FEBS Letters 579 (2005) 5940–5949 FEBS 29975

Minireview

RNA silencing in *Drosophila*

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Received 20 June 2005; revised 26 August 2005; accepted 28 August 2005

Available online 20 September 2005

Edited by Shou-Wei Ding

Abstract Knowledge of the role of RNA in affecting gene expression has expanded in the past several years. Small RNAs serve as homology guides to target messenger RNAs for destruction at the post-transcriptional level in the experimental technique known as RNA interference and in the silencing of some transgenes. These small RNAs are also involved in sequence-specific targeting of chromatin modifications for transcriptional silencing of transgenes, transposable elements, heterochromatin and some cases of Polycomb-mediated gene silencing. RNA silencing processes in *Drosophila* are described.

Keywords: RNAi; Transgene silencing; Transposons; Heterochromatin; Cosuppression; RNA editing

1. Introduction

The idea of an involvement of an RNA moiety in gene expression processes grew out of studies of transgene silencing in a variety of plant species. The first case involved the finding that transgenes that shared the same promoter in tobacco would silence each other when brought together in a genetic cross [1]. This case was shown to involve transcriptional silencing. Subsequent experiments suggest that the basis of this silencing involves an RNA molecule homologous to the promoter sequences that target them for methylation and therefore silencing [2]. At about the same time, others found that transgenes introduced into Petunia in an attempt to increase flower pigment intensity in fact caused a null mutant phenotype [3,4]. This type of silencing was subsequently found to be post-transcriptional and likely to involve a double-stranded RNA (dsRNA) structure in the process [5]. Transgene silencing was found to occur in the animal kingdom with experiments carried out in *Drosophila*, the initial case being of the transcriptional type [6,7]. The finding in work conducted with *Caenorhabditis elegans*, that the active agent in the technique of RNA interference was a dsRNA [8], stimulated biochemical studies on the nature of this process [9–11]. It is now recognized that RNA plays a role in many aspects of gene expression both transcriptionally and post-transcriptionally [12]. The status of studies on RNA silencing in *Drosophila* will be discussed here.

2. Transgene silencing

The first described case of transgene silencing in *Drosophila* involved a hybrid transgene between the regulatory region of the *white* (*w*) eye color gene and the structural portion of the *Alcohol dehydrogenase* (*Adh*) locus [6]. This transgene was produced as a promoter-reporter construct to study *trans*-acting modifiers of *white* [13]. This *white-Adh* (*w-Adh*) transgene exhibits pairing sensitive silencing in that a paired copy at any one location is expressed less than a single copy at the same genomic site. The finding of interest, however, was that two unpaired copies were not additive in their cumulative expression, but rather the total amount of RNA expressed declined with increasing numbers of transgenes. The endogenous *Adh* gene is also silenced in the process. This silencing mechanism is modified by mutations in the Polycomb complex of repressive chromatin proteins and the silenced copies become associated with the complex as visualized on the salivary gland polytene chromosomes. This type of silencing operates at the transcriptional level [7].

Post-transcriptional silencing is also observed for different transgenes in *Drosophila* [7]. A dosage series of the full length *Adh* gene will show a linear increase in total expression up to about five copies after which there is a departure from linearity. This change is not reflected in transcriptional assays and the silenced copies accumulate small interfering RNAs (siRNA) of 21–23 bp in length as is typical of post-transcriptional silencing.

Interestingly, the *w-Adh* transgenes will silence a reciprocally constructed *Adh-w* transgene despite the fact that they share no homology [14]. This type of silencing is Polycomb dependent and at the transcriptional level [7]. The paradox that silencing occurs between non-homologous sequences was resolved by the realization that the endogenous *Adh* gene was in the silencing pool and could serve as an homology bridge. This connection was demonstrated by deleting the endogenous *Adh* gene and finding that the silencing interaction could not occur in these flies [14]. An analogous case of silencing of the I transposable element has been documented in which non-homologous sequences can silence each other using an endogenous bridge [15].

The known genes involved with RNA interference processes in *Drosophila* are listed in Table 1. Of these, the *piwi* mutation of the argonaute gene family is one example in which the

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0014-5793/$30.00
doi:10.1016/j.febslet.2005.08.069

Table 1

Gene	Role in *Drosophila* RNAi pathway
Dcr-1	Generates miRNA; stable intermediate in the RISC assembly pathway
Dcr-2	Generates siRNA; efficient delivery of siRNA to the RISC complex
AGO1	Generates mature miRNA; associates with Dcr-1
AGO2	Component of the RISC; catalytic core of the RISC complex
AGO3	Unknown
piwi	Argonaute family protein; implicated in heterochromatin and transposon silencing
aub	Argonaute family protein; implicated in germline gene repression
spn-E (homeless)	RNA helicase; implicated in heterochromatin and transposon silencing
armi	RNA helicase; stabilization of the active RISC complex implicated in germline gene repression
Rm62 (*Lip*)	RNA helicase required for RNAi in cultured cells; implicated in heterochromatin and transposon silencing
r2d2	Binds to the 5′ end of the thermodynamically stable strand of the siRNA duplex which is then degraded
vig	Component of the active RISC complex
Tudor-SN	Component of the active RISC complex
Fmr1 (*dFXR*)	Component of the active RISC complex; Fragile X orthologue
	Consult Flybase for further details (http://flybase.net)

