REVIEW ARTICLE

Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others?

Katarzyna JARONCZYK^{*1}, Jon B. CARMICHAEL^{*1} and Tom C. HOBMAN^{*†2}^{*}Department of Cell Biology, University of Alberta, Edmonton, AB, Canada T6G 2H7, and [†]Medical Microbiology and Immunology, University of Alberta, Edmonton, AB, Canada T6G 2H7

PPD (PAZ Piwi domain) proteins and the Dicer family have been the subjects of intense study over the last 6 years. These proteins have well-established roles in RNAi (RNA interference), a process that relies on siRNAs (small interfering RNAs) or miRNAs (microRNAs) to mediate specificity. The development of techniques for applying RNAi as a laboratory tool and a molecular therapeutic technique has rapidly outpaced our understanding of the biology of this process. However, over the last 2 years, great strides have been made towards elucidating how PPD proteins and Dicer regulate gene-silencing at the pre- and post-transcriptional

levels. In addition, evidence is beginning to emerge that suggests that these proteins have additional siRNA-independent roles as cell-cycle regulators. In the present review, we summarize the well-known roles of these two classes of proteins in gene-silencing pathways, as well as explore the evidence for novel roles of PPD and Dicer proteins.

Key words: Dicer, gene expression, gene silencing, PAZ Piwi domain protein (PPD protein), RNA-induced silencing complex (RISC), RNA interference (RNAi).

INTRODUCTION

RNAi (RNA interference) is a process utilized by eukaryotes to modulate gene expression at pre- and post-transcriptional levels [1]. In its original form, RNAi may have evolved as a genome immune system that acted to maintain the integrity of eukaryotic genomes. For example, in lower eukaryotes and plants, components of the RNAi apparatus are required for transposon silencing, resistance to viruses and maintenance of chromosome stability. Since the discovery 7 years ago that introduction of dsRNA (double-stranded RNA) into cells initiates sequence-specific gene silencing [2], the use of RNAi as a laboratory tool has revolutionized the study of eukaryotic gene function. Moreover, it holds great promise as a molecular therapeutic technique for the treatment of cancers and infectious diseases [3–5].

Specificity in RNAi-dependent gene silencing is provided by small (21–26 nt) guide RNAs. Two main types of small RNAs that differ in origin and function are known to play integral roles in RNAi: siRNAs (small interfering RNAs) and miRNAs (microRNAs). siRNAs are the cleavage products of longer dsRNAs that are generated by RNA-dependent RNA polymerases, or from bi-directional transcription of genes or transposable elements. The siRNAs usually guide the RNAi machinery to carry out mRNA degradation and/or chromatin modification. Endogenous transcripts that contain near-complementary inverted repeats fold back on themselves to form dsRNA hairpins, which, after a two-step maturation process involving two RNase III enzymes, give rise to miRNAs. miRNAs mediate translational repression, although, in some cases, they can also direct mRNA degradation. For example, in plants, miRNAs mainly function in the cleavage of cognate mRNAs, while, in animals, miRNAs predominantly

inhibit translation by targeting partially complementary sequences in the 3' UTR (untranslated region) of mRNAs [6].

Based on genetic and biochemical studies, the canonical RNAi pathway has been divided into two stages: initiation and effector. The initiation stage involves the generation of siRNAs [7–10] and miRNAs [11,12] from long dsRNA or hairpin RNA precursors respectively. Cleavage of precursors into si/miRNAs is mediated by the type III RNase Dicer [13]. The effector stage requires the transfer of the si/miRNAs into ribonucleoprotein complexes known as RISCs (RNA-induced silencing complexes) [14]. Functional RISCs contain only single-stranded siRNAs or miRNAs [15]. Gene silencing by RISC is accomplished via homology-dependent mRNA degradation [16–18], translational repression [19] or transcriptional gene silencing [20–22]. PPD (PAZ Piwi domain) proteins form the core of RISCs [23–26], which are then guided by the si/miRNAs to specific mRNA targets or genomic loci. A third core component of the RNAi pathway, RNA-dependent RNA polymerase, is utilized by a number of lower eukaryotes, including fission yeast and nematodes [20,22,27]. Its purported function in gene-silencing pathways is to amplify siRNAs and possibly miRNAs in order to increase the speed and/or the magnitude of the RNAi response after detection of the dsRNA trigger. Interestingly, this enzyme is not required for RNAi in mammals or *Drosophila melanogaster*.

CORE COMPONENTS OF THE RNAI APPARATUS

PPD proteins

PPD proteins form a highly conserved superfamily of proteins that are found in diverse organisms ranging from archaea to humans.

Abbreviations used: ds, double-stranded; FXR, fragile X mental retardation protein; GFP, green fluorescent protein; miRNA, microRNA; miRNP, miRNA-containing ribonucleoprotein; MVH, mammalian Vasa homologue; PPD, PAZ Piwi domain; RISC, RNA-induced silencing complex; RITS, RNA-induced initiation of transcriptional gene silencing; RNAi, RNA interference; siRNA, small interfering RNA; ss, single-stranded; UTR, untranslated region; VIG, Vasa intronic gene protein.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (email Tom.Hobman@ualberta.ca).

Figure 1 PPD protein and Dicer domain structures

PPD proteins are classically defined by the presence of PAZ and Piwi domains. The PAZ domain binds to siRNAs, whereas the Piwi domain serves as the binding site for Dicer. The Piwi domains of some PPD proteins possess endonuclease activity. The two ribonuclease III domains of Dicer dimerize to form the catalytic centre that is responsible for cleaving long dsRNA. Some Dicers, such as *Schiz. pombe* Dcr1, *A. thaliana* DCL4 and *D. melanogaster* Dcr1 do not have recognizable PAZ domains. One or more nuclear localization signal can be found in *A. thaliana* DCL1 and DCL4, *D. melanogaster* Dcr1, and mouse and human Dicers.

By definition, members of this family contain two signature domains [28], a centrally located PAZ domain and a C-terminally located Piwi domain (Figure 1). The 100-amino-acid PAZ domain binds the 2 nt 3'-overhang of the siRNA duplex, and facilitates anchoring of this guide RNA into the effector complex [29]. The Piwi domain, comprising approx. 300 amino acids, has been shown to mediate the interaction of PPD proteins with Dicer [30,31]. Recent structural and bioinformatic analyses suggest that Piwi domains share similarity with endonucleases, an observation that led to the discovery that PPD proteins are directly involved in cleavage of targeted mRNAs during the effector stage of RNAi [32–35]. The mechanisms by which PPD proteins mediate translational suppression and chromatin silencing are not known at this time.

Functions of PPD proteins in siRNA and miRNA pathways

The genes encoding *Arabidopsis thaliana* AGO1 and ZWILLE were the first PPD family members to be characterized [36,37]. Before the discovery that PPD proteins are RNAi effectors, AGO1 and ZWILLE were shown to have overlapping functions in plant development [38]. Although it has yet to be proved that ZWILLE has RNAi-related functions, it is well-documented that AGO1 is integral to gene silencing [39–41].

The *Caenorhabditis elegans* genome encodes 24 PPD proteins, a striking number when compared with the fact that mice and humans each contain only eight PPD genes. It appears that, in nematodes at least, PPD proteins perform highly specialized functions. For example, the PPD proteins RDE-1 and PPW-1 are required for efficient siRNA-mediated mRNA cleavage [40,42,43], whereas ALG-1 and ALG-2 are not required for this process [19]. However, the last two PPD proteins function in maturation and translational inhibition activities of miRNAs that regulate developmental timing pathways [19]. In *D. melanogaster*, Argonaute2 (*dAgo2*) is required for the incorporation of siRNAs into RISC and subsequent targeting of cognate mRNAs for destruction [44]. Fly embryos that lack *dAgo2* activity are defective for siRNA-targeted mRNA cleavage. In contrast, *dAgo1* is required for miRNA biogenesis [44], but not siRNA-mediated RISC activities. In humans, four PPD proteins (Argonautes 1–4) were shown to bind miRNAs,

but only hAgo2 is associated with the catalytic activity required for mRNA cleavage [45]. These examples serve to reinforce the idea that in metazoans, PPD proteins exhibit isoform-specific functions in gene-silencing pathways. Since sequence conservation among family members is greatest in the C-termini, it is likely that their N-terminal domains determine the isoform-specific roles of PPD proteins.

In unicellular organisms, it seems that PPD proteins are multi-functional. The *Schizosaccharomyces pombe* PPD protein Ago1 is required for silencing of pericentric chromatin, and for accurate chromosome segregation during mitosis and meiosis [20,22,46–48]. Ago1 is also purported to function in concert with short heterochromatic RNAs, which are derived from repetitive sequences, to guide chromatin-silencing machinery to sites of heterochromatin formation [22,49]. It was later demonstrated that this protein also functions in the classic RNAi pathway that culminates in siRNA-directed cleavage of mRNAs [50].

PPD proteins and development

As mentioned above, genetic studies first pointed to the importance of PPD proteins in developmental pathways in plants and animals [36,37,51,52]. For instance, AGO1 and ZWILLE are required for leaf development in *A. thaliana*, whereas Piwi and *dAgo1* are essential for normal development of gametes and nervous system tissues respectively in *D. melanogaster* [51,52]. PPD protein activity is also required for maintaining pools of stem cells in developing plants [37,38] and animals [51]. Conversely, loss of a given PPD protein activity can result in differentiation and ultimately depletion of stem cell populations, whereas over-expression of the same protein results in increased proliferation of these cell types [53].

