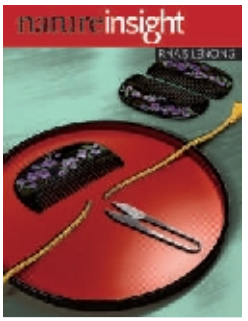

Cover illustration
In RNA silencing, one strand of a small duplex RNA (combs) enters a silencing complex (platter) that contains a catalytic Argonaute (pincers) to cleave a target RNA (cord). (Courtesy of M. Inudo and Y. Tomari. Artwork by N. Spencer)

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RNA SILENCING

When *Nature* published the first Insight on RNA interference (RNAi), in September 2004, it was clear that RNAi was going to have a broad impact on biology, even though only six years had passed since the seminal paper by Andrew Fire, Craig Mello and colleagues was published.

But who would have imagined how far we would come in the next four years in terms of understanding and exploiting this fundamental system of gene regulation? There is now a much clearer picture of how the small non-coding RNAs involved in this type of regulation are generated, drawn from the static images provided by crystallographic studies, together with the kinetic and mechanistic details gleaned through biochemical assays. From large-scale efforts to map how gene expression is affected by just one class of these small RNAs, microRNAs, it is easy to reach the conclusion that when studying any biological process, researchers must consider how it is regulated by small RNAs. Relationships between small RNAs and development are also being uncovered almost daily. And nimble biotechnology firms have, with breathtaking speed, aggressively translated this knowledge into therapeutic candidates.

It was no surprise that the researchers who opened this Pandora's box were awarded the Nobel Prize in Physiology or Medicine in 2006. As Göran Hansson stated in his presentation speech for the award, RNAi "has added a new dimension to our understanding of life and provided new tools for medicine". However, the story is far from complete even now. With advances in sequencing technology, for example, more classes of small RNA are being identified, and their functions are likely to continue to entice and surprise us.

With these reviews, we hope to convey some of the excitement driving this rapidly evolving field forward. We are pleased to acknowledge the financial support of Alnylam Pharmaceuticals and Roche in producing this Insight. As always, *Nature* carries sole responsibility for editorial content and peer review.

Angela K. Eggleston, Senior Editor

REVIEWS

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On the road to reading the RNA-interference code

Haruhiko Siomi[1] & Mikiko C. Siomi[1,2]

The finding that sequence-specific gene silencing occurs in response to the presence of double-stranded RNAs has had an enormous impact on biology, uncovering an unsuspected level of regulation of gene expression. This process, known as RNA interference (RNAi) or RNA silencing, involves small non-coding RNAs, which associate with nuclease-containing regulatory complexes and then pair with complementary messenger RNA targets, thereby preventing the expression of these mRNAs. Remarkable progress has been made towards understanding the underlying mechanisms of RNAi, raising the prospect of deciphering the 'RNAi code' that, like transcription factors, allows the fine-tuning and networking of complex suites of gene activity, thereby specifying cellular physiology and development.

The discovery of RNA interference (RNAi)[1] heralded a revolution in RNA biology. Researchers uncovered 'hidden' layers of regulation of gene expression, in which many previously unidentified families of small RNAs (consisting of ~20–30 nucleotides) mediate gene silencing. A diverse set of gene-regulatory mechanisms were found to use key steps in the RNAi process, including mechanisms that silence endogenous genes and mechanisms that restrain the expression of parasitic and pathogenic invaders such as transposons and viruses[2–5].

The basic RNAi process can be divided into three steps[6,7]. First, a long double-stranded RNA (dsRNA) that is expressed in, or introduced into, the cell (for example, as a result of the base-pairing of sense and antisense transcripts or the formation of stem–loop structures) is processed into small RNA duplexes by a ribonuclease III (RNaseIII) enzyme known as Dicer. Second, these duplexes are unwound, and one strand is preferentially loaded into a protein complex known as the RNA-induced silencing complex (RISC). Third, this complex effectively searches the transcriptome and finds potential target RNAs. The loaded single-stranded RNA (ssRNA), called the guide strand, then directs an endonuclease that is present in the RISC (sometimes called the 'slicer' and now known to be an Argonaute protein[8–11]) to cleave messenger RNAs that contain sequence homologous to the ssRNA, over many rounds. In this way, the guide strand determines the sequence specificity of the RNAi response.

In different organisms, the RNAi pathways comprise different proteins and mechanisms, but they operate by strikingly convergent strategies. In all organisms that have been studied, RNAi involves two main components: small RNAs, which determine the specificity of the response; and Argonaute proteins, which carry out the repression. Depending on both the nature of the Argonaute in the RISC and the degree of complementarity between the small RNA and the target sequence in the mRNA, the association of the RISC with target mRNAs has been shown to have different outcomes: it can control protein synthesis and mRNA stability, maintain genome integrity or produce a specific set of small RNAs[8,12]. Analyses of the biogenesis of small RNAs and their targeting mechanisms have benefited from the advent of high-throughput sequencing technologies and sophisticated bioinformatics[13]. The picture emerging from these studies is that RNAi systems in different organisms have been refined in many ways, and such modifications include built-in molecular 'rulers' that define the size of small RNAs, structures that determine which strand of a small RNA is selected, mechanisms that direct further rounds of small RNA amplification, or safeguards against off-target (unrestricted and unrelated) silencing.

Another emerging finding in the field is that the activity of RNAi pathways is subject to intense regulation at various levels, from the level of biogenesis of small RNAs to the silencing mode of the RISC. In this Review, we describe the biogenesis of the guide strand of small RNAs and the formation and actions of the RISC, and we discuss the current understanding of the molecular mechanisms of RNAi in the light of recent insights into how silencing pathways are specified and regulated.