mutant flies are viable and could be tested for an effect on post-transcriptional silencing of *Adh* transgenes [7]. In the homozygous mutant flies, this type of silencing is inhibited. The *piwi* mutation will also strongly inhibit the transcriptional silencing of *Adh-w* by *w-Adh*. To a lesser extent, the *aubergine* mutation will also reduce the magnitude of this silencing [16]. These findings established a link among post-transcriptional transgene silencing, transcriptional transgene silencing and the RNAi machinery, which implicates a small RNA as a *trans*-acting mediator of sequence-specific silencing.

Transgene silencing will also occur in a dosage series of *engrailed-white* (*en-w*) constructs illustrating the generality of such silencing [17]. This construct was the one in which pairing sensitive silencing was first recognized [18]. Interestingly, when this transgene was combined with *piwi* or *homeless*, the pairing sensitive silencing was intensified. The expression of the unpaired transgene was not affected by the "RNAi" mutations, but the paired situation showed an effect. If the silencing requires a small RNA as a sequence-specific guide, one might expect a suppression of silencing, but the opposite result was found, illustrating a novel type of action of the RNAi machinery with regard to pairing sensitive silencing.

3. Biochemistry of RNAi in *Drosophila*

As a reverse genetic technique, the RNAi process involves the targeted degradation of the endogenous mRNA by a specific dsRNA [19]. Biochemical studies performed to investigate RNAi have complemented the genetic studies and provided valuable information about the mechanism. A series of experiments have led to the isolation, purification and functional characterization of the molecular components of the RNAi process, which is illustrated in Fig. 1.

The trigger for RNAi in all cases studied to date is a dsRNA molecule, which is cleaved to siRNAs [20–23]. The specificity of RNAi is achieved by the nucleotide complementation between the target mRNA and the dsRNA. The source of dsRNA can be endogenous, such as RNA derived from heterochromatin, transposon repeats and other types of aberrant RNA or it can be exogenous such as injected or viral dsRNA. The most potent source of dsRNA is an expressed inverted repeat, which might give rise to a hairpin loop structure [24]. These types of repeat elements can be introduced into the cell via a plasmid vector containing a part of the target gene in an inverted orientation [25]. A DNA template of about 500–700 bp as multiple copies or as inverted repeats is usually sufficient to trigger RNAi. RNAi can also be produced by 21–23 nt siRNA, when introduced into the cell externally via microinjection, liposomes or other methods.

The enzyme Dicer acts upon the dsRNA trigger. It is a ribonuclease III type of enzyme having an N-terminal DEXH-box RNA helicase domain, a domain of unknown function (DUF283), a PAZ domain, two ribonuclease domains (RIIIa and RIIIb) and a dsRNA binding domain [11]. Dicer acts upon the dsRNA and cleaves it into 21–23 nt small RNAs. Depletion of Dicer inhibits the production of siRNA and hence the entire RNAi mechanism [23,26,27]. Dicer homologs are found in all biological systems where RNAi occurs (e.g., fission yeast, mice, humans, *Arabidopsis*, *Drosophila*, etc.). *Drosophila* has two dicers: *dcr-1* and *dcr-2*. The *dcr-1* gene plays a role in miRNA biogenesis, whereas *dcr-2* is involved in siRNA production [28], although some overlap in function occurs. The role of Dicer is not merely confined to cleavage of the template to produce dsRNA but it also plays a role in the delivery of siRNA molecules to the RISC complex.

The structural integrity of the siRNA functional groups is extremely important for effective RNAi to occur. The siRNA has a 5′-PO_4 group and a 3′-OH group with two nucleotide overhangs at the 3′ end. A series of biochemical experiments have demonstrated that removal or substitution of the 5′-PO_4 from the antisense strand results in complete abolishment of RNAi. A recent biochemical study using human AGO-2 (hAGO2) and siRNA indicates that the phosphate group is not absolutely essential for cleavage of the target mRNA. The presence of a 5′-PO_4 on the siRNA stabilizes its interaction with hAGO-2 and provides fidelity to the RISC with respect to the cleavage site on the target [29]. However, mutations at the 3′ end of the antisense strand as well as on the sense strand of siRNA are relatively well tolerated [30]. It has been experimentally demonstrated that the antisense strand with a 5′-PO_4 serves as the guide strand and is incorporated into the active RISC complex to cleave the target mRNA. The increase or decrease in the number of 3′ end nucleotide overhangs also reduces the efficiency of the RNAi process. Mutations that affect the major groove of the A-Form helix substantially reduce the degradation of the target mRNA because it is believed that the interaction between the siRNA