In vertebrates, PPD proteins appear to be important for regulating multiple developmental pathways, and certain family members are highly expressed in undifferentiated cells and germline tissues. For example, levels of Hiwi mRNA expression are high in Cdc34⁺ haematopoietic progenitor bone marrow stem cells, but, as these cells differentiate, Hiwi expression levels decrease dramatically [54]. Similarly, Mili is preferentially expressed in

germ cells, and is essential for spermatogenesis in mice. Interestingly, *Mili*^{-/-} strains of mice resemble *MVH*^{-/-} strains in that they are developmentally normal, but are sterile owing to arrest of spermatogenesis at the spermatocyte stage [55]. MVH is the mammalian homologue of Vasa, an ATP-dependent DEAD-box RNA helicase that was previously found to co-localize with the PPD protein Aub in germline-specific granules [56], and in a RISC-like complex [26]. Expression and localization of Vasa mRNA is an important determinant of germline fate commitment [57,58]. Accordingly, since Mili associates with MVH, it is likely that these two proteins function together as a complex that regulates cell differentiation. Finally, mouse Ago2 was demonstrated recently to play a critical role in embryogenesis. Targeted disruption of the *ago2* gene results in embryonic lethality owing to multiple developmental abnormalities, the most severe of which are defects in neural crest tube closure and cardiac failure [33]. Taken together, these data suggest that it is not only the level of expression and/or activity of PPD family members, but also their intracellular localization, that dictates specific developmental processes.

Bioinformatic analyses have revealed that many of the miRNA targets in plants are transcription factors [59,60]. Accordingly, it seems likely that the majority of the developmental defects exhibited by PPD mutants result from aberrant expression of transcription factors that determine cell fate. However, it is important to consider the possibility that at least some of the developmental roles of PPD proteins are unrelated to gene silencing. In *A. thaliana*, for instance, some allelic mutants of *ago1* are defective for RNAi, but are developmentally normal [61]. This study indicates that the gene-silencing functions of PPD proteins can be genetically uncoupled from their roles in development. *D. melanogaster* has also served as an excellent experimental system to study the roles of PPD proteins in signalling pathways that control cell fate and developmental patterning. For example, *dAGO1* was isolated in a genetic screen for dominant activators of the Wingless signal transduction pathway [52]. Moreover, the phenotypes of *piwi* mutants can be rescued by overexpression of Hedgehog, a secreted signalling protein that is not implicated in RNAi [62–65]. Of course, these results do not discount the possibility that Wingless and Hedgehog function downstream of *dAgo1* and *Piwi* respectively. In this case, overexpression of Hedgehog may simply bypass the need for *Piwi* at an upstream miRNA-dependent step.

RNase III enzyme family

Dicer was first identified as the ribonuclease that functions at the initiation stage of RNAi [13]. It is a member of the RNase III family, a group of enzymes that exhibit specificity for dsRNA [66]. The RNase III family is divided into three structural classes. Class I enzymes contain one catalytic endonuclease domain (RIII) and a dsRNA-binding domain, and are involved in numerous RNA-processing reactions, including maturation of rRNAs, tRNAs and mRNAs [67]. Enzymes such as *Escherichia coli* RNase III are part of the class I subgroup. The second class of enzymes is represented by the nuclear protein Drosha. Class II enzymes comprise two RIII domains, a dsRNA-binding domain, and long N-termini that are rich in proline, serine and arginine residues [68]. The latter regions are presumably involved in protein–protein interactions. Drosha homologues are encoded in the *D. melanogaster*, *C. elegans*, mouse and human genomes [69–71], and are the nucleases that are responsible for processing primary miRNAs into stem-loop precursor miRNAs in the nucleus [72].

Drosha exists in two multiprotein complexes. One is a large complex that displays weak primary miRNA-processing activity

as well as non-specific RNase activity, contains DEAD-box RNA helicases, dsRNA-binding proteins, heterogeneous nuclear ribonucleoproteins and the Ewing's sarcoma family of proteins [73]. This complex may function in other Drosha-mediated RNA processing pathways, such as pre-ribosomal RNA processing [71]. The smaller complex, named the Microprocessor, displays high primary miRNA-processing activity and contains a dsRNA-binding protein, CG1800 in *D. melanogaster* or DGCR8 in *C. elegans* and mammals [73–75]. The function of DGCR8 may be to recognize and present the primary miRNA transcripts to Drosha. It interacts with the middle region and the two RNase III domains of Drosha, and is required for efficient primary miRNA processing [75]. Its function may be to help determine the specificity of Drosha cleavage [75]. The RNA cleavage products of Drosha are then exported to the cytoplasm, where Dicer processes them further to miRNAs. Nuclear export of the miRNA precursors is dependent upon Exportin 5 and RanGTP [76–78].

Dicer belongs to the third class of RNase III enzymes, which contain an N-terminal DEXH-box RNA helicase/ATPase domain, two RIII domains and a dsRNA-binding domain (Figure 1). Most, but not all, Dicer proteins also contain PAZ domains. Whereas *C. elegans* and *D. melanogaster* Dicer activities are stimulated by ATP [79–82], mammalian Dicer enzymes do not require ATP for activity, and are instead strongly stimulated by limited proteolysis *in vitro* [83]. Fascinatingly, some Dicer mRNAs are subject to negative-feedback regulation by miRNA-dependent mechanisms [84].

Based on the structural comparisons with *Aquifex aeolicus* RNase III enzyme and biochemical analyses of residues within the catalytic domains of *E. coli* RNase III and human Dicer [85], the current model predicts that Dicer molecules possess one RNA-processing centre. Within the processing centre, each RIII domain cleaves one strand of the dsRNA. Intramolecular dimerization of the two RNase III domains in co-operation with two RNA-binding domains, PAZ and dsRNA-binding domain, results in a processing centre that generates products with the characteristic two-nucleotide 3' overhangs [85]. Cleavage of RNA substrates by Drosha is also thought to involve the formation of an intramolecular dimer [75]. The first RIII domain cuts the RNA strand bearing a 3'-hydroxy group, approx. 21 nucleotides from the end. In conjunction with the PAZ domain, it is probably responsible for determining the distance from the terminus of the RNA to the cleavage site [85]. By analogy with its role in PPD protein function, the PAZ domain of Dicer may also be involved in RNA substrate recognition [86–88]. If this is the case, it raises the question as to whether Dicer enzymes that lack PAZ domains function in a similar manner.

Functional diversification and characteristics of Dicer proteins

The number of Dicer genes in a given organism varies from one to four. For example, *C. elegans*, *Schiz. pombe* and vertebrates each appear to encode only one Dicer protein [19,22,79,80,89–91]. Dicer enzymes are essential for the normal development of zebrafish [89] and mice [79], as well as for the formation of heterochromatin in vertebrate cells [92]. Curiously, *Neurospora crassa* expresses two Dicer proteins that are functionally redundant [93], whereas the two Dicer paralogs in *D. melanogaster* (Dcr1 and Dcr2) have unique functions. The former enzyme, which is essential for miRNA processing and miRNA-directed translational repression [94], contains a PAZ domain, but lacks a functional helicase domain. Surprisingly, a complex that includes Dcr1 and Dcr2 is required for siRNA-directed mRNA cleavage. This points to a role for Dicer enzymes at the effector stage of RNAi [94]. The *A. thaliana* genome encodes four Dicer

homologues, at least three of which have unique functions: DCL1 processes miRNA precursors [12,49,95], DCL2 is required for processing virus-specific dsRNA [96] and, finally, DCL3 processes endogenously generated siRNAs [96]. To date, the role of DCL4 in gene silencing has not been elucidated. Lastly, one or more predicted nuclear localization signals are present in DCL1 and DCL4 [95]. *D. melanogaster* Dcr1 and mouse and human Dicers also contain nuclear localization signals [95]; however, it is unlikely that these motifs are functional, since, to date, these proteins have not been detected in the nucleus. In contrast, the nuclear localization signal of *A. thaliana* DCL1 does indeed appear to function in mediating nuclear localization [97].

PROTEIN-PROTEIN INTERACTIONS IN RNAi PATHWAYS

Interactions between PPD proteins and Dicer

Shortly after PPD and Dicer proteins were determined to be central to RNAi, an obligatory interaction between these two groups of proteins was predicted to be important for the transfer of si/miRNAs from Dicer to RISC. Initially, heterotypic PAZ-PAZ interactions were thought to mediate PPD protein-Dicer binding [98]; however, the discovery of PAZ-less Dicer isoforms [95] cast doubt upon that hypothesis. Subsequently, two independent studies revealed that PPD protein-Dicer interactions are mediated through the Piwi domains of PPD proteins [30,31]. Moreover, PAZ domains are not required at all for stable PPD protein-Dicer complex formation. Instead, it appears to be the Piwi-box region of PPD proteins that binds to the first RIII domain of human Dicer [31]. Interestingly, the stability of mammalian PPD protein-Dicer complexes is dependent on the activity of Hsp90 (heat-shock protein 90), suggesting that assembly of RNAi components is regulated at multiple steps [31,99]. Finally, PPD proteins and Dicer were shown to be present in soluble and membrane-associated fractions [31], a fact that may indicate that RISCs are present at multiple intracellular locations.