Biogenesis of small RNAs

A hallmark of RNAi is that short (~20–30 nucleotide) dsRNAs — known as small RNAs — are generated by the activity of RNaseIII enzymes (either Dicer alone or Drosha and Dicer). Two main categories of small RNAs have been defined on the basis of their precursors. The cleavage of exogenous long dsRNA precursors in response to viral infection or after artificial introduction generates short interfering RNAs (siRNAs), whereas the processing of genome-encoded stem–loop structures generates microRNAs (miRNAs). Using high-throughput sequencing technology, several new classes of endogenous small RNA species have recently been uncovered, and these include PIWI-interacting RNAs (piRNAs) and endogenous siRNAs (endo-siRNAs or esiRNAs).

A common feature of all of these small RNAs is that they are loaded onto Argonaute proteins to effect their targeting function (discussed further in the section 'Loading and sorting of small RNAs by the RISC'). An overview of the generation of small RNAs is presented in Fig. 1.

siRNA biogenesis

Dicer (Table 1) processes long RNA duplexes and generates siRNAs. These small RNAs are ~21–25-nucleotide duplexes with a phosphate group at both 5′ ends, and hydroxyl groups and two-nucleotide overhangs at both 3′ ends, all hallmarks of RNaseIII-mediated cleavage. The Dicer protein contains a PAZ domain, which binds to the 3′ end of an siRNA, and two RNaseIII domains, which have the catalytic activity. It functions as a monomer[14], but the RNaseIII domains associate with each other to

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form an 'internal dimer' (see page 405). The distance between the PAZ domain and the two RNaseIII domains is the length spanned by 25 base pairs (bp) of RNA[15]. Thus, Dicer itself is a molecular ruler.

miRNA biogenesis

Similarly, miRNAs are short (~21–25-nucleotide) RNA molecules[16]; however, their biogenesis differs markedly from that of siRNAs. The primary precursors of miRNAs (pri-miRNAs) are encoded in the genome, and the relevant genomic regions are mostly transcribed by RNA polymerase II (ref. 17). The pri-miRNAs contain stem–loop structures that harbour the miRNA in the 5′ or 3′ half of the stem. During miRNA production in plants, one type of RNaseIII, Dicer-like protein 1 (DCL1), generates the miRNA–miRNA* duplex in the nucleus (miRNA* being the sequence in the stem–loop that pairs with the miRNA, equivalent to the passenger strand of siRNA duplexes; discussed later). By contrast, in animals, miRNAs are derived in a two-step process, in which the nuclear-localized RNaseIII Drosha defines one end of the miRNA–miRNA* duplex and releases a precursor miRNA (pre-miRNA) of ~65–70 nucleotides. The pre-miRNA hairpin is then exported to the cytoplasm, where Dicer completes the processing.