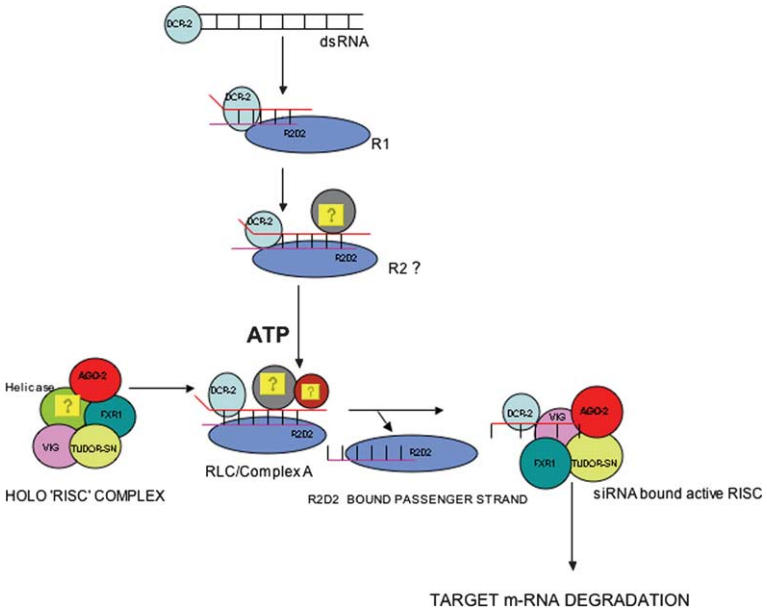

Fig. 1. Assembly of cleavage competent RISC complex. The degradation of dsRNA in the cell by DCR-2 results in 21–23 nt siRNAs (see text for details). Only one strand in the siRNA duplex enters the RNAi pathway and the other strand known as the 'passenger strand' is degraded. Two proteins, DCR-2 and R2D2, form a heterodimeric complex and recognize the thermodynamic asymmetry in the siRNA duplex. The strand, which is thermodynamically unstable, forms the guide strand and is a part of the active RISC complex. siRNA duplex bound by DCR-2/R2D2 constitutes the R1 complex which then forms a transient complex R2. This complex is then activated by ATP to form RISC Loading Complex (RLC) (also known as complex A). It has been shown that AGO2 is exchanged for R2D2 and the passenger strand bound by R2D2 is destroyed. The active RISC complex contains single stranded siRNA bound by AGO2, vasa intronic gene (VIG), TSN (Tudor staphylococcus nuclease) and dFXR1 (Fragile X-related).

loaded RISC complex and the target mRNA involves the recognition of the A-Form helix [31]. The introduction of certain functional groups such as fluoro or phosphorothioates at the 2′ position of the pentose sugar increases the stability of siRNA but is not known to increase the activity relative to the unmodified siRNA found in vivo [30].

The highly conserved argonaute family of highly basic proteins, which have been strongly implicated in RNAi and related phenomenon such as cosuppression and transgene silencing in a wide variety of organisms, has five members in *Drosophila*: *piwi*, *aubergine*, *argonaute-1*, *argonaute-2* and *argonaute-3* (see Table 1). The argonaute proteins have a characteristic PAZ and PIWI domain. The C-terminal PIWI domain interacts with the ribonuclease domain of Dicer, while the PAZ domain is used for promoting other protein–protein interactions. X-ray diffraction studies of the AGO-2 crystal structure from *Pyrococcus furiosus* has indicated that the 5′-PO_4 group of siRNA binds to the PIWI domain while the 3′ end of the siRNA tightly interacts with AGO-2 in such a way that it is not available directly for binding with the complementary target mRNA [32,33]. Genetic and biochemical evidence has demonstrated that *ago-2* is involved in siRNA-directed target RNA cleavage, while *ago-1* is involved in miRNA-directed target RNA cleavage. The structural studies of AGO-2 crystals have identified unique features of the PAZ and PIWI domains, which increasingly point towards the role of AGO-2 as the 'catalytic engine' of the RISC complex cleaving the target mRNA. The role of AGO-2 as the 'catalytic engine' of RISC has been demonstrated experimentally in the human T-293 cell line [32]. The PAZ domain has conserved aromatic residues and forms a characteristic oligonucleotide-binding fold, which binds the 3′ end of the siRNA. The PIWI domain has a structure similar to RNase H and binds to the 5′ end of the guide siRNA. RNAse H produces cleavage products with a 5′-PO_4 and 3′-OH and also requires metal ions for its activity; both of these properties are shared by RISC. Mutations in the PIWI domain residues that are similar to RNase H abolish the target cleavage activity, thus strongly implicating hAGO-2 in the cleavage of the target mRNA. The crystal structure of *Aquifex aeolicus* AGO-2 identifies a highly basic pocket adjacent to the PIWI domain called the Mid-Domain. It has been shown that the 5′-PO_4 group of the guide siRNA binds to this basic pocket. The crystal structures of *Pfu* and *Aae* AGO2 revealed the catalytic motif of amino acids DDH (Asp Asp His), which is involved in the cleavage of target mRNA. It is postulated that certain paralogs of hAGO2, for example hAGO4, lack the target cleavage ability due to the absence of the DDH motif [34]. The structural studies have been complemented by biochemical experiments showing that only hAGO-2 is able to form cleavage competent RISC. In *Drosophila*, AGO-2 plays a central role in the RNAi mechanism by bringing about protein–protein interaction among siRNA, the RISC complex and Dicer-2 via its different domains [35]. In addition to its central role in the RNAi machinery, *ago-2* has been implicated in germ cell formation, nuclear division and assembly of centric heterochromatin during early embryogenesis in *Drosophila* [36].