Curiously, binding of PPD proteins to Dicer inhibits its RNase activity *in vitro* [31]. One of the possible consequences of this effect is to prevent Dicer from releasing si/miRNAs before they associate with other RISC components that mediate mRNA target cleavage or translational repression. This would be expected to maintain the specificity of a given RISC for a single mRNA species.

Interactions between Dicer and dsRNA-binding proteins

In addition to PPD proteins, several dsRNA-binding proteins (RDE-4, R2D2 and HYL1) are known to associate with Dicer [81,100–102]. These proteins may function as physical links between the initiation and effector stages of RNAi. For example, R2D2 was identified as a dsRNA-binding protein that co-fractionated with dsRNA-processing activity from *D. melanogaster* S2 cell extracts [81]. This protein forms a stable complex with Dcr2 and dsRNA. Maximum stimulation of dsRNA-initiated RISC activity was observed in the presence of Dcr2–R2D2 complexes. In contrast, Dcr2 does not bind detectable amounts of dsRNA in the absence of R2D2. Furthermore, RISC activity was abolished when the dsRNA-binding domains of R2D2 were ablated by site-directed mutagenesis. Similar results were obtained when pre-cleaved siRNAs were used to trigger RISC activity, indicating that R2D2 is needed for RISC to efficiently utilize siRNAs [81]. Finally, the Dcr2–R2D2 complex is required for efficient siRNA transfer and binding to dAgo2 in RISC [102a].

The R2D2 sequence is approx. 33% similar to that of the *C. elegans* dsRNA-binding protein RDE-4, which interacts with Dicer, PPD proteins and Dicer-related helicase [100]. RDE-4 was

first identified as a protein necessary for the initiation step of RNAi in *C. elegans* [43,103], but its activity is not required for miRNA processing or development [43,100]. It binds to long dsRNA molecules, but not to ds or ss (single-stranded) siRNAs [100]. The PPD protein RDE-1, whose activity was previously shown to be required for the effector stage of RNAi [102], is necessary for the accumulation of long dsRNAs which are then bound by RDE-4 [100]. Thus the function of RDE-4 may be to recognize long dsRNAs, and, together with RDE-1, mediate their transfer to DCR1 for processing into siRNAs. The helicase activity of Dicer-related helicase is likely to be necessary to unwind dsRNA in order to facilitate the movement of the RDE-4–DCR1–RDE-1 complex along the molecule. Alternatively, it may be involved in the transfer of dsRNA from RDE-4 to DCR1 [100].

Recently, it was discovered that a nuclearly localized dsRNA-binding protein, HYL1, is required for miRNA accumulation and development in *A. thaliana*, but not for post-transcriptional gene silencing [101]. Interestingly, the developmental defects of *hyll* mutants overlap with those of *hen1* and *dcl1*. HEN1 is a nuclear protein that functions in plant development, presumably through its roles in miRNA metabolism and siRNA-mediated post-transcriptional gene silencing [12,39]. *hyll*-, *hen1*- and *dcl1*-null mutants also share common RNAi defects, specifically reduced miRNA accumulation and a concomitant reduction in targeted mRNA cleavage. In addition, the reported or predicted nuclear localizations of HYL1, HEN1 and DCR1 are consistent with the possibility that these proteins function together as a complex in the nucleus [101].

RISC

Protein components of RISC

The first published reports of RISC purification were difficult to reconcile, since preparations that displayed sequence-specific nuclease activity varied in size from 160 kDa [15] to 500 kDa [14,25,82]. One common thread between all of these studies was that the RISC-like complexes always purified with 21–25-nt-long RNAs. The earliest identified protein component of RISCs were PPD proteins, specifically dAgo2 [13,25,82] and hAgo1 and hAgo2 [15].

Additional RISC components are continually being identified, a situation that makes the precise determination of RISC composition difficult. Two putative RNA-binding proteins, the *D. melanogaster* homologue of the fragile X mental retardation protein, dFXR, and VIG (Vasa intronic gene protein) were recently identified as components of RISC [26]. Although the specific roles of these proteins in RISC function are not known, it is interesting to note that the dFXR-associated RISC also contains the ribosomal proteins L5 and L11, as well as 5 S RNA, dAgo2 and p68 RNA helicase [104]. Furthermore, dFXR was found to bind Dicer and p68 RNA helicase, the latter protein being important for RNAi in *D. melanogaster* [104]. Given that one of the functions of dFXR is to repress translation of specific mRNAs, it is reasonable to assume that dFXR-containing complexes function in siRNA- and miRNA-initiated pathways [104].

Characterization of an miRNP (miRNA-containing ribonucleoprotein) complex revealed that it contained components similar to those in siRNA-containing RISC [23,105]. The 15 S miRNP complex isolated from HeLa cells contains hAgo2, Gemin3, a DEAD-box helicase, Gemin4, and is associated with multiple miRNAs [23]. In a human retinoblastoma cell line, a let-7b-containing miRNP complex was shown to bind to the 3' UTR of its lin-28 mRNA target in polyribosome-containing fractions. The levels of a reporter protein, but not its corresponding mRNA

Figure 2 Model of RISC assembly in *D. melanogaster*

The principal siRNA-generating enzyme complex in *D. melanogaster* is composed of Dcr2 and R2D2. Although R2D2 does not affect the ability of Dcr2 to cleave long dsRNA, it is important for binding to the siRNA product after cleavage. R2D2 binds to the 3' end of the guide strand (red) of the siRNA duplex, while Dcr2 binds to the 3' end of the passenger strand. R1 forms after Dcr2-mediated RNA cleavage of long dsRNAs. The R2 complex may be an intermediate that derives from R1 and ultimately matures into R3. Alternatively, it has been suggested that R3 may form directly from R1. The R3 complex is large (80 S) and associates with ribosomes (not shown). Ago2 displaces the Dcr2–R2D2 complex from the siRNA duplex by binding to the 3' end of the guide siRNA strand. Formation of this complex is dependent upon ATP. The siRNAs in the R3 complex are single-stranded, having possibly been unwound by the helicase Armitage. R3 binds the cognate mRNA target, which is then cleaved by the Ago2 subunit. It is possible that there are other unidentified proteins (?) associated with each of the intermediate complexes.

containing the lin28 3' UTR, were significantly lower in the presence of let-7b-miRNP, suggesting that the miRNP functions through translational suppression [106]. In contrast, human let-7-programmed RISC was shown to be a multiple turnover enzyme complex, capable of catalysing the cleavage of more than ten target mRNA molecules [24]. These findings are not surprising, since miRNAs that are perfectly complementary to mRNAs can mediate targeted mRNA degradation similarly to siRNAs and vice versa [107,108]. In general, perfect complementarity between a miRNA and its cognate mRNA results in degradation of the target rather than translational repression, which occurs when base pairing is not perfectly matched [107].

Dynamics of RISC formation

Recently, the application of native gel electrophoresis to the study of RISC dynamics [109] has led to the identification of three stages of RISC formation defined by three distinct siRNA-containing complexes: R1, R2 and R3 (Figure 2). A role for Dicer in RISC function is indicated by the fact that Dcr2 is found in all of these RNP complexes. The R1 complex probably corresponds to the 360 kDa RISC-like structure described previously [82]. It consists of Dcr2, R2D2, and one or more as yet unidentified proteins. As mentioned above, both Dcr2 and R2D2 are required for association of siRNAs with RISC. The latter protein is known to contact siRNAs directly in the R1 complex. The function of R1 may be to process long dsRNA and initiate RISC assembly by serving as a precursor to R2 and R3 [109]. The high rate at which R2 is formed suggests that it may be derived from the binding of R1 to an as yet unidentified pre-assembled complex. The R2 complex is thought to function in siRNA duplex unwinding.

Thermodynamic differences in the base-pairing of the two siRNA strands determine which siRNA strand is assembled into RISC: the one whose 5' end is less tightly paired to its complement [110]. The Dcr2–R2D2 complex senses the thermodynamic stabilities of the ends of an siRNA duplex and selects which RNA strand becomes associated with Ago2. R2D2 binds to the 3' end of the siRNA guide strand that enters RISC, as well as to the 5' phosphate of the non-incorporated siRNA strand. Dcr2 binds to the opposite end of the siRNA duplex. As siRNA unwinding

proceeds, the Dcr2–R2D2 complex is replaced with Ago2, which binds the 2 nt 3' overhang of the guide strand. The unwinding of the siRNA is initiated by the Dcr2–R2D2 complex, but can proceed only in the presence of Ago2 [102a].