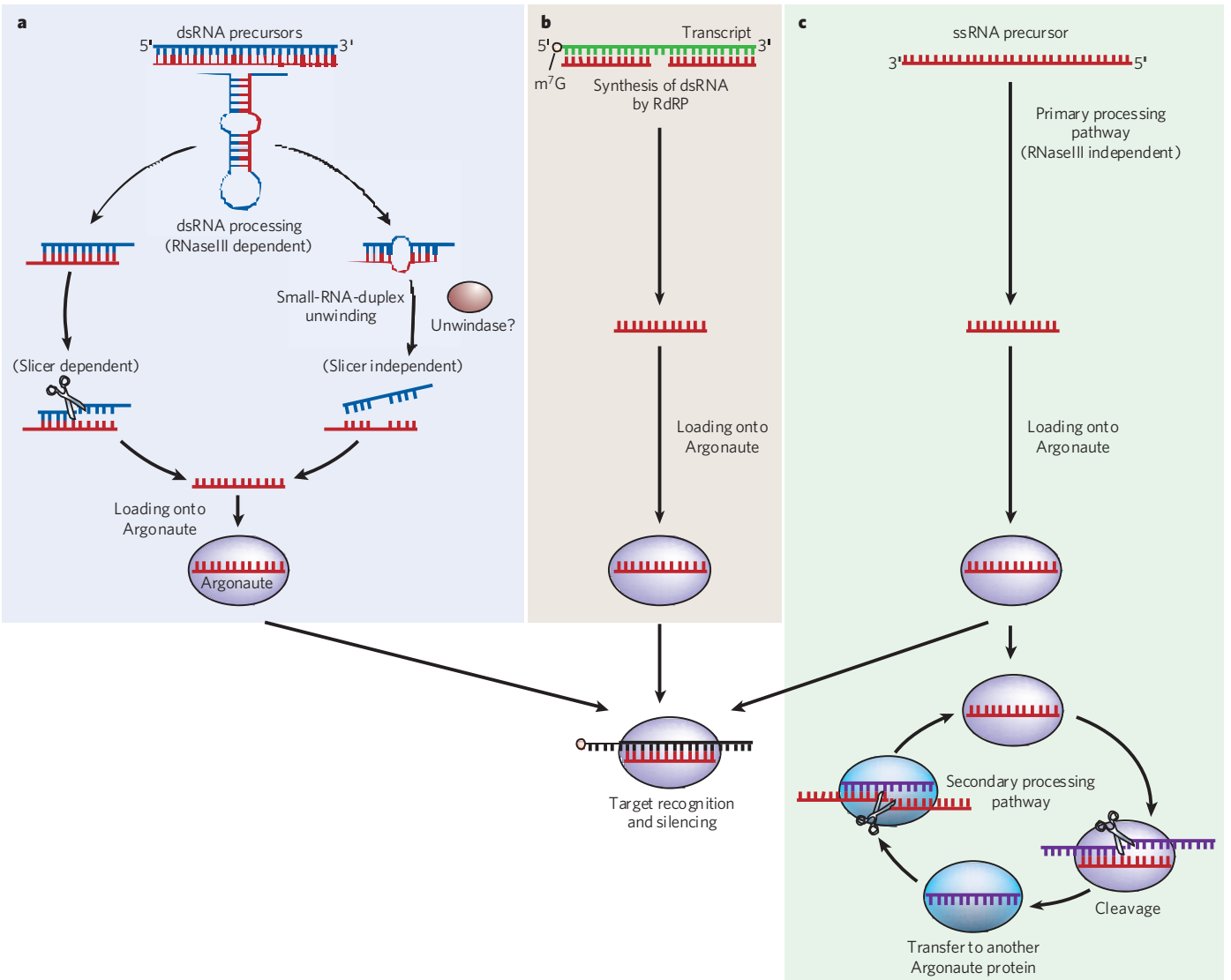

Figure 1 | Small RNA production and RNA silencing. a, Natural transcripts that form dsRNAs and hairpin-shaped structures can be sources of small RNAs. These precursors are processed by an RNaseIII enzyme (such as Drosha or Dicer), yielding small RNA duplexes. Duplexes with a perfect match (left pathway) are further processed by an enzyme with slicer activity (an Argonaute protein) into single-stranded small RNAs. By contrast, small RNA duplexes with a mismatch or bulge in the centre (right pathway) are not substrates for the slicer and thus become single-stranded in a cleavage-independent manner. The identity of the protein that carries out this unwinding is unknown. Single-stranded small RNAs are then loaded onto Argonaute proteins. The particular strand that is selected (sense or antisense) depends on thermodynamic stability. The loaded Argonaute proteins are guided to target mRNAs containing complementary sequence, and the expression of the corresponding genes is silenced. The mode of action of this silencing — whether the mRNA is cleaved or whether translation is just repressed — largely depends on the degree of complementarity between the target mRNAs and the Argonaute-associated small RNAs. **b**, Some small RNAs found in *Caenorhabditis elegans* and plants are known to be produced in an RNA-dependent RNA polymerase (RdRP)-dependent manner. Natural transcripts (often aberrant RNAs) can be substrates for this type of small RNA synthesis. This does not occur in organisms that lack RdRP activity, such as mammals and *Drosophila melanogaster*. Single-stranded small RNAs generated in this way can then be loaded onto Argonaute proteins and silence gene expression. **c**, The PIWI subfamily of Argonaute proteins, which are germline specific, are loaded with piRNAs. These complexes function to silence transposons. Single-stranded precursors give rise to piRNAs, through a mechanism called the primary processing pathway. The proteins required for this pathway are unknown. The silencing of transposons by PIWI proteins simultaneously amplifies piRNAs in germ cells. This pathway is known as the secondary processing pathway (or the ping-pong amplification loop) and is conserved in a variety of organisms, including mice and zebrafish. In this pathway, the slicer activity of the PIWI proteins reciprocally forms the 5′ ends of piRNAs by cleaving transposon transcripts (piRNA precursors). Proteins required to form the 3′ end of piRNAs remain unidentified.

Table 1 | Key proteins in RNA silencing in various organisms

Protein	Yeast (*Schizosaccharomyces pombe*)	Plant (*Arabidopsis thaliana*)	Nematode (*Caenorhabditis elegans*)	Fruitfly (*Drosophila melanogaster*)	Mammal	
					Mouse	Human
RNaseIII	Dcr1	DCL1	DCR-1	DCR-1	DICER1	DICER1
		DCL2	DRSH-1	DCR-2	DROSHA	DROSHA
		DCL3		DROSHA		
		DCL4				
Argonaute: AGO subfamily	Ago1	AGO1	ALG-1	AGO1	AGO1	AGO1
		AGO2	ALG-2	AGO2	AGO2	AGO2
		AGO4	3 others		AGO3	AGO3
		AGO5			AGO4	AGO4
		AGO6			AGO5 (possibly a pseudogene)	
		AGO7 (ZIPPY)				
		AGO10 (ZLL, PNH)				
		3 others				
Argonaute: PIWI subfamily	None	None	ERGO-1	AGO3	MILI (PIWIL2)	HILI (PIWIL2)
			PRG-1	PIWI	MIWI (PIWIL1)	HIWI (PIWIL1)
			PRG-2	AUB	MIWI2 (PIWIL4)	HIWI2 (PIWIL4)
						PIWIL3 (HIWI3)
Argonaute: WAGO subfamily	None	None	RDE-1	None	None	None
			SAGO-1			
			SAGO-2			
			PPW-1			
			PPW-2			
			CSR-1			
			NRDE-3			
			11 others			
Double-stranded-RNA-binding domain (dsRBD)-containing cofactor of RNaseIII	None	HYL1	PASH-1	PASHA	DGCR8	DGCR8
			RDE-4	R2D2	TRBP (TARBP2)	TRBP (TARBP2)
				LOQS	PACT (PRKRA)	PACT (PRKRA)
RNA-dependent RNA polymerase (RdRP)	Rdp1	RDR1	EGO-1	None	None	None
		RDR2 (SMD1)	RRF-1			
		RDR6 (SDE1, SGS2)	RRF-3			
		3 others	1 other			

Molecules that belong to these categories but have unknown functions are not listed but are indicated as 'others'. Common synonyms are indicated in parentheses. Data were taken from refs 8, 12, 29, 50 and 98.