RISC is a multiprotein complex, reported to be in the range of 200–500 kDa, which ultimately brings about the cleavage of the target mRNA. The different protein components of RISC aid in the assembly of the active complex and loading of the single stranded 'guide' siRNA, which is competent for target cleavage. Biochemical purification of

Drosophila embryo lysates has identified AGO-2, dFXR (*Drosophila* ortholog of Fragile X mental retardation protein), TUDOR-SN (a nuclease with a tudor domain and bearing five nuclease domains homologous to *Staphylococcus* nuclease domain) and VIG (vasa intronic gene) [37]. The active RISC complex loaded with guide siRNA has been shown to require Mg^{2+} for its activity and depletion of Mg^{2+} drastically reduces the catalytic activity of the complex [38]. The biochemical evidence indicates that RISC is an endonuclease whose cleavage products have a 5′-phosphomonoester and a 3′-hydroxyl group [39]. RISC cleaves the target RNA at the scissile phosphate group (i.e., phosphodiester cleavage). The scissile phosphate group on the target has been shown to base pair with the 11th and 12th nucleotide starting from the 5′ end of the guide (antisense) siRNA strand. The rate-limiting step for RISC endonuclease cleavage has been demonstrated to be due to steric hindrance/conformation binding. The substitution of a 2′-hydroxyl group by a deoxy group at the same position does not affect the catalysis by the RISC complex. On the other hand, substitution at the 2′ end of the ribose sugar by a more bulky methyl group or a phosphorothioate group severely affects the catalysis by the RISC complex, underscoring the importance of steric hindrance during the cleavage of the target by the RISC complex.

A hallmark of the RNAi pathway is the specificity with which the target mRNA is degraded by the siRNA. The template/trigger sequence is acted upon by Dicer-2 to produce 21–23 nt siRNA, which are double-stranded. It has been shown experimentally that only one strand of the siRNA is associated with the active RISC complex. A series of biochemical experiments have shown that the Dcr-2/R2D2 heterodimeric protein complex detects the asymmetry in the siRNA duplex [40]. R2D2 was identified as a protein which co-immunoprecipitates with Dcr-2 during biochemical fractionation of *Drosophila* embryo extracts. R2D2 is able to bind siRNA produced by the activity of Dcr-2 by virtue of its two dsRNA binding domains. The heterodimeric Dcr-2/R2D2 complex not only stabilizes each component but also brings about stabilization of the nascent siRNA delivered to the RISC complex. The efficiency of RNAi was severely compromised in r2d2 mutant flies due to decreased delivery of siRNA to the RISC complex. The role of R2D2 protein has been further extended to the identification of the bonafide siRNA strand referred to as the 'guide strand' and degradation of the 'passenger strand' that does not enter the RISC complex. It has been shown that the R2D2/DCR-2 heterodimer recognizes the thermodynamic instability in the 5′ region of the siRNA duplex [41,42]. The R2D2 protein binds to the 5′ end of the thermodynamically stable end and this strand is degraded. The 5′ end of the thermodynamically unstable strand is bound by DCR-2 and this becomes the 'guide strand' that is loaded onto the RISC complex. The assembly of the activated RISC complex takes place in a stepwise manner that requires ATP [43]. The formation of DCR-2/R2D2 heterodimer bound siRNA constitutes the R1 complex, which then forms a transient R2 complex. This process is ATP independent and it is believed that R1 and R2 phases of the RISC assembly process constitute the initiation process. The formation of the active RISC complex is preceded by the formation of RLC (RISC Loading Complex) also known previously as complex A. This assembly of RLC is ATP dependent and in addition to DCR-2, R2D2 and siRNA, it also contains some other unknown components. The exact relationship between R1, R2 and RLC is not understood completely. The active RISC complex, also known as 'holo RISC', or the 80S complex, associates with other RISC components such as AGO-2, VIG, dFXR etc. The 'holo RISC' complex contains a 'guide strand' of the siRNA, which interacts with the PAZ domains of AGO-2. At this stage of assembly, the complex is cleavage competent and degrades the specific target mRNA.