The 80 S R3 effector complex whose formation is enhanced by ATP, contains siRNAs, Dcr1, Dcr2, VIG, Tudor staphylococcal nuclease, Ago2, dFXR and R2D2. The R3 complex co-purifies with rRNA from small and large ribosomal subunits, suggesting that it is ribosome-associated [109]. This possibility is supported by the fact that only RNAs undergoing translation are susceptible to RNAi [112], and that dFXR-containing RISC contains ribosomal proteins and RNA [104]. Recently, Tomari et al. [111] reported that an additional, ATP-dependent step occurs in RISC assembly after siRNA duplex unwinding. This process requires the PPD protein Aub, and an ATP-dependent DEA(H/D)-box helicase Armitage [111,113]. Because R3 is so large, and smaller active components of this complex can be purified, it has been proposed that R3 is a 'holoenzyme', which includes regulatory factors that are not absolutely necessary for mRNA cleavage *in vitro* [109].

Slicer

Slicer is the term given to the RISC-associated component that catalyses cleavage of siRNA-targeted mRNAs. The Slicer entity was predicted to be an endoribonuclease that cleaves dsRNA molecules. In their search for Slicer, Hannon's group identified Tudor staphylococcal nuclease in RISC preparations from *C. elegans*, *D. melanogaster* and mammalian cells [114]. However, data from other studies were inconsistent with the likelihood that Tudor staphylococcal nuclease was the Slicer in RISC. Specifically, this enzyme is a calcium-dependent nuclease that generates 3'-phosphomono- and di-nucleotides through hydrolysis of DNA or RNA [115]. In contrast, the RISC-associated endonuclease is magnesium-dependent, and its products contain 5' phosphomonoesters [116,117]. Although the Tudor staphylococcal nuclease inhibitor, 2'-deoxythymidine 5',3'-biphosphate, was found to inhibit RISC activity in one report [114], a later study found that it had no such effect [117].

Interestingly, Dicer enzymes share a number of characteristics with the RISC Slicer. For example, Dicer cleavage products are similar to those of Slicer in that they contain 3'-hydroxide 5'-phosphate termini [116,117]. In addition to functioning at the initiation stage, Dicer is also essential for the effector phase of RNAi. For example, in *D. melanogaster* embryos, Dcr2 is required for both siRNA formation and RISC assembly [94]. Similarly, knockdown of Dicer activity in mammalian cells results in failure of siRNA-mediated gene silencing [30]. Further evidence that Dicer associates with RISC stems from the observation that siRNAs can be UV-cross-linked to both Dcr1 and Dcr2 in the *D. melanogaster* RISC [109].

Recent findings from a number of laboratories revealed that a subset of Argonaute proteins contribute the Slicer activity of RISCs [32,33,45]. Whereas all four human Ago proteins bind to miRNAs, only hAgo2-containing complexes are able to cleave mRNA substrates [6,33]. Furthermore, genetic studies support the biochemical evidence that Ago2 is the Slicer. Specifically, mouse fibroblasts with homozygous null mutations in *ago2* are unable to repress gene expression via RNAi [33]. The nuclease activity of Ago2 appears to be highly regulated, since it does not function in the absence of ss siRNAs, nor does it exhibit significant activity in the presence of dsRNA [33]. This suggests that before targeted mRNA cleavage can occur, Ago2 requires an accessory protein, such as Dicer or an R2D2-like protein, to present it with siRNA. It is puzzling that not all hAgo2-associated miRNAs are able to direct cleavage of complementary target mRNAs. This may indicate that some of the Argonaute-protein-containing complexes function in translational repression. Indeed, this scenario is supported by a recent report showing that miRNA-independent tethering of hAgo2, hAgo3 and hAgo4 proteins to the 3' UTRs of a reporter RNA mediates translational repression without affecting mRNA levels [118]. Although it is unknown how PPD proteins mediate translation repression, it seems that the major role of the miRNAs is simply to guide PPD-protein-containing complexes to homologous mRNAs.

Implications of Argonaute structure for the Slicer function

Recent structural studies have greatly advanced our understanding of how PPD proteins function in RNAi. For example, resolution of the PAZ structure indicated that this domain adopts an OB (oligosaccharide/oligonucleotide-binding)-like fold that binds to the 3' overhangs of siRNAs [29,86,87]. Next, the three-dimensional structure of the archaeon *Pyrococcus furiosus* Argonaute protein was solved [32], and data from this study further strengthen the argument for the Slicer function of Argonaute proteins. In particular, the finding that the Piwi domain structure is similar to endonucleases such as RNase H and endonuclease V was key to understanding the function of PPD proteins [32,34,35]. The magnesium-dependent activities of RNase H and endonuclease V are in agreement with the reported characteristics of RISC [116,117]. Moreover, two aspartate residues that are required for mRNA target cleavage are spatially conserved between the archaeal Ago protein catalytic centre and the active sites of endonucleases [32,33]. Interestingly, the three human Argonaute proteins hAgo1, hAgo3 and hAgo4, which are unable to form cleavage-competent complexes, contain the same amino acid residues that are found in the catalytic centre of hAgo2. This indicates that in addition to the conserved catalytic carboxylates, other residues (in the Piwi domain) or interacting factors are needed to promote the endonucleolytic activity of hAgo2.

The current model of Argonaute Slicer activity, based on the crystal structure of archaeal Argonaute protein is as follows: the siRNA guide interacts with the PAZ cleft, while the mRNA

substrate enters a binding groove formed by the N-terminal, middle and Piwi domains. The 5' end of the mRNA is predicted to lie between the PAZ and N-terminal domains of Argonaute, the latter domain functioning as an 'mRNA grip' [32]. The mRNA is positioned so that the active site, located in the Piwi domain, is 9 nt from the 5' end of the siRNA-mRNA ds region, allowing for the cleavage of mRNA target between 11 and 12 nt from the 3' end of the siRNA guide. The predicted length of the groove is enough to accommodate the entire siRNA. A putative hinge region connects the PAZ domain to the rest of the protein. Upon binding to other proteins, this hinge may function to lift PAZ away from the crescent base, allowing binding of ss siRNAs and the completion of RISC assembly [32].

NON-RISC-DEPENDENT FUNCTIONS OF RNAi CORE PROTEINS

RNA-induced initiation of transcriptional gene silencing

Recently, the importance of RNAi in nuclear events such as genome rearrangement [119], chromatin silencing [20,22,48,120] and chromosome segregation [47,121] has come to light. The latter two processes are directly related to one another in that accurate chromosome segregation, which involves cohesin-mediated sister chromatid association [122–125], is contingent upon the assembly of centromeric heterochromatin [126]. In turn, binding of the cohesin complex to silent centromeric sites is dependent upon recruitment of the chromodomain-containing protein Swi6 to these regions [127,128]. Histone H3 Lys⁹ methylation-dependent Swi6 localization to centromeric, telomeric and the silent mating-type loci requires the function of three core components of the RNAi pathway, Ago1, Dcr1 and RNA-dependent RNA polymerase (Rdp1) [20,22,120].

Targeting of Swi6 to sites of heterochromatin formation in the *Schiz. pombe* genome is reportedly mediated by a novel type of RNP complex, named the RITS (RNA-induced initiation of transcriptional gene silencing) complex [129]. The functional specificity of the RITS complex requires incorporation of siRNAs derived from centromeric transcripts [49]. Deletion of any one of the core RNAi genes, *ago1*, *dcr1* or *rdp1*, or genes that encode the RITS-associated proteins Chp1 and Tas1, results in the loss of histone H3 Lys⁹ methylation and the absence of Swi6 from centromeric chromatin [20,22,47,48,120,129]. This is presumably due to an inability to target the methyltransferase Ctr4 to sites of heterochromatin formation. In turn, loss of centromeric heterochromatin leads to chromosomal segregation defects as a result of reduced cohesin binding to centromeres.

Subcellular localization of RITS and other RNAi components

Analysis of the RNAi components in *Schiz. pombe* using ChIP (chromatin immunoprecipitation) revealed that both Rdp1 and Ago1 are associated with centromeric chromatin [22,130]. In addition, Ago1 can be co-purified with chromatin derived from telomeric and silent mating type loci [130]. Indeed, fluorescence-microscopy studies demonstrated that pools of tagged Rdp1, Ago1 and Chp1 are localized to the nuclei of *Schiz. pombe* cells [130,131]. Ago1 was also detected in the cytoplasm where it presumably functions in siRNA-directed mRNA degradation. Although Dcr1 is an integral component of chromatin silencing, it was not detected in association with any heterochromatic regions [22]. As yet, the localization of Dcr1 is not known. Whereas it was previously assumed that most siRNA processing and RISC-mediated silencing were restricted to the cytoplasm [132,133], a number of studies are consistent with the possibility that at least some PPD proteins and Dicer homologues are present in the nucleus [20,22,47,48,120,129]. In addition, more direct evidence

to support the notion that the initiation and/or effector stages of RNAi take place in the nucleus has recently emerged. For instance, the discovery of Droscha offered a plausible mechanism to account for nucleus-restricted processing of primary miRNAs to precursor miRNAs [72]. Moreover, studies in *A. thaliana* using nuclear and cytoplasmic variants of the viral suppressor of post-transcriptional gene silencing P19 and DCL1-GFP (green fluorescent protein) fusion proteins provided evidence for nuclear production of siRNAs and miRNAs from precursor molecules [97]. Finally, in *D. melanogaster*, the nuclearly localized PPD protein Piwi is required for RNA-dependent silencing of specific loci such as *gypsy* [134].