Drosha is present in a large complex, known as the microprocessor complex, which functions like a molecular ruler to determine the cleavage site in the pri-miRNAs[18,19]. In this complex, Drosha interacts with its cofactor, known as DGCR8 or Pasha (depending on the species), which also binds to dsRNA (through its dsRNA-binding domain; dsRBD)[20,21]. A typical metazoan pri-miRNA consists of a 33-bp stem, a terminal loop and ssRNA flanking segments. The flanking segments are crucial for binding to DGCR8, and the 33-bp stem is also required for efficient binding. Drosha can interact transiently with the stem of this 'pre-cleavage' complex, and the processing centre of the enzyme, located at ~11 bp from the ssRNA–dsRNA junction, makes a staggered pair of breaks in the RNA to create the ~65–70-nucleotide pre-miRNA. Thus, DGCR8 might function as the molecular anchor that measures the distance from the ssRNA–dsRNA junction. It is possible that the microprocessor complex could recognize the terminal loop as ssRNA and bind to the stem–loop structure in the opposite orientation. In this case, abortive cleavage can occur at an alternative site ~11 bp from the terminal loop. However, most pri-miRNAs contain internal bulges or weakly paired bases ~11 bp from the terminal loop that mitigate processing from this direction[18].

Although many of the sequences encoding miRNAs are located within introns, clusters encoding miRNAs that are processed directly by the spliceosome, instead of Drosha, were recently identified[22,23]. The 3′ end of the stem–loop precursor of these intronic miRNAs (known as mirtrons) coincides with the 3′ splice site of a small annotated intron and is cleaved in the same splicing pathway as pre-mRNA in the nucleus instead of by Drosha. Subsequently, the mirtron precursors, which are released by the spliceosome in the shape of a lariat (lasso), are linearized by a de-branching enzyme. They then enter the miRNA-processing pathway directly (by mimicking the structural features of pre-miRNA hairpins) and are therefore exported to the cytoplasm and processed by a Dicer protein, bypassing Drosha-mediated cleavage.

The imprecision of Drosha or Dicer cleavage could result in the production of a set of miRNA–miRNA* duplexes with a variety of 5′ and 3′ ends. Most miRNAs in animals form imperfect hybrids with sequences in the target mRNA, with most of the pairing specificity being provided by the 5′-proximal region of the miRNA (that is, positions 2–8; also known as the seed region)[24,25]. Imprecise cleavage either alters the seed sequence or inverts the relative stabilities of the 5′ and 3′ ends of the duplex (see the section 'Loading and sorting of small RNAs by the RISC'). The results of recent deep-sequencing studies of small RNAs, however, indicate that human cells might take advantage of such imprecise cleavage, because the generation of a diverse set of miRNAs from a single precursor could be a way of broadening the network of factors and processes that are regulated by miRNAs[26–28].

RNaseIII-independent pathways of small RNA biogenesis

In some systems, small RNAs do not seem to be produced in response to dsRNA, but silencing signals are still amplified. Because these small RNAs do not arise from dsRNA precursors, RNaseIII enzymes cannot be involved in their generation. These findings therefore call into question the definition of RNAi. In this subsection, we describe the known RNaseIII-independent pathways of small RNA production, including those that generate piRNAs, 21U-RNAs, and secondary siRNAs in *Caenorhabditis elegans*.

The small RNAs known as piRNAs were named for their ability to bind to a group of Argonaute proteins known as PIWI proteins. As noted earlier, members of the Argonaute family bind directly to small guide RNAs and lie at the core of all known RISCs[8]. Argonaute proteins consist of a variable amino-terminal domain and three conserved domains (the PAZ, middle (MID) and PIWI domains)[8,29,30]. The 3′ end of a small RNA interacts with the PAZ domain, whereas the phosphate group at the 5′ end of small RNAs binds to a cleft bridging the MID domain and the PIWI domain[29,30] (see page 405). The PIWI domain has an RNaseH-like folded structure[10] and slicer activity (although some Argonaute proteins seem to have no slicer activity). There are three phylogenetic groups of Argonaute proteins[29]: the AGO subfamily (or AGO clade), named after the founding member *Arabidopsis thaliana* ARGONAUTE 1 (AGO1); the PIWI subfamily, named after *D. melanogaster* PIWI (*P*-element-induced wimpy testis); and the WAGO (worm-specific Argonaute) subfamily of *C. elegans*-specific proteins. PIWI-subfamily proteins bind to piRNAs[31–37] (Table 1). These small RNAs have been found only in germ cells, and they are important for germline development and suppress transposon activity in the germline cells of mammals, fish and *D. melanogaster*. They are ~24–31 nucleotides (slightly longer than miRNAs), usually have a uridine at the 5′ end and carry a 5′ monophosphate. Unlike mammalian miRNAs, but similarly to plant miRNAs, piRNAs have a 2′-*O*-methyl (2′-*O*-Me) modification on the nucleotide at the 3′ end, a modification that is carried out by a HEN1-like methyltransferase[38–42]. If Dicer is mutated, the production of piRNAs is not affected, indicating that their biogenesis is distinct from that of miRNAs and siRNAs and does not involve dsRNA precursors[31,42].