The site of target mRNA degradation is believed generally to be the cytoplasm. A recent study in mammalian cells proposes specific cytoplasmic bodies to be the site of target mRNA degradation by the activated RISC complex [44]. Fluorescence microscopy and immunoprecipitation studies indicated that AGO-2 is localized in these cytoplasmic bodies. The authors propose that AGO-2/RISC shuttles between the cytoplasm and cytoplasmic bodies. The AGO-2/RISC bound by siRNA can bind target mRNA in the cytoplasm and then this complex is directed to the cytoplasmic bodies. The issue of the site of target mRNA degradation by the RISC complex requires further study.

4. Transposable element silencing in *Drosophila*

Hybrid dysgenesis in *Drosophila melanogaster* occurs when laboratory-maintained strains are crossed to those from the wild. The syndrome is caused by paternally introducing a potentially active transposable element, e.g. P, I or hobo element, to a maternal strain devoid of the element. In the progeny, the element will be transiently activated resulting in high mutation rate, chromosomal abnormalities and sterility. Subsequently, the transposition of the elements will cease after several generations [45,46]. Accumulating evidence suggests that the repression of I element transposons is mediated by both PTGS (post-transcriptional gene silencing) and TGS (transcriptional gene silencing), which depend on the RNAi machinery, while the involvement of RNA silencing processes with P and hobo elements is not known (see Fig. 2).

Either the DNA transposons, P and hobo, or the LINE-like non-LTR (long terminal repeat) retrotransposon I can trigger hybrid dysgenesis. An inducer strain contains multiple insertions of an element, called I strain for the I element or P strain for the P element. A reactive strain, called R or M strain, respectively, is devoid of intact I or P elements. The copies of the element in the inducer strain germline remain inactive. The silent state can be maternally transmitted through female gametes for one (P strain) or many generations (I strain). Thus a female inducer mated with a reactive male does not show dysgenic syndromes, because the incoming transposon is readily repressed. However, the reciprocal cross causes dysgenesis. The germline of the progeny from the cross has a burst of transposition of the element, which will last for generations until a new silent state is reached. On the other hand, the maternal control of the repression is not stable: the effect is lost quickly after one generation for a P strain and is reduced gradually for an I strain; the long-term maintenance of repression needs a zygotic factor, namely the presence of the elements.

The I element dysgenesis has been investigated in light of RNA silencing mechanisms. Inducer strains with several copies of the I element suggest that cosuppression may be responsible

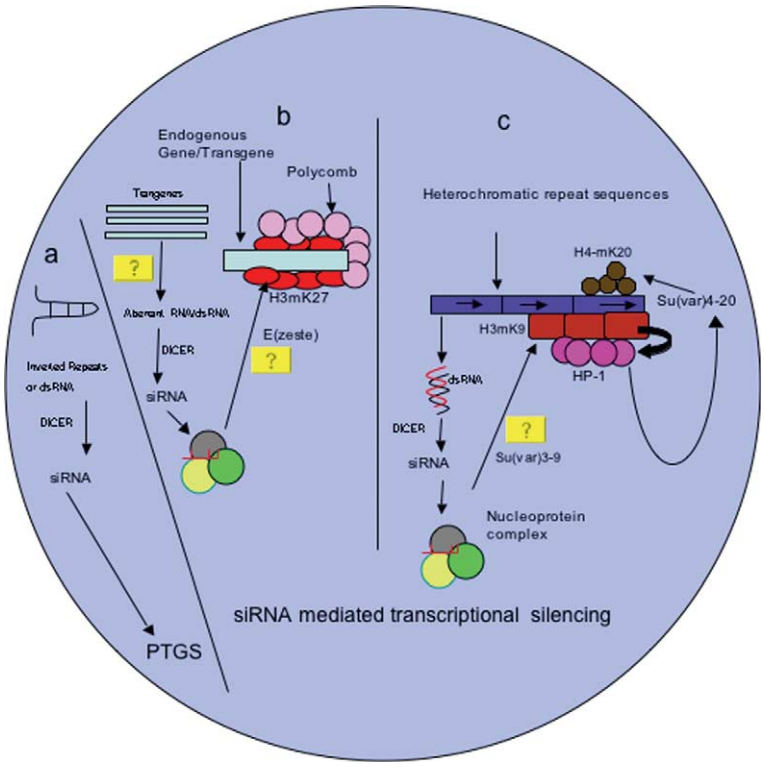

Fig. 2. Potential connections between transcriptional and post-transcriptional gene silencing in *Drosophila*. (a) When a dsRNA is produced, Dicer generates siRNA. These small RNAs target cytoplasmic mRNAs for degradation, which results in post-transcriptional gene silencing (PTGS). (b) With transgene transcriptional silencing, siRNAs are thought to associate with histone methyltransferases, such as Enhancer of zeste [E(z)], to bring about H3-mK27 modification, which provides docking sites for Polycomb complexes on the silenced gene copies. (c) The siRNAs originating from the heterochromatic repeat sequences are thought to associate with Su(var)3-9, which catalyzes H3-mK9 modifications, which in turn recruit HP-1. Subsequently, Su(var)4-20 is recruited to provide the H4-mK20 modification. The deposition of HP-1 and various histone modifications at the heterochromatic repeats constitute transcriptional silencing.