A recent study reported that miRNA-directed cleavage activity is present in both nuclear and cytoplasmic extracts of HeLa cells [6]. Although cleavage appeared to occur more efficiently in the cytoplasmic fraction, these data suggest that at least a portion of the cytoplasmic miRNA-associated RISC is able to translocate to the nucleus or that there is a stable nuclear pool of RISC. In support of this, another group reported the involvement of RDR2, DCL3, AGO4 and HEN1 in chromatin-modification events in *A. thaliana* [96]. In this study, DCL3-GFP, HEN-1-GFP and GFP-AGO4 proteins were detected exclusively in the nuclei [96].

Involvement of RNAi core components in cell cycle events

The regulation of chromosome structure and segregation is central to the successful completion of cell cycle events. The heterochromatin-assembly defects observed in *Schiz. pombe ago1*, *dcr1* and *rdp1* mutants are associated with frequent lagging DNAs and chromosome loss during mitosis, presumably owing to incorrect sister chromatin orientation and defective kinetichore attachment to spindles [47,121]. While these data support a model in which chromatin architecture is regulated by the RNAi pathway, evidence is also mounting to suggest that RNAi core proteins perform functions that are not directly related to gene silencing [61,135]. In fact, homologues of RNAi core components are now known to regulate cell cycle events in organisms that do not employ RNAi. For example, in the budding yeast *Saccharomyces cerevisiae*, it was recently discovered that the type III RNase Rnt1p, a Dcr1 homologue, is required for cell-cycle progression [136]. While nuclear localization of Rnt1p is essential for this function, the catalytic activity is dispensable for its role in cell-cycle progression [136]. Although the classical RNAi pathway is not present in *S. cerevisiae*, this result does support the notion of cell-cycle-related functions for RNase III proteins that are independent of their nuclease activity. By extension, the role of the RNase III Dcr1 in cell cycle events may be independent of its ability to generate siRNAs [137].

In *Schiz. pombe*, Ago1 and Dcr1, but not Rdp1, are required for regulated hyperphosphorylation of Cdc2 when encountering genotoxic insults [137]. Under normal circumstances, agents that inhibit DNA replication or cause DNA damage activate signalling cascades that lead to inhibitory phosphorylation of the mitotic regulator Cdc2, thereby resulting in cell-cycle arrest [138–140]. After the cells are deemed free of DNA damage or have become replication competent, alleviation of the inhibitory phosphorylation of Cdc2 by the mitotic stimulatory phosphatase Cdc25 initiates resumption of the cell cycle [141,142]. *ago1*- and *dcr1*-null mutants fail to block mitosis in response to DNA damage or replication inhibitors such as hydroxyurea, presumably as a result of their inability to effect hyperphosphorylation of Cdc2. These cells continue to divide unchecked, resulting in unequal division of genomic material between mother and daughter cells. Interestingly, overexpression of hAgo2 was shown to complement the checkpoint deficiency in *Schiz. pombe ago1*

mutants [137]. This suggests that, in humans, the core catalytic subunit of RISC may also play a role in cell cycle checkpoint regulation [33]. Moreover, ectopic overexpression of Ago1 in a *dcr1*-null strain rescued the checkpoint deficiencies, but not the chromosome-segregation defects [137]. Since the biogenesis of siRNAs is completely dependent upon Dcr1 [129], this argues that Ago1 and Dcr1 function in siRNA-independent pathways. Finally, it appears that Ago1 functions downstream of Dcr1 in cell-cycle regulation. Figure 3 depicts a model of how of PPD proteins and Dicer function in the classic RNAi pathway, RNAi-mediated chromatin restructuring and siRNA-independent cell-cycle regulation.

PPD PROTEINS, DICER AND CANCER

Cancer has often been referred to as a disease caused by the failure of cells to maintain genome stability, which in turn is dependent upon accurate chromosome segregation and cell cycle checkpoints that prevent or delay DNA replication/cell division in response to DNA damage [143]. Not surprisingly, mutations in human genes encoding checkpoint proteins are associated with familial predisposition to cancer [144]. In lower eukaryotes at least, the RNAi apparatus functions to maintain genome stability [119]. Of course, it is not hard to imagine how RNAi effector proteins may indirectly affect cell-cycle progression through their involvement in gene-silencing pathways that regulate expression of transcription factors and other proteins. However, it has recently become evident that PPD proteins and Dicer also function in siRNA-independent pathways that regulate cell cycle events [137]. Accordingly, it is important to consider the aberrant expression of PPD proteins and Dicer in human cancers.

Of particular significance is the observation that seminoma tumours are often associated with increased levels of Hiwi mRNA [145]. Coincidentally, *Hiwi* was first reported as a gene that is expressed in undifferentiated haematopoietic stem cells, but not in differentiated cells [54]. Likewise, expression of the *D. melanogaster* orthologue of Hiwi, Piwi, had previously been reported to promote mitosis in stem cells [53]. In addition, the chromosomal locus 12q24.33 that includes the Hiwi gene has been linked to testicular germ cell tumours [146,147]. Conversely, deletion of this region is associated with hypogonadism [148]. Together, these observations are consistent with a scenario in which overexpression of certain PPD proteins is associated with increased mitosis in undifferentiated cells. The role of other PPD protein family members in this process is less clear. For example, we have observed that the expression of Argonaute mRNAs is elevated in multiple human tumour cell lines (K. Jaromczyk, J. B. Carmichael and T. C. Hobman, unpublished work). Paradoxically, there is evidence to suggest that some Argonaute proteins function as tumour suppressors. Specifically, chromosome 1 region p34-35, which is often lost in Wilms' tumours [149,150], contains three human Argonaute genes (*hAgo1*, *hAgo3* and *hAgo4*) [151]. In addition, *miR15* and *miR16* are located in chromosome 13q14, a region lost in more than half of B-cell chronic lymphocytic leukaemias. Both genes are deleted or down-regulated in the majority of chronic lymphocytic leukaemia cases [152]. Finally, overexpression of hAgo2 in a *Schiz. pombe* strain lacking the *ago1* gene was shown to correct the cell cycle checkpoint deficiencies of this mutant [137]. These data point to a role for mammalian PPD proteins in enacting checkpoints in response to genotoxic stress.

There is compelling, but indirect, evidence that Dicer may also have a role in human cancer development. For example, in yeast, lack of Dicer function results in chromosome segregation defects [22,121], as well as failure to enact S–M cell cycle checkpoints

Figure 3 Multiple functions of PPD and Dicer proteins

PPD proteins and Dicer are proposed to function in three different pathways. On the right-hand side of the diagram, si/miRNA-dependent functions of Dicer and PPD proteins are illustrated. si/miRNA-directed mRNA cleavage or translational repression does not require Chp1 and Tas1-like proteins and is proposed to occur in the cytoplasm. In contrast, the heterochromatin-specific functions take place in the nucleus. On the left-hand side, we outline a model to account for the cell cycle functions of Dicer and PPD proteins. DNA damage or inhibition of replication results in a cascade of signalling events that lead to cell-cycle arrest via a Cdc2-dependent checkpoint. Although the exact points of PPD or Dicer proteins action along this pathway are unknown (red arrows), it is thought that these functions are independent of si/miRNAs. In contrast, RISC-mediated mRNA degradation and translational suppression (green arrows) require Dicer-dependent si/miRNA production and their inclusion into RISC or RISC-like complexes. Likewise, heterochromatin formation (purple arrows) requires the production and inclusion of si/miRNAs into a specialized complex named RITS. Association of RITS with chromatin results in methylation of histone H3 Lys⁹, followed by Swi6 binding and subsequent spreading of silencing to neighbouring regions.

[137]. Both of these defects would be expected to contribute to the development of cancer in humans. Human Dicer can partially complement the loss of Dcr1 activity in yeast [121], and thus it is possible that the human enzyme also functions in cell-cycle regulation. Interestingly, human Dicer is also known to bind to 5-lipoxygenase [135], an enzyme whose activity is deregulated in pancreatic tumours [153]. Lipoxygenase inhibitors are a promising new class of anticancer reagents [154] that have been shown to prevent lung tumours in mice and slow progression of adenomas to carcinomas [155]. Unfortunately, it is not known if or how Dicer affects the activity of lipoxygenase. In addition, the Burkitt's lymphoma-derived cell line, EB-3, was found to possess 4-fold higher expression of Dicer mRNA than normal human lymphocytes, and 2-fold higher activity of RNA polymerase III [156]. It is also important to note that Burkitt's lymphoma is characterized by increased activity of c-myc, a transcription factor that regulates genes involved in cell-cycle progression and apoptosis [157]. These observations suggest that RNA polymerase III, whose promoter sequences are present in exon1/intron1 of the c-myc gene [157], produces siRNAs that silence the negative-regulatory elements of c-myc expression [156]. Finally, the core RNAi proteins undoubtedly play a role in signalling and cancer that can be attributed to miRNA-regulated alteration of transcription factor expression [158].

OUTLOOK

In a very short period of time, through a combination of genetic and biochemical approaches, we have learned a great deal about how PPD and Dicer family members function in gene-silencing

processes. This is particularly true of siRNA-directed cleavage of mRNAs or classical RNAi. The challenges that must be met now are to understand how PPD proteins function in other gene-silencing mechanisms including translational suppression and chromatin remodelling. Moreover, elucidation of how both PPD proteins and Dicer function in cell-cycle modulation, either directly or indirectly, will go a long way to aid our understanding of how the RNAi machinery positively and/or negatively affects tumorigenesis.