The sequencing of small RNAs associated with *D. melanogaster* PIWI-subfamily proteins (PIWI, Aubergine (AUB) and AGO3)[43,44] showed that piRNAs associated with AUB and PIWI are derived mainly from the antisense strand of retrotransposons, whereas AGO3-associated piRNAs arise mainly from the sense strand. AUB- and PIWI-associated piRNAs show a strong preference for uridine at their 5′ ends, whereas AGO3-associated piRNAs show a preference for adenosine at nucleotide 10. The first ten nucleotides of AUB-associated piRNAs can be complementary to the first ten nucleotides of AGO3-associated piRNAs. In addition, PIWI-subfamily proteins have slicer activity that allows them to cleave an RNA substrate opposite position 10 of their bound piRNA[32,44]. These observations suggest that piRNAs have a self-amplifying loop (Fig. 1), in which sense piRNAs associated with AGO3 cleave long antisense transcripts and guide the formation of the 5′ end of antisense piRNAs bound to AUB or PIWI, and vice versa. Thus, in this amplification loop, which is called the ping-pong cycle[43], transposons are both a source of piRNAs and a target of piRNA-mediated silencing. After the resultant cleavage products have been loaded onto another member of the PIWI subfamily, further (as yet unidentified) nuclease activity generates the 3′ end of the piRNA, with the specific size of the piRNA determined by the footprint of the PIWI-subfamily protein on the RNA, a step that seems to precede 2′-*O*-Me modification[38]. In each PIWI-subfamily protein, the PAZ domain might be positioned at a distance from the MID domain that corresponds to the length of each piRNA. Thus, the PAZ domain might function as part of a molecular ruler for processing piRNAs of a defined size. Signatures of this amplification cycle are also apparent in zebrafish (*Danio rerio*) germ cells and in mammalian germ cells before the pachytene stage of meiosis during spermatogenesis[42,45].

PIWI-subfamily proteins and, presumably, their associated piRNAs are loaded into embryos from the ova[8], implying that the piRNAs that initiate an amplification cycle of piRNA biogenesis (which generates secondary piRNAs) could be supplied by germline transmission. But several findings indicate that there must be mechanisms of piRNA biogenesis other than amplification induced by maternal piRNAs. First, the amplification cycle in *D. melanogaster* engages mainly AGO3 and AUB[43,44], but piRNAs are still loaded onto PIWI, which is spatially separated from these proteins at the subcellular and cell-type levels[32,43,44]. Second, piRNAs derived from a particular piRNA cluster in the genome (the *flamenco* locus) associate almost exclusively with PIWI[43]. These findings indicate that *flamenco*-derived piRNAs are produced by a pathway independent of the amplification loop. Whether such a piRNA-biogenesis pathway exists remains to be determined.

What at first seemed to be another type of small RNA, 21U-RNA, is found in *C. elegans*. These small RNAs are precisely 21 nucleotides and have a bias towards uridine at the 5′ end (but not in the remaining 20 nucleotides), and the genetic regions that encode them contain a characteristic sequence motif ~42 bp upstream of the first nucleotide of the small RNA[46]. It is possible that these RNAs are derived from thousands of separate, autonomously expressed, loci that are broadly scattered in two large regions of one chromosome. They are expressed solely in the germ line and interact with the PIWI-subfamily protein PRG-1 (refs 47, 48); therefore, 21U-RNAs are the *C. elegans* equivalent of piRNAs by definition. Like piRNAs, they depend on PRG-1 activity for their accumulation and are independent of DCR-1 (the *C. elegans* Dicer protein) for their production. *C. elegans* with mutations in *prg-1* have a smaller brood and a temperature-sensitive sterile phenotype, which is consistent with the idea that PIWI-subfamily proteins are involved in germline maintenance. Like the piRNAs found in mammalian germ cells in pachytene[33,34], 21U-RNAs have remarkable sequence diversity but lack obvious targets.

Small RNAs with a similar role to piRNAs have also been found in the ciliate *Tetrahymena thermophila*. These scan RNAs (scnRNAs) direct the elimination of transposon-like DNA sequences and associate with a PIWI-subfamily protein, TWI1 (ref. 8) but, in contrast to piRNAs and 21U-RNAs, are produced by a Dicer-dependent pathway[49].

These three examples (piRNAs, 21U-RNAs and scnRNAs) indicate that the core PIWI and piRNA machinery might have evolved to produce small RNAs and silence targets by different strategies.

RNA silencing pathways include mechanisms that downregulate endogenous genes and restrain the expression of selfish or exogenous genetic material, and these pathways often share common components such as Dicer. Therefore, there should be competition between different silencing pathways for particular components. Ways to overcome such competition should also exist; for example, by amplifying a weak silencing signal. In *C. elegans*, distinct Argonaute proteins operate at different stages of RNAi, directing gene silencing in a sequential manner[50] — the second stage of which involves RNaseIII-independent biogenesis of small RNAs. First, a primary Argonaute protein (such as RDE-1 for exogenous siRNAs (exo-siRNAs) and ERGO-1 for endo-siRNAs) is guided by 'primary' siRNAs (that is, a first round of siRNAs), which have been generated from long dsRNAs by DCR-1. Second, the silencing signal is amplified by the production of 'secondary' siRNAs by the action of RNA-dependent RNA polymerases (RdRPs) (Fig. 1). These secondary siRNAs then bind differentially to secondary Argonaute proteins (SAGOs, members of the WAGO subfamily), which mediate downstream silencing. In plants, RNAs with aberrant features, including lack of a poly(A) tail and lack of a 5′ cap, are copied into double-stranded forms by RdRPs and become substrates for Dicer, which converts them into siRNA duplexes[12]. By contrast, the *C. elegans* somatic RdRP mostly produces 21-nucleotide, single-stranded, 5′-triphosphorylated small RNAs directly from the target mRNA in a primer-independent manner without the need for Dicer-mediated cleavage of dsRNA[51–53]. Such recruitment of an RdRP directly to the target mRNA allows dsRNA synthesis without consuming the siRNAs generated in response to the original trigger, although it is unclear how the 3′ end of these secondary siRNAs is formed and what the molecular ruler is that determines their size.