for the repression of the element. To test this point, Chaboissier et al. [47] found that increasing the copy number of the 5′-UTR (untranslated region) in the genome repressed the I promoter driven expression and the activity of the element. Jensen and colleagues [48] transferred a fragment of the I element under control of a heat shock promoter to the reactive genome. The reactivity of the transgenic strains was measured by crossing to an inducer male and then counting the dead embryos of the progeny as a measure of dysgenesis. The suppression effect was detected with only one copy of the transgene and was stronger when the copy number increased. The effect requires the I fragment to be transcribed, but not translated, and is not limited to the 5′-UTR. Different fragments from the I element were effective and the combined length was additive. Furthermore, expression of sense or antisense fragments of I was equally efficient [49]. The data strongly suggest that an RNAi-based cosuppression mechanism inactivates I element expression.

Although a role of RNA silencing is unknown for P-M dysgenesis, there is one unique type of P strain in which only one or two copies of the P element inserted in the telomeric region of the X chromosome (1A) can have repression effects equal to the multiple copies in typical P strains [50–52] and has an implicated connection with RNA silencing [53]. The telomeric region is comprised of TAS (telomere-associated sequence) repeat sequences [54]. Transgenic insertions of the P element with a marker gene in the TAS repeats are able to repress the expression of the P insertions in the euchromatin with the same marker gene but with different promoters. This *trans* silencing effect was shown to be homology-dependent and possibly heterochromatin-dependent. The recent observation that *aubergine*, an RNAi component in *Drosophila*, is essential for the P repression in this kind of P strain indicates that the RNAi process is involved [53]. To explain this epigenetic P repression by RNAi, one might assume that siRNAs homologous to the TAS repeats would be processed and extend to the P element insertion sequences. The latter siRNA would silence the *trans*-expressed genes or the homologous transposons.

Aravin et al. [55] have shown that in the male germline, several retrotransposons are regularly silenced. When the RNAi component gene *homeless* is mutated, new insertions were observed associated with a significant increase of the transposon transcripts. Subsequently, the *argonaute* protein, *piwi*, was shown to be involved with retrotransposon silencing [56]. These authors also showed that the RNAi machinery repressed the expression of a copia LTR driven reporter gene, suggesting a transcriptional level control.

An endogenous case of regulation via RNA involves the *Stellate–Suppressor of Stellate* interaction [55,57]. The *Stellate (Ste)* gene on the X chromosome in *Drosophila* is downregulated in males by *Su*(*Ste*), which is composed of tandem repeats located on the Y chromosome. This silencing is required for male fertility. Each *Su*(*Ste*) repeat is homologous to *Ste* with regions sharing 90% identity. The analysis of the

transcripts of *Su*(*Ste*) indicates that sense and antisense RNA was expressed [55]. The 5′ end of the antisense RNA originates from a nearby copy of the transposon *hoppel* (also known as *1360*). siRNA is detected that would result from dsRNA homologous to the region of overlap. Reporter constructs of *Ste-lacZ* with a *Ste* sequence as short as 134 bp were silenced in the testes of transgenic males. The *homeless* and *aubergine* mutations relieved this silencing. A recently identified gene in *Drosophila* named '*loquacious*' has also been implicated in silencing of *Ste* by *Su*(*Ste*). The *loqs* mutants are female sterile and fail to maintain germline stem cells. The *loqs* gene product has been identified as a binding partner of *dicer-1* and has three dsRNA binding domains. It plays an important role in miRNA biogenesis [58].

The endogenous retrovirus (similar to LTR retrotransposons) *gypsy* is silenced in the ovaries owing to an X-heterochromatin locus named *flamenco*. Recent data [59] suggest the repression may resemble telomeric P *trans*-silencing. The authors suggest a cosuppression mechanism, probably mediated by inactive copies inserted in heterochromatic regions. They demonstrated that *flamenco* repression is not restricted to a specific UTR sequence and is *gypsy*-promoter independent. More importantly, they observed siRNA for the *gypsy* element and that functional product from *piwi* is needed for the silencing. Two other endogenous retroviruses, ZAM and Idefix, whose expression is similarly controlled by a locus close to *flamenco* [60], may also be silenced by RNAi.

Whether RNAi is involved in transposon regulation in somatic tissues is not well investigated. It is known that many alleles of the *white* eye color gene have a variety of phenotypes due to transposable element insertions. By attempting to understand how the elements regulate the *w* phenotype, many modifier loci were found affecting this regulation [61–63]. Some of the modifiers appear to regulate a wide range of the retroelement insertion alleles. Further investigation of these genes might reveal additional biochemical functions that intersect with the RNAi machinery.