This work was funded by grants from the Canadian Institutes of Health Research (CIHR) and the Natural Sciences and Engineering Research Council (T. C. H.). T. C. H. is the recipient of a Senior Medical Scholarship from the Alberta Heritage Foundation for Medical Research (AHFMR). K. J. is supported by a pre-doctoral studentship award from AHFMR.

REFERENCES

- Hannon, G. J. (2002) RNA interference. *Nature (London)* **418**, 244–251
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature (London)* **391**, 806–811
- Kapadia, S. B., Brideau-Andersen, A. and Chisari, F. V. (2003) Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2014–2018
- Wilson, J. A., Jayasana, S., Khvorova, A., Sabatino, S., Rodrigue-Gervais, I. G., Arya, S., Sarangi, F., Harris-Brandts, M., Beaulieu, S. and Richardson, C. D. (2003) RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2783–2788
- Fuchs, U., Damm-Welk, C. and Borkhardt, A. (2004) Silencing of disease-related genes by small interfering RNAs. *Curr. Mol. Med.* **4**, 507–517

- 6 Meister, G., Landthaler, M., Dorsett, Y. and Tuschl, T. (2004) Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* **10**, 544–550
- 7 Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J.* **21**, 4671–4679
- 8 Lee, R. C. and Ambros, V. (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294**, 862–864
- 9 Lau, N. C., Lim, L. P., Weinstein, E. G. and Bartel, D. P. (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294**, 858–862
- 10 Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853–858
- 11 Reinhardt, B. J., Weinstein, E. G., Rhoades, M. W., Bartel, B. and Bartel, D. P. (2002) MicroRNAs in plants. *Genes Dev.* **16**, 1616–1626
- 12 Park, W., Li, J., Song, R., Messing, J. and Chen, X. (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr. Biol.* **12**, 1484–1495
- 13 Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature (London)* **409**, 363–366
- 14 Hammond, S. M., Bernstein, E., Beach, D. and Hannon, G. J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature (London)* **404**, 293–296
- 15 Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. and Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* **110**, 563–574
- 16 Tuschl, T., Zamore, P. D., Lehmann, R., Bartel, D. P. and Sharp, P. A. (1999) Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev.* **13**, 3191–3197
- 17 Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33
- 18 Hamilton, A. J. and Baulcombe, D. C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**, 950–952
- 19 Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire, A., Ruvkun, G. and Mello, C. C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106**, 23–34
- 20 Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A. and Grewal, S. I. (2002) Establishment and maintenance of a heterochromatin domain. *Science* **297**, 2232–2237
- 21 Pal-Bhadra, M., Bhadra, U. and Birchler, J. A. (2002) RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in *Drosophila*. *Mol. Cell* **9**, 315–327
- 22 Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. and Martienssen, R. A. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**, 1833–1837
- 23 Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. and Dreyfuss, G. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev.* **16**, 720–728
- 24 Hutvagner, G. and Zamore, P. D. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Science* **297**, 2056–2060
- 25 Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. and Hannon, G. J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* **293**, 1146–1150
- 26 Caudy, A. A., Myers, M., Hannon, G. J. and Hammond, S. M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev.* **16**, 2491–2496
- 27 Dalmay, T., Hamilton, A., Rudd, S., Angell, S. and Baulcombe, D. C. (2000) An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. *Cell* **101**, 543–553
- 28 Cerutti, L., Mian, N. and Bateman, A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem. Sci.* **25**, 481–482
- 29 Ma, J. B., Ye, K. and Patel, D. J. (2004) Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature (London)* **429**, 318–322
- 30 Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., UiTei, K. and Saigo, K. (2003) Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. *Curr. Biol.* **13**, 41–46
- 31 Tabbaz, N., Kolb, F. A., Zhang, H., Jaronczyk, K., Filipowicz, W. and Hobman, T. C. (2004) Characterization of the interactions between mammalian PAZ PIWI domain proteins and Dicer. *EMBO Rep.* **5**, 189–194
- 32 Song, J. J., Smith, S. K., Hannon, G. J. and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**, 1434–1437
- 33 Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L. and Hannon, G. J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**, 1437–1441
- 34 Rand, T. A., Ginalski, K., Grishin, N. V. and Wang, X. (2004) Biochemical identification of Argonaute 2 as the sole protein required for RNA-induced silencing complex activity. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14385–14389
- 35 Parker, J. S., Roe, S. M. and Barford, D. (2004) Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *EMBO J.* **23**, 4727–4737
- 36 Bohmert, K., Camus, I., Bellini, C., Bouchez, D., Caboche, M. and Benning, C. (1998) AGO1 defines a novel locus of *Arabidopsis* controlling leaf development. *EMBO J.* **17**, 170–180
- 37 Moussian, B., Schoof, H., Haecker, A., Juergens, G. and Laux, T. (1998) Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. *EMBO J.* **17**, 1799–1809
- 38 Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P. and Barton, M. K. (1999) The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. *Development* **126**, 469–481
- 39 Boutet, S., Vazquez, F., Liu, J., Beclin, C., Fagard, M., Gratias, A., Morel, J. B., Crete, P., Chen, X. and Vaucheret, H. (2003) *Arabidopsis* HEN1: a genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. *Curr. Biol.* **13**, 843–848
- 40 Fagard, M., Boutet, S., Morel, J. B., Bellini, C. and Vaucheret, H. (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11650–11654
- 41 Vaucheret, H., Vazquez, F., Crete, P. and Bartel, D. P. (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* **18**, 1187–1197
- 42 Tijsterman, M., Okihara, K. L., Thijssen, K. and Plasterk, R. H. (2002) PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of *C. elegans*. *Curr. Biol.* **12**, 1535–1540
- 43 Tabara, H., Sarkissian, M., Kelly, W. G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. and Mello, C. C. (1999) The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* **99**, 123–132
- 44 Okamura, K., Ishizuka, A., Siomi, H. and Siomi, M. C. (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes Dev.* **18**, 1655–1666
- 45 Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. and Tuschl, T. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol. Cell* **15**, 185–197
- 46 Ekwall, K. (2004) The RITS complex – a direct link between small RNA and heterochromatin. *Mol. Cell* **13**, 304–305
- 47 Hall, I., Noma, K. and Grewal, S. I. (2003) RNA interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 193–198
- 48 Volpe, T., Schramke, V., Hamilton, G. L., White, S. A., Teng, G., Martienssen, R. A. and Allshire, R. C. (2003) RNA interference is required for normal centromere function in fission yeast. *Chromosome Res.* **11**, 137–146
- 49 Reinhardt, B. J. and Bartel, D. P. (2002) Small RNAs correspond to centromere heterochromatic repeats. *Science* **297**, 1831
- 50 Sigova, A., Rhind, N. and Zamore, P. D. (2004) A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in *Schizosaccharomyces pombe*. *Genes Dev.* **18**, 2359–2367
- 51 Cox, D. N., Chao, A., Baker, J., Chang, L., Qiao, D. and Lin, H. (1998) A novel class of evolutionarily conserved genes defined by piwi are essential for stem cell self-renewal. *Genes Dev.* **12**, 3715–3727
- 52 Kataoka, Y., Takeichi, M. and Uemura, T. (2001) Developmental roles and molecular characterization of a *Drosophila* homologue of *Arabidopsis* Argonaute1, the founder of a novel gene superfamily. *Genes Cells* **6**, 313–325
- 53 Cox, D. N., Chao, A. and Lin, H. (2000) piwi encodes a nucleoplasmic factor whose activity modulates the number and division rate of germline stem cells. *Development* **127**, 503–514
- 54 Sharma, A. K., Nelson, M. C., Brandt, J. E., Wessman, M., Mahmud, N., Weller, K. P. and Hoffman, R. (2001) Human CD34⁺ stem cells express the hiwi gene, a human homologue of the *Drosophila* gene piwi. *Blood* **97**, 426–434
- 55 Kuramochi-Miyagawa, S., Kimura, T., Yomogida, K., Kuroiwa, A., Tadokoro, Y., Fujita, Y., Sato, M., Matsuda, Y. and Nakano, T. (2001) Two mouse piwi-related genes: miwi and mili. *Mech. Dev.* **108**, 121–133
- 56 Findley, S. D., Tamanaha, M., Clegg, N. J. and Ruohola-Baker, H. (2003) Maelstrom, a *Drosophila* spindle-class gene, encodes a protein that colocalizes with Vasa and RDE1/AGO1 homolog, Aubergine, in nuage. *Development* **130**, 859–871