Blurring of the boundaries between small RNA types

As described above, the three main classes of small RNA — siRNAs, miRNAs and piRNAs — are distinct in their biogenesis and cellular roles. However, recent findings blur these distinctions and show that there are even more-complex interactions between factors involved in small RNA biogenesis. Deep sequencing of small RNAs from somatic tissues and cultured somatic cells in *D. melanogaster* has uncovered another class of small RNA, consisting of 3′-methylated, 21-nucleotide RNAs derived from the *D. melanogaster* genome. These endogenous RNAs are derived from transposons and from several loci, including

loci that encode *cis*-natural antisense transcript pairs, and long stem–loop structures containing many mismatched pairs in their stems[54–57]. In *D. melanogaster*, distinct Dicer-containing complexes produce exo-siRNAs and miRNAs[58,59]. DCR-1 generates miRNAs, acting with its dsRNA-binding protein partner, Loquacious (LOQS)[60,61], and the miRNAs are loaded onto AGO1. By contrast, DCR-2, together with its dsRNA-binding protein partner, R2D2 (ref. 62), generates exo-siRNAs, which are loaded onto AGO2. Like exo-siRNAs, the recently discovered endogenous small RNAs are produced by the DCR-2-dependent pathway and are loaded onto AGO2, and they are therefore called endo-siRNAs. However, the generation of many endo-siRNAs requires LOQS[54,56], the dsRBD-containing partner of DCR-1 in the miRNA pathway[60,61], but not R2D2, the partner of DCR-2 (ref. 62). In *D. melanogaster* deficient in DCR-2 or AGO2, the expression of transposons increases, so endo-siRNAs might be the main mechanism for silencing 'selfish' genetic elements in somatic cells, which lack the piRNA pathway. Therefore, endo-siRNAs and piRNAs are fundamentally similar in that they defend organisms against nucleic-acid-based 'parasites'. This finding also shows that *D. melanogaster* has two RNAi pathways that repress transposon expression. Mouse oocytes have also been shown to contain endo-siRNAs. These RNAs are derived from various sources, including transposons[63,64]; however, some are processed from overlapping regions of functional genes and their cognate pseudogenes. This finding suggests that pseudogenes, which have been thought to be non-functional protein 'fossils', might regulate the expression of their founder genes.

Although siRNAs and miRNAs are categorized in terms of their origin rather than their size or function[7,12], the discovery of endo-siRNAs makes it difficult to distinguish between siRNAs and miRNAs. This blurring of the boundaries between the different types of small RNA has interesting evolutionary implications. The long stem–loop structures that are processed to form endo-siRNAs are reminiscent of the pre-miRNAs in plants. One hypothesis for the evolutionary origin of plant miRNAs is that new plant miRNA loci might evolve from the inverted duplication of founder loci, which when transcribed would result in hairpin RNAs[12]. These hairpin RNAs would have almost perfect self-complementarity and might be processed by Dicer-like enzymes other than DCL1, the main miRNA-processing enzyme in plants, because DCL1 has limited activity against such substrates. Subsequent acquisition of mutations as a result of genetic drift would produce a hairpin with imperfect complementarity, which could then be processed by DCL1. Thus, the stem–loop structures from which endo-siRNAs are derived could be evolutionary intermediates that are gradually transformed into miRNA precursors. It is possible that such an adaptive switch could also occur during the evolution of miRNA-encoding genes in *D. melanogaster*, in which DCR-1 would then generate miRNAs instead of endo-siRNAs being generated by DCR-2.

Loading and sorting of small RNAs by the RISC

In gene silencing pathways initiated by dsRNA precursors, Dicer-mediated cleavage yields small dsRNA intermediates (small RNA duplexes). These small RNA duplexes must be dissociated into 'competent' single strands in order to function as guides for RISCs. For each small RNA duplex, only one strand, the guide strand, is loaded onto a specific Argonaute protein and assembled into the active RISC; the other strand, the passenger strand, is destroyed. Many eukaryotes express more than one Argonaute protein, and these proteins bind to small RNAs in a sequence-independent manner. So how are small RNAs sorted and loaded onto a specific Argonaute protein?

Loading

A small RNA generated from dsRNA precursors is converted from a duplex into a single-stranded form as it is loaded into the RISC. The key steps in converting the RISC from its precursor form (the pre-RISC), which contains the small RNA duplex, to its mature form (the holo-RISC), which contains the guide strand, are small RNA strand unwinding and preferential strand selection. The prevalent view of RISC loading is that thermodynamic asymmetry along small RNA duplex determines which RNA strand is retained and which is discarded. More specifically, the strand that has its 5′ end at the thermodynamically less stable end of the small RNA duplex is preferentially loaded into the RISC as the guide strand, a phenomenon referred to as the asymmetry rule[65,66].

For siRNAs, the known interactions between Dicer and the Argonaute proteins[8] indicate that the production of the small RNA and the assembly of the RISC might be physically coupled. For example, in *D. melanogaster*, DCR-2 does not simply transfer siRNAs to a distinct RISC but, instead, forms part of the RISC together with the siRNAs, indicating that the role of DCR-2 extends beyond the initiation phase. The loading of siRNA duplexes onto AGO2 is facilitated by the RISC-loading complex, which contains DCR-2 and its dsRBD-containing partner, R2D2 (refs 62, 67). The particular strand of the siRNA duplex that is loaded onto AGO2 seems to be determined by the orientation of the DCR-2–R2D2 heterodimer on the siRNA duplex[68]. R2D2 is thought to sense the thermodynamic stability of the siRNA duplexes and bind to the more stable end of the siRNA, whereas DCR-2 is recruited to the less stable end. The heterodimer probably recruits AGO2 through an interaction between DCR-2 and AGO2. Previous models have proposed that the transition from a double-stranded silencing trigger to a single-stranded one is mediated by an unidentified ATP-dependent RNA helicase. However, the unwinding of the siRNA duplex and the loading of a single strand into the RISC are facilitated by the slicing of the unincorporated (passenger) strand by AGO2, a process that does not require ATP[69–71] (Fig. 1). Cleavage in the middle of the passenger strand, as though the passenger strand were an mRNA target, would be expected to reduce the annealing temperature and the free energy of duplex formation, which in turn facilitates the separation of the siRNA strands. These data support a model in which siRNAs are initially loaded as duplexes onto an AGO2-containing pre-RISC (Fig. 2).