RNAi is also used for antiviral defense. Viruses encode virulence genes that counteract RNAi silencing. Such a suppressor gene was found in the flock house virus (FHV) in *Drosophila*. This gene (B2) was able to suppress RNAi silencing in plants, indicating the similarity of the silencing machineries between different kingdoms [64]. Using a *Drosophila* S2 cell line, FHV siRNAs were present after infection and their production was dependent on *AGO-2* function. The suppressor B2 gene was able to decrease the amount of the siRNA and increase the amount of viral mRNA. The data confirmed that RNAi-based antiviral defense also exists in *Drosophila* cells [65].

5. RNAi, heterochromatin, RNA editing and DNA repair

The formation of heterochromatin was initially shown to be associated with the RNAi machinery in fission yeast. Deletion of genes involved in RNAi, *argonaute*, *dicer* and *RNA-dependent RNA polymerase*, resulted in the derepression of transgenes that were otherwise silenced by their heterochromatic location [66]. In addition, histone H3 lysine-9 methylation (H3-mK9), which is a characteristic feature of heterochromatin structure, was greatly reduced in the RNAi mutants. It was also shown that centromeric repeats were transcribed. These results, along with other studies that found siRNAs matching centromeric repeats [67], illustrated the importance of the RNAi machinery to heterochromatin formation. Subsequent to these studies came the identification of a complex, termed RITS (RNA-Induced initiation of Transcriptional gene Silencing), which was shown to be associated with siRNAs and was necessary for heterochromatin assembly [68,69]. In further study involving RNA PolII C-terminal domain (CTD) mutants in fission yeast, it was shown that association of AGO-1 with centromeric repeats required transcription by RNA PolII and a physical association occurs between AGO-1 and RNA PolII largest subunit CTD. This study highlights the role of RNA PolII CTD in stabilizing interactions between the RNAi machinery components and the nascent transcripts to induce heterochromatin modifications [70,71].

While these discoveries of the importance of RNAi to heterochromatin formation have been characterized in most detail in yeast, similar findings have been demonstrated in *Drosophila* [72]. The effects of mutation of three genes involved in RNA interference were examined: *aubergine*, *piwi* and *homeless* (*hls*). It was shown that mutation of these loci resulted in the suppression of silencing of a marker gene inserted into pericentric chromatin, which under normal circumstances shows a variegated phenotype. Similarly, tandem repeats of a transgene, which would also normally show a variegated phenotype, exhibit suppression of silencing in the RNAi mutants.

The effects of the loss of these RNAi components on heterochromatin protein 1 (HP1) localization and H3-mK9 were also examined. In normal flies, HP1 is heavily concentrated on pericentric heterochromatin by binding to the methylated form of H3; however loss of *hls* function resulted in HP1 redistribution, concurrent with the sharp reduction of H3-mK9. In *aub* and *piwi* mutants, there was a partial loss of H3-mK9; however, the effects on HP1 and HP2 (another heterochromatin-associated protein) were minimal, in contrast to *hls*. These results demonstrated the importance of components of the RNAi machinery to heterochromatin formation.

Another RNAi gene implicated in heterochromatin formation is *Rm62* (also known as *Lighten-up*). This gene encodes a dsRNA helicase that was found associated with fragile X protein and argonaute-2 in *Drosophila* tissue culture cells [73]. When its function was eliminated, RNAi of a GFP construct was not supported. Previous studies on *Lighten-up* had shown that *Lip* mutations caused upregulation of several retrotransposons and a suppression of position effect variegation caused by heterochromatin [62]. The combined results provide further support for an involvement of RNAi in heterochromatin functions.

It was recently reported that the transposable element *1360* (*hoppel*) was responsible for initiating the formation of heterochromatin on chromosome 4 and involved the RNAi apparatus [74]. A *white* eye color gene reporter construct had been inserted at many sites along the fourth chromosome, which contains interspersed heterochromatic and euchromatic regions. It was found through deletion studies that proximity to the *1360* element determined whether there was silencing of the reporter. This element was necessary for the initiation of a heterochromatic domain, which could spread for approximately 10 kb. The silencing was dependent on HP1. The authors proposed that the RNAi apparatus targeted the *1360* element for initiation of a heterochromatic domain.

A recent study examining the functions of the multi-KH-domain protein, DDP1 (*Drosophila* dodeca-satellite binding protein 1), introduced another potential partner of the RNAi machinery in the formation of heterochromatin [75]. The KH domain is a single stranded nucleic acid binding motif. DDP1 had previously been shown to bind single stranded nucleic acids with high affinity in vitro [76]. Huertas et al. [75] showed that DDP1 mutations suppressed PEV. They also demonstrated that *ddp1* mutants have strongly reduced H3-mK9 and HP1 deposition at pericentric heterochromatin. These results, along with the fact that multi KH-domain proteins have been shown to have numerous roles in RNA metabolism [77–79], suggest that DDP1 might cooperate with the RNAi machinery in the assembly of heterochromatin.