- 57 Yoon, C., Kawakami, K. and Hopkins, N. (1997) Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. *Development* **124**, 3157–3165
- 58 Knaut, H., Pelegri, F., Bohmann, K., Schwarz, H. and Nusslein-Volhard, C. (2000) Zebrafish vasa RNA but not its protein is a component of the germ plasm and segregates asymmetrically before germline specification. *J. Cell Biol.* **149**, 875–888
- 59 Wang, J. F., Zhou, H., Chen, Y. Q., Luo, Q. J. and Qu, L. H. (2004) Identification of 20 microRNAs from *Oryza sativa*. *Nucleic Acids Res.* **32**, 1688–1695
- 60 Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B. and Bartel, D. P. (2002) Prediction of plant microRNA targets. *Cell* **110**, 513–520
- 61 Morel, J. B., Godon, C., Mourrain, P., Beclin, C., Boutet, S., Feuerbach, F., Proux, F. and Vaucheret, H. (2002) Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* **14**, 629–639
- 62 King, F. J., Szakmary, A., Cox, D. N. and Lin, H. (2001) Yb modulates the divisions of both germline and somatic stem cells through piwi- and hh-mediated mechanisms in the *Drosophila* ovary. *Mol. Cell* **7**, 497–508
- 63 Barnes, E. A., Kong, M., Ollendorff, V. and Donoghue, D. J. (2001) Patched1 interacts with cyclin B1 to regulate cell cycle progression. *EMBO J.* **20**, 2214–2223
- 64 Roy, S. and Ingham, P. W. (2002) Hedgehogs tryst with the cell cycle. *J. Cell Sci.* **115**, 4393–4397
- 65 Tabata, T. and Kornberg, T. B. (1994) Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. *Cell* **76**, 89–102
- 66 Lamontagne, B., Larose, S., Boulanger, J. and Elela, S. A. (2001) The RNase III family: a conserved structure and expanding functions in eukaryotic dsRNA metabolism. *Curr. Issues Mol. Biol.* **3**, 71–78
- 67 Nicholson, A. W. (1999) Function, mechanism and regulation of bacterial ribonucleases. *FEMS Microbiol. Rev.* **23**, 371–390
- 68 Kay, B. K., Williamson, M. P. and Sudol, M. (2000) The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB J.* **14**, 231–241
- 69 Filippov, V., Solov'yev, V., Filippova, M. and Gill, S. S. (2000) A novel type of RNase III family proteins in eukaryotes. *Gene* **245**, 213–221
- 70 Fortin, K. R., Nicholson, R. H. and Nicholson, A. W. (2002) Mouse ribonuclease III. cDNA structure, expression analysis, and chromosomal location. *BMC Genomics* **3**, 26
- 71 Wu, H., Xu, H., Miraglia, L. J. and Crooke, S. T. (2000) Human RNase III is a 160-kDa protein involved in preribosomal RNA processing. *J. Biol. Chem.* **275**, 36957–36965
- 72 Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. and Kim, V. N. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature (London)* **425**, 415–419
- 73 Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. and Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature (London)* **432**, 235–240
- 74 Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F. and Hannon, G. J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature (London)* **432**, 231–235
- 75 Han, J., Lee, Y., Yeom, K. H., Kim, Y. K., Jin, H. and Kim, V. N. (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* **18**, 3016–3027
- 76 Yi, R., Qin, Y., Macara, I. G. and Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17**, 3011–3016
- 77 Bohnsack, M. T., Czaplinski, K. and Gorlich, D. (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* **10**, 185–191
- 78 Lund, E., Guttlinger, S., Calado, A., Dahlberg, J. E. and Kutay, U. (2004) Nuclear export of microRNA precursors. *Science* **303**, 95–98
- 79 Bernstein, E., Kim, S. Y., Carmell, M. A., Murchison, E. P., Alcorn, H., Li, M. Z., Mills, A. A., Elledge, S. J., Anderson, K. V. and Hannon, G. J. (2003) Dicer is essential for mouse development. *Nat. Genet.* **35**, 215–217
- 80 Ketting, R. F., Fischer, S. E., Bernstein, E., Sijen, T., Hannon, G. J. and Plasterk, R. H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev.* **15**, 2654–2659
- 81 Liu, Q., Rand, T. A., Kalidas, S., Du, F., Kim, H. E., Smith, D. P. and Wang, X. (2003) R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. *Science* **301**, 1921–1925
- 82 Nykanen, A., Haley, B. and Zamore, P. D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309–321
- 83 Zhang, H., Kolb, F., Brondani, V., Billy, E. and Filipowicz, W. (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO J.* **21**, 5875–5885
- 84 Xie, Z., Kasschau, K. D. and Carrington, J. C. (2003) Negative feedback regulation of Dicer-Like1 in *Arabidopsis* by microRNA-guided mRNA degradation. *Curr. Biol.* **13**, 784–789
- 85 Zhang, H., Kolb, F. A., Jaskiewicz, L., Westhof, E. and Filipowicz, W. (2004) Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**, 57–68
- 86 Lingel, A., Simon, B., Izaurralde, E. and Sattler, M. (2003) Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. *Nature (London)* **426**, 465–469
- 87 Song, J. J., Liu, J., Tolia, N. H., Schneiderman, J., Smith, S. K., Martienssen, R. A., Hannon, G. J. and Joshua-Tor, L. (2003) The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat. Struct. Biol.* **10**, 1026–1032
- 88 Yan, K. S., Yan, S., Farooq, A., Han, A., Zeng, L. and Zhou, M. M. (2003) Structure and conserved RNA binding of the PAZ domain. *Nature (London)* **426**, 468–474
- 89 Wienholds, E., Koudijs, M. J., van Eeden, F. J., Cuppen, E. and Plasterk, R. H. (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat. Genet.* **35**, 217–218
- 90 Knight, S. W. and Bass, B. L. (2001) A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in *Caenorhabditis elegans*. *Science* **293**, 2269–2271
- 91 Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. and Zamore, P. D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**, 834–838
- 92 Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T. and Oshimura, M. (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat. Cell Biol.* **6**, 784–791
- 93 Catalanotto, C., Pallotta, M., Refalo, P., Sachs, M. S., Vayssie, L., Macino, G. and Cogoni, C. (2004) Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. *Mol. Cell. Biol.* **24**, 2536–2545
- 94 Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J. and Carthew, R. W. (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell* **117**, 69–81
- 95 Schauer, S. E., Jacobsen, S. E., Meinke, D. W. and Ray, A. (2002) DICER-LIKE1: blind men and elephants in *Arabidopsis* development. *Trends Plant Sci.* **7**, 487–491
- 96 Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., Jacobsen, S. E. and Carrington, J. C. (2004) Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol.* **2**, E104
- 97 Papp, I., Mette, M. F., Aufsatz, W., Daxinger, L., Schauer, S. E., Ray, A., van der Winden, J., Matzke, M. and Matzke, A. J. (2003) Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol.* **132**, 1382–1390
- 98 Baulcombe, D. (2001) Diced defence. *Nature (London)* **409**, 295–296
- 99 Tabbaz, N., Carmichael, J. B. and Hobman, T. C. (2001) GERP95 belongs to a family of signal-transducing proteins and requires Hsp90 activity for stability and Golgi localization. *J. Biol. Chem.* **276**, 43294–43299
- 100 Tabara, H., Yigit, E., Siomi, H. and Mello, C. C. (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DEXH-box helicase to direct RNAi in *C. elegans*. *Cell* **109**, 861–871
- 101 Vazquez, F., Gascoli, V., Crete, P. and Vaucheret, H. (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr. Biol.* **14**, 346–351
- 102 Parrish, S. and Fire, A. (2001) Distinct roles for RDE-1 and RDE-4 during RNA interference in *Caenorhabditis elegans*. *RNA* **7**, 1397–1402
- 102a Tomari, Y., Matranga, C., Haley, B., Martinez, N. and Zamore, P. D. (2004) A protein sensor for siRNA asymmetry. *Science* **306**, 1377–1380
- 103 Grishok, A., Tabara, H. and Mello, C. C. (2000) Genetic requirements for inheritance of RNAi in *C. elegans*. *Science* **287**, 2494–2497
- 104 Ishizuka, A., Siomi, M. C. and Siomi, H. (2002) A *Drosophila* fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev.* **16**, 2497–2508
- 105 Schwarz, D. S., Hutvagner, G., Haley, B. and Zamore, P. D. (2002) Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. *Mol. Cell* **10**, 537–548
- 106 Nelson, P. T., Hatzigeorgiou, A. G. and Mourelatos, Z. (2004) miRNP:mRNA association in polyribosomes in a human neuronal cell line. *RNA* **10**, 387–394
- 107 Doench, J. G., Petersen, C. P. and Sharp, P. A. (2003) siRNAs can function as miRNAs. *Genes Dev.* **17**, 438–442
- 108 Zeng, Y., Yi, R. and Cullen, B. R. (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 9779–9784
- 109 Pham, J. W., Pellino, J. L., Lee, Y. S., Carthew, R. W. and Sontheimer, E. J. (2004) A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in *Drosophila*. *Cell* **117**, 83–94
- 110 Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P. D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199–208