By contrast, in humans, pre-miRNAs are known to bind to a preformed trimeric complex of AGO2, DICER1 and DICER1's dsRBD-containing partner, TRBP[72]. This complex can cleave target RNAs using pre-miRNA and can distinguish miRNA from miRNA*, in the absence of ATP hydrolysis[72,73], suggesting that DICER1-mediated cleavage and sensing of thermodynamic stability occur in series in the AGO2–DICER1–TRBP complex.

This process by which a pre-RISC is converted to a holo-RISC can also occur by a slicer-independent mechanism. Three of the four Argonaute proteins in humans (AGO1, AGO3 and AGO4) lack slicer activity but are nonetheless loaded with single-stranded guide siRNAs[9,11,28]. Similarly, single-stranded miRNAs are found associated with AGO2 in humans, despite the expectation that mismatches in the unwound pre-miRNA should block the passenger-strand cleavage activity of AGO2. Thus, a cleavage-independent (bypass) mechanism for RISC assembly must exist. RNA helicase A has been identified as a candidate for unwinding the duplex in this process[74].

Sorting

Once assembled, RISCs mediate a range of the effector steps in all RNA silencing mechanisms, from repressing translation to maintaining genome stability. The specialized functions of RISCs are likely to result from the particular proteins that associate with each Argonaute protein. In other words, the different RISC variants are distinguished by their constituent Argonaute protein. Thus, it is crucial that a specific set of small guide RNAs is directed to a specific Argonaute protein. Analyses of how different types of small RNA are channelled to different Argonaute proteins show that there are multiple mechanisms: the determinants for small RNA sorting vary from the structure of the small RNA duplex to the identity of the 5′ nucleotide and the presence and extent of modifications to this nucleotide.

In *D. melanogaster*, pre-miRNAs are processed by DCR-1, whereas exo-siRNA duplexes are produced by DCR-2 from long dsRNAs[58] (Fig. 2a). Small RNAs then seem to be loaded onto either AGO1 or AGO2, depending on the structure of a small intermediate RNA duplex[75]. If the duplex has a bulge in the middle (frequently observed in miRNA