A subsequent study by Wang and colleagues [80] examining the functions of the mammalian homolog of DDP1, vigilin, provided further evidence for the importance of these KH domain containing proteins to heterochromatin structure and function. In addition, their results suggested an overlap among RNAi, RNA editing, and DNA repair involvement in heterochromatin formation. Site-specific RNA editing by adenosine deaminases (ADARs), which only edit a small number of residues in an mRNA molecule, allows multiple proteins to be synthesized from the same primary RNA molecule [81]. However, promiscuous editing occurs when dsRNA is targeted non-specifically, resulting in extensive deamination of adenosines to inosines, and unwinding of the RNA double helix [82,83]. These promiscuously edited RNA molecules are then retained in the nucleus, in a protein complex [84]. The study by Wang et al. [80] showed that vigilin was associated with heterochromatin, and that it also had the activity of binding to promiscuously edited inosine containing RNA molecules. This finding led to the proposition that vigilin played a role in heterochromatin formation and structure in conjunction with the RNAi apparatus.

This binding of vigilin to edited RNA molecules highlights an interaction between the processes of RNA editing, carried out by ADARs, and RNA interference. Scadden and Smith [85] used *Drosophila* cell extracts to show that RNAi was antagonized by promiscuous editing. This was found to result from an inhibition of the production of siRNAs, suggesting that in vivo, the two processes might have some antagonistic effect. These implications were subsequently supported by Knight and Bass [86], who showed that transgenes expressed in somatic tissues of *C. elegans* were silenced in strains that were mutant for the two ADAR genes, *adr-1* and *adr-2*. The editing of dsRNA transcribed from the transgenes in wild-type animals prevented RNAi from occurring, whereas in the ADAR mutants this editing did not happen, and allowed silencing to occur.

Another study by Tonkin and Bass [87] demonstrated that mutation of an RNAi component corrected an aberrant phenotype produced by mutation of the ADAR genes in *C. elegans*, suggesting a role for ADARs in regulating dsRNA molecules entering the RNAi pathway. Indeed, it was shown more recently that ADAR1 and ADAR2 in *C. elegans* are bound to siRNAs, reducing the silencing conferred by them [88]. To add further complexity to this emerging picture, it was also recently shown that the RISC subunit Tudor-SN binds to promiscuously (or "hyper") edited dsRNA in *Xenopus laevis* extracts, and promoted their cleavage [89]. Thus, evidence is accumulating that ADARs play a role in the regulation of RNAi in some way, and that this might also relate to heterochromatin formation, suggested by the binding activities and localization of vigilin in *Drosophila,* as outlined by Wang and colleagues [80].

Further characterization of the proteins involved in the inosine containing RNA binding complex by Wang et al. [80] demonstrated the inclusion of DNA-dependent protein kinase (DNA-PK), an important DNA repair protein. The finding that a DNA repair protein was involved inferred a link among RNAi, heterochromatin formation and DNA repair. A number of DNA repair proteins have roles in heterochromatin structure and function. These include PARP-1, which in mammalian cells is crucial to the immediate response to DNA damage, and which the *Drosophila* homolog has been shown to be necessary for proper heterochromatin formation during development [90]. Another gene crucial to the DNA damage response is the protein kinase ATM. The *Drosophila* homolog has been found to interact with HP1 [91]. Examples from other organisms include proliferating cell nuclear antigen (PCNA), which has been shown in mammalian cells to act in concert with chromatin assembly factor 1 (CAF-1), in response to DNA damage [92]. CAF-1 and PCNA were shown to be required for the proper deposition of HP1 during heterochromatin replication in mammalian cells [93]. A direct link between transcriptional gene silencing and DNA repair was found in *Chlamydomonas reinhardtii*, where mutations that reactivated silenced transgenes were more sensitive to DNA damaging agents [94].

The involvement of RNAi with heterochromatin structure and function has so far been characterized in most detail in fission yeast. However, studies in *Drosophila* have indicated that there are most likely similar mechanisms involved. In addition, there is increasing evidence that DNA repair proteins are also involved in this process, and in time it is likely that the precise nature of the interactions among these proteins and pathways will be uncovered.

6. Concluding remarks

As the narrative above indicates, the RNAi machinery serves a role in the cell to eliminate aberrant RNAs. These might originate from foreign sources such as viruses, from repeated sequences in the genome or from aberrant messenger RNAs. For example, in a study using *Drosophila* S2 cell lines it was demonstrated that depletion of AGO2 and DCR-2 resulted in the shortening of poly(A) tails and increased expression of the monitored transgene, thus implicating polyadenylation in RNAi-mediated transgene silencing phenomena [95]. Although not covered in this review, but in others in this volume, microRNAs serve to modulate translation of specific mRNAs during development. Further potential functions of small RNAs include an involvement in sequence-specific targeting of chromatin modifications that delimit various regions of the chromosome. These include the centromere, various types of heterochromatin and the telomeric regions as suggested by the profile of siRNAs found in flies [96]. Furthermore, RNAs are implicated in at least some cases of Polycomb silencing [6]. These diverse findings illustrate that small RNAs are increasingly found to play sequence-specific targeting roles in establishing different domains of chromosome structure and function as well as a genomic defense against viruses and transposable elements.

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