- 111 Tomari, Y., Du, T., Haley, B., Schwarz, D. S., Bennett, R., Cook, H. A., Koppetsch, B. S., Theurkauf, W. E. and Zamore, P. D. (2004) RISC assembly defects in the *Drosophila* RNAi mutant armitage. *Cell* **116**, 831–841
- 112 Kennerdell, J. R., Yamaguchi, S. and Carthew, R. W. (2002) RNAi is activated during *Drosophila* oocyte maturation in a manner dependent on aubergine and spindle-E. *Genes Dev.* **16**, 1884–1889
- 113 Cook, H. A., Koppetsch, B. S., Wu, J. and Theurkauf, W. E. (2004) The *Drosophila* SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell* **116**, 817–829
- 114 Caudy, A. A., Ketting, R. F., Hammond, S. M., Denli, A. M., Bathoorn, A. M., Tops, B. B., Silva, J. M., Myers, M. M., Hannon, G. J. and Plasterk, R. H. (2003) A micrococcal nuclease homologue in RNAi effector complexes. *Nature (London)* **425**, 411–414
- 115 Reddi, K. K. (1960) Mode of action of micrococcal phosphodiesterase. *Nature (London)* **187**, 74–75
- 116 Martinez, J. and Tuschl, T. (2004) RISC is a 5' phosphomonoester-producing RNA endonuclease. *Genes Dev.* **18**, 975–980
- 117 Schwarz, D. S., Tomari, Y. and Zamore, P. D. (2004) The RNA-induced silencing complex is a Mg²⁺-dependent endonuclease. *Curr. Biol.* **14**, 787–791
- 118 Pillai, R. S., Artus, C. G. and Filipowicz, W. (2004) Tethering of human Ago proteins to mRNA mimics the miRNA-mediated repression of protein synthesis. *RNA* **10**, 1518–1525
- 119 Mochizuki, K., Fine, N. A., Fujisawa, T. and Gorovsky, M. A. (2002) Analysis of a piwi-related gene implicates small RNAs in genome rearrangement in *Tetrahymena*. *Cell* **110**, 689–699
- 120 Pal-Bhadra, M., Leibovitch, B. A., Gandhi, S. G., Rao, M., Bhadra, U., Birchler, J. A. and Elgin, S. C. (2004) Heterochromatic silencing and HP1 localization in *Drosophila* are dependent on the RNAi machinery. *Science* **303**, 669–672
- 121 Provost, P., Silverstein, R., Dishart, D., Walfridsson, J., Djupedal, I., Kniola, B., Wright, A., Samuelsson, B., Radmark, O. and Ekwall, K. (2002) Dicer is required for chromosome segregation and gene silencing in fission yeast cells. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 16648–16653
- 122 Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998) An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93**, 1067–1076
- 123 Guacci, V., Koshland, D. and Strunnikov, A. (1997) A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*. *Cell* **91**, 47–57
- 124 Michaelis, C., Ciosk, R. and Nasmyth, K. (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**, 35–45
- 125 Lee, J. Y. and Orr-Weaver, T. L. (2001) The molecular basis of sister-chromatid cohesion. *Annu. Rev. Cell Dev. Biol.* **17**, 753–777
- 126 Grewal, S. I. and Elgin, S. C. (2002) Heterochromatin: new possibilities for the inheritance of structure. *Curr. Opin. Genet. Dev.* **12**, 178–187
- 127 Bernard, P., Maure, J. F., Partridge, J. F., Genier, S., Javerzat, J. P. and Allshire, R. C. (2001) Requirement of heterochromatin for cohesion at centromeres. *Science* **294**, 2539–2542
- 128 Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S. I. and Watanabe, Y. (2002) Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. *Nat. Cell Biol.* **4**, 89–93
- 129 Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I. and Moazed, D. (2004) RNAi-mediated targeting of heterochromatin by the RITS complex. *Science* **303**, 672–676
- 130 Noma, K., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D. and Grewal, S. I. (2004) RITS acts in *cis* to promote RNA interference-mediated transcriptional and post-transcriptional silencing. *Nat. Genet.* **36**, 1141–1142
- 131 Motamedi, M. R., Verdel, A., Colmenares, S. U., Gerber, S. A., Gygi, S. P. and Moazed, D. (2004) Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. *Cell* **119**, 789–802
- 132 Zeng, Y. and Cullen, B. R. (2002) RNA interference in human cells is restricted to the cytoplasm. *RNA* **8**, 855–860
- 133 Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14428–14433
- 134 Sarot, E., Payen-Groschene, G., Bucheton, A. and Pelisson, A. (2004) Evidence for a piwi-dependent RNA silencing of the gypsy endogenous retrovirus by the *Drosophila melanogaster* *flamenco* gene. *Genetics* **166**, 1313–1321
- 135 Provost, P., Samuelsson, B. and Radmark, O. (1999) Interaction of 5-lipoxygenase with cellular proteins. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 1881–1885
- 136 Catala, M., Lamontagne, B., Larose, S., Ghazal, G. and Elela, S. A. (2004) Cell cycle-dependent nuclear localization of yeast RNase III is required for efficient cell division. *Mol. Biol. Cell* **15**, 3015–3030
- 137 Carmichael, J. B., Provost, P., Ekwall, K. and Hobman, T. C. (2004) ago1 and dcr1, two core components of the RNA interference pathway, functionally diverge from rdp1 in regulating cell cycle events in *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **15**, 1425–1435
- 138 Raleigh, J. M. and O'Connell, M. J. (2000) The G₂ DNA damage checkpoint targets both Wee1 and Cdc25. *J. Cell Sci.* **113**, 1727–1736
- 139 Rhind, N., Furnari, B. and Russell, P. (1997) Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. *Genes Dev.* **11**, 504–511
- 140 Rhind, N. and Russell, P. (1998) Tyrosine phosphorylation of cdc2 is required for the replication checkpoint in *Schizosaccharomyces pombe*. *Mol. Cell. Biol.* **18**, 3782–3787
- 141 Russell, P. and Nurse, P. (1986) cdc25⁺ functions as an inducer in the mitotic control of fission yeast. *Cell* **45**, 145–153
- 142 Millar, J. B., McGowan, C. H., Lenaers, G., Jones, R. and Russell, P. (1991) p80^{cdc25} mitotic inducer is the tyrosine phosphatase that activates p34^{cdc2} kinase in fission yeast. *EMBO J.* **10**, 4301–4309
- 143 Nyberg, K. A., Michelson, R. J., Putnam, C. W. and Weinert, T. A. (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annu. Rev. Genet.* **36**, 617–656
- 144 Hoeijmakers, J. H. (2001) Genome maintenance mechanisms for preventing cancer. *Nature (London)* **411**, 366–374
- 145 Qiao, D., Zeeman, A. M., Deng, W., Looijenga, L. H. and Lin, H. (2002) Molecular characterization of hiwi, a human member of the piwi gene family whose overexpression is correlated to seminomas. *Oncogene* **21**, 3988–3999
- 146 Skotheim, R. I., Kraggerud, S. M., Fossa, S. D., Stenwig, A. E., Gedde-Dahl, Jr, T., Danielsen, H. E., Jakobsen, K. S. and Lothe, R. A. (2001) Familial/bilateral and sporadic testicular germ cell tumors show frequent genetic changes at loci with suggestive linkage evidence. *Neoplasia* **3**, 196–203
- 147 Summersgill, B., Osin, P., Lu, Y. J., Huddart, R. and Shipley, J. (2001) Chromosomal imbalances associated with carcinoma *in situ* and associated testicular germ cell tumours of adolescents and adults. *Br. J. Cancer* **85**, 213–220
- 148 Sathya, P., Tomkins, D. J., Freeman, V., Paes, B. and Nowaczyk, M. J. (1999) *De novo* deletion 12q: report of a patient with 12q24.31q24.33 deletion. *Am. J. Med. Genet.* **84**, 116–119
- 149 Dome, J. S. and Coppes, M. J. (2002) Recent advances in Wilms tumor genetics. *Curr. Opin. Pediatr.* **14**, 5–11
- 150 Koesters, R., Ridder, R., Kopp-Schneider, A., Betts, D., Adams, V., Niggli, F., Briner, J. and von Knebel Doeberitz, M. (1999) Mutational activation of the β -catenin proto-oncogene is a common event in the development of Wilms' tumors. *Cancer Res.* **59**, 3880–3882
- 151 Carmell, M., Xuan, Z., Zhang, M. and Hannon, G. (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev.* **16**, 2733–2742
- 152 Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K. et al. (2002) Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 15524–15529
- 153 Ding, X. Z., Hennig, R. and Adrian, T. E. (2003) Lipoxygenase and cyclooxygenase metabolism: new insights in treatment and chemoprevention of pancreatic cancer. *Mol. Cancer* **2**, 10
- 154 Kennedy, T. J., Chan, C. Y., Ding, X. Z. and Adrian, T. E. (2003) Lipoxygenase inhibitors for the treatment of pancreatic cancer. *Expert Rev. Anticancer Ther.* **3**, 525–536
- 155 Gunning, W. T., Kramer, P. M., Steele, V. E. and Pereira, M. A. (2002) Chemoprevention by lipoxygenase and leukotriene pathway inhibitors of vinyl carbamate-induced lung tumors in mice. *Cancer Res.* **62**, 4199–4201
- 156 Kaul, D. and Sikand, K. (2004) Defective RNA-mediated c-myc gene silencing pathway in Burkitt's lymphoma. *Biochem. Biophys. Res. Commun.* **313**, 552–554
- 157 Hecht, J. L. and Aster, J. C. (2000) Molecular biology of Burkitt's lymphoma. *J. Clin. Oncol.* **18**, 3707–3721
- 158 Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. and Burge, C. B. (2003) Prediction of mammalian microRNA targets. *Cell* **115**, 787–798