006; Girard *et al*., 2006). It will be interesting to see whether pachytene piRNAs can trigger stable transgenerational silencing of foreign sequences.

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VI. APPENDIX

VI. APPENDIX

Table A. Worm strains used in this study.

Genotype	Name	Supplied by
Wild-type	N2 Bristol	Inhouse
21Usensor II	mjIs144	Eric Miska
prg-1 (pk2298) I; 21Usensor II;	RFK0049	Inhouse
prg-1 (pk2298) I; 21Usensor (RNAe) II	-	Inhouse
mut-7 (pk204) III	NL917	Inhouse
nrde-1 (gg088) III	YY160	CGC
nrde-2 (gg091) II	YY186	CGC
nrde-3 (gg066) X	YY158	CGC
nrde-4 (gg129) IV	YY453	CGC
smg-2 (e2008) I; him-5 (e1490) V	CB4043	CGC
smg-5 (r860) I	TR1335	CGC
hpl-2 (ok916) III	RB995	CGC
wago-9 (tm1200) III	-	Craig Mello
wago-10 (tm1332) V	-	Craig Mello
wago-11 (tm1127) II	WM157	CGC
C04F12.1 (tm1637) I	WM153	CGC

Table B. Strains created in this study.

Genotype	Germline eGFP
21Usensor II; mut-7 (pk204) III	+
prg-1 (pk2298) I; 21Usensor II; mut-7 (pk204) III	+
21Usensor II; wago-9 (tm1200) III	+
prg-1 (pk2298) I; 21Usensor II; wago-9 (tm1200) III	+
21Usensor II; wago-10 (tm1332) V	-
prg-1 (pk2298) I; 21Usensor II; wago-10 (tm1332) V	-
prg-1 (pk2298) I; wago-11 (tm1127) II; 21Usensor II	-
C04F12.1 (tm1637) I; 21Usensor II	-
prg-1 (pk2298) I; wago-11 (tm1127) II; 21Usensor II; wago-10 (tm1332) V	-
C04F12.1 (tm1637) I; wago-11 (tm1127) II; 21Usensor II	-
21Usensor II; nrde-1 (gg088) III	+
prg-1 (pk2298) I; 21Usensor II; nrde-1 (gg088) III	+
prg-1 (pk2298) I; nrde-2 (gg091) II; 21Usensor II	+
21Usensor II; nrde-3 (gg066) X	-
prg-1 (pk2298) I; 21Usensor II; nrde-3 (gg066) X	-
smg-5 (r860) I; 21Usensor II	-
smg-2 (e2008) I; 21Usensor II	-
21Usensor II; hpl-2 (ok916) III	-
prg-1 (pk2298) I; 21Usensor II; hpl-2 (ok916) III	-

Table C. Primers used in this study

Sequence	Use
CTGCTTCAAAGACGTTTTCTCG	Genotype 21Usensor. Forward
GCATGACGTCTCTTCTTTCCG	Genotype 21Usensor. Reverse
TTCTTGAAGACGACGAGCCACTTG	Genotype wild-type without 21Usensor. Forward
ACGCCCAGGAGAACACGTTAGTTT	Genotype wild-type without 21Usensor. Reverse
TGTAAAACGACGGCCAGTCCCATCCTTCACGAATTCAC	Genotype *prg-1 (pk2298)* by sequencing. Forward
TCAACTGGGGAGATTCTCGT	Genotype *prg-1 (pk2298)* by sequencing. Reverse
TGTGTTGGGGCTAGGGATAC	Genotype *wago-10 (tm1332)*. Forward
GGATGGCCGAATTTTCATAA	Genotype *wago-10 (tm1332)*. Reverse
CCCTTGTCGTCCTTCATCAT	Genotype *wago-9 (tm1200)*. Forward
TTCGTGTCGAAGTTGTCTCG	Genotype *wago-9 (tm1200)*. Reverse
GGAGGAGAGCGTCAATTCAG	Genotype *wago-11 (tm1127)*. Forward
TCTCTGTGGACCCATTTTCC	Genotype *wago-11 (tm1127)*. Reverse
TCACGCCACCATACTTCAGA	Genotype *C04F12.1 (tm1637)*. Forward
GCACACTTGGTCATGGACTG	Genotype *C04F12.1 (tm1637)*. Reverse
TGCAGAAAATTTGTCGATGC	Genotype mutant *hpl-2 (ok916)*. Forward
CAGTCGGTGAGTTTGGGAAT	Genotype *hpl-2 (ok916)*. Reverse
TCCTCAATCGAACGCTTCTT	Genotype wild-type *hpl-2*. Forward
AGCAAATATCCGTCATTGCA	Genotype wild-type *nrde-1*. Forward
AGCAAATATCCGTCAAAGTA	Genotype mutant *nrde-1 (gg088)*. Forward
CGTTTTCGGCATTGGTAAAT	Genotype nrde-1 (gg088). Reverse
ACCACGTACAAATGTTACGG	Genotype wild-type *nrde-3*. Forward
ACCACGTACAAATGTTTCAG	Genotype mutant *nrde-3 (gg066)*. Forward
AGTTCAAAGCGACGTCCATC	Genotype *nrde-3 (gg066)*. Reverse
GATATTATCTAGATTCTACC	Genotype wild-type *nrde-4*. Forward
GATATTATCTAGATTCAATC	Genotype mutant *nrde-4 (gg129)*. Forward
CAAATATTCGAAACCCTTAGTCC	Genotype *nrde-4 (gg129)*. Reverse
TGAGAATCCAGATCTTATTA	Genotype wild-type *nrde-2*. Forward
TGAGAATCCAGATCTTTTAA	Genotype mutant *nrde-2 (gg091)*. Forward
ATTTTCGCGTCTCTCCTGAA	Genotype *nrde-2 (gg091)*. Reverse