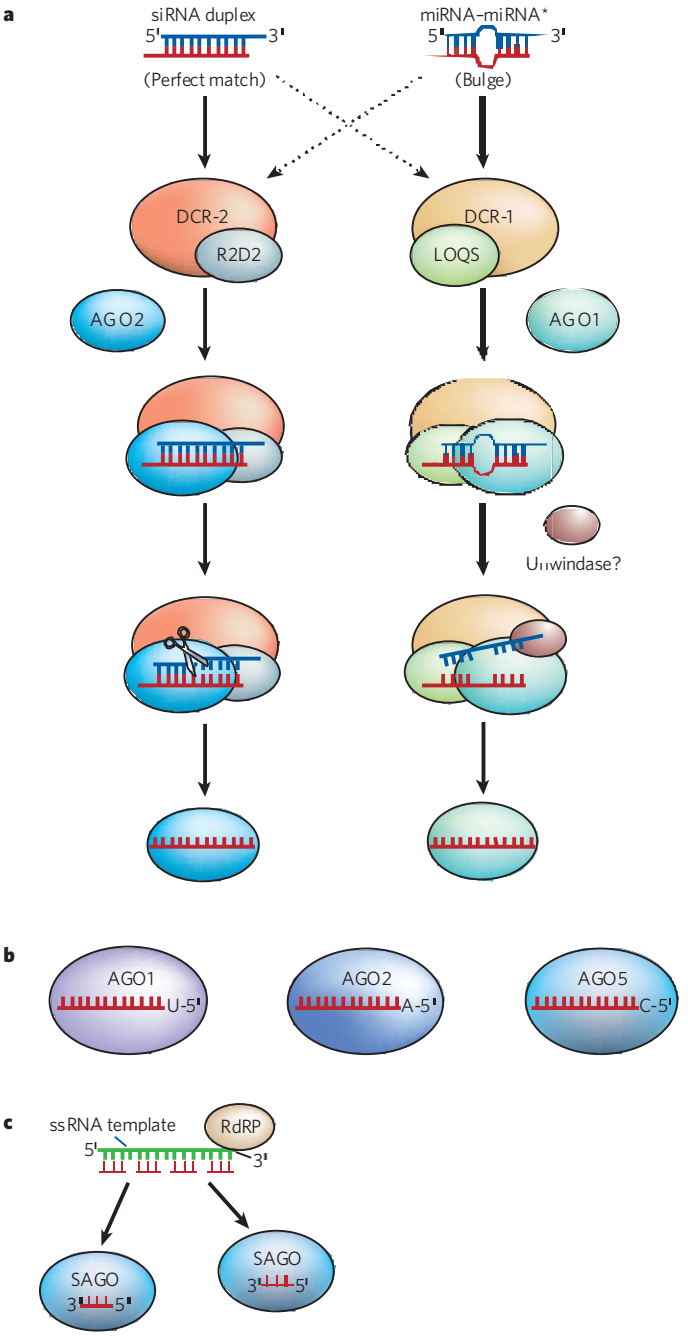

Figure 2 | Sorting of small RNAs onto distinct Argonaute proteins. Small RNAs are sorted onto specific Argonaute proteins, and this process occurs by several mechanisms. **a**, In *Drosophila melanogaster*, small RNAs originating from a duplex are loaded onto one of two Argonaute proteins (AGO1 or AGO2), on the basis of the structure of the small RNA duplex. If the duplex has a mismatch or a bulge in the centre (as miRNAs do), then the RNA is routed to AGO1. If the duplex is perfectly matched (as siRNAs are), then the small RNA is routed to AGO2. This selectivity occurs because the small RNAs are loaded onto Argonaute proteins from a Dicer-containing complex, and the two forms of Dicer, DCR-1 and DCR-2, associate with different RNA structures. DCR-2 pairs with R2D2, and this heterodimer binds to highly paired small RNA duplexes but recognizes small RNA duplexes with a central mismatch only poorly. AGO2 favours binding to DCR-2–R2D2 over binding to the other Dicer-containing complex, DCR-1–LOQS, which binds to small RNAs with bulges. Further processing into single-stranded small RNAs is described in Fig. 1. **b**, *Arabidopsis thaliana* miRNAs and *trans*-acting siRNAs (ta-siRNAs) have a 5′ uridine and preferentially associate with AGO1. By contrast, AGO2 and AGO5 show preferences for small RNAs containing 5′ adenosines and 5′ cytidines, respectively. However, it is unlikely that the 5′ nucleotide is the sole determinant of selective loading in *A. thaliana*. **c**, Secondary endo-siRNAs in *Caenorhabditis elegans*, as well in *Schizosaccharomyces pombe*, have a striking strand bias in which only the antisense siRNA is loaded onto Argonaute proteins. These siRNAs correspond to the RNA strand synthesized by RdRP. In *C. elegans*, RdRP produces small RNAs directly from the target mRNA in a primer-independent manner. Thus, these secondary small RNAs show negative polarity, and this mechanism reinforces the silencing carried out by the primary small RNAs.

precursors), the small RNA is routed to AGO1. If the duplex is perfectly matched, the small RNA is channelled to AGO2. This is because the DCR-2–R2D2 heterodimer, which recruits AGO2 to form the pre-RISC, binds well to highly paired small RNA duplexes but poorly to duplexes with central mismatches. Thus, the DCR-2–R2D2 heterodimer not only determines the polarity of siRNA loading on the basis of thermodynamic stability rules but also functions as a gatekeeper for AGO2-containing RISC assembly, promoting the incorporation of siRNAs over miRNAs. These observations suggest that each siRNA duplex dissociates from the active site of the Dicer protein after it is produced and is subsequently recaptured by the DCR-2–R2D2 heterodimer. However, although AGO1 favours binding to small RNA duplexes with central mismatches, a large proportion of miRNA–miRNA* duplexes with a base-paired central region still enter into AGO1-containing RISCs[55], suggesting that the AGO1-loading pathway is selective and not a default pathway for small RNAs rejected by the AGO2 pathway.

The identity of the nucleotide at the 5′ end and the extent to which this nucleotide is phosphorylated also influence which Argonaute protein the small RNA associates with. In contrast to what is observed in *D. melanogaster*, processing by Dicer may be uncoupled from association with Argonaute proteins in *A. thaliana* because, in this species, the miRNAs are all generated by one particular Dicer protein, DCL1, but are still sorted and loaded onto different Argonaute proteins. In *A. thaliana*, miRNAs and *trans*-acting siRNAs (ta-siRNAs), a class of small RNAs that regulate plant development[12], generally have a 5′ uridine and preferentially associate with AGO1 (ref. 76) (Fig. 2b). By contrast, AGO2 associates preferentially with small RNAs containing 5′ adenosines, and AGO5 prefers 5′ cytidines. Interestingly, if the opposite strand of a miRNA (that is, miRNA*) has a 5′ adenosine or a 5′ cytidine, it is bound to AGO2 or AGO5, respectively. These findings have led to the hypothesis that the binding affinity of Argonaute proteins for small RNAs is determined by the nucleotide at the 5′ end. Although these 5′-nucleotide preferences generally hold true for these Argonaute proteins in plants, exceptions have been observed: the *A. thaliana* miRNA known as miR-172 has a 5′ adenosine but preferentially associates with AGO1 (ref. 77); and AGO7 preferentially associates with

miR-390, which has a 5′ adenosine[77]. Therefore, the 5′ nucleotide does not seem to be the sole determinant of Argonaute association.

Another mechanism might operate for secondary siRNAs in *C. elegans*. These small RNAs are specifically loaded onto SAGOs[50]. Secondary siRNAs carry a 5′-triphosphate modification[51,52], the hallmark of RdRP products, which might function as a recognition element for SAGO binding while excluding binding by a primary Argonaute, such as RDE-1.

Endo-siRNAs in *C. elegans* (including the secondary siRNAs just mentioned) and *Schizosaccharomyces pombe* (fission yeast) have a striking strand bias in which only the antisense siRNA strand, corresponding to the RNA strand synthesized by RdRP, is loaded into Argonaute-containing complexes. Because *C. elegans* RdRPs produce small RNAs directly from the target mRNA, in a primer-independent manner (Fig. 2c), all secondary siRNAs have a negative polarity and function to reinforce the silencing of the target mRNA[50–52]. In *S. pombe*, the strand bias is probably the result of a different mechanism. The physical association of Dicer with an RdRP-containing complex known as RDRC and an Argonaute-containing complex known as the RNA-induced transcriptional silencing complex (RITS) (see page 413) may facilitate the loading of siRNAs onto Argonaute proteins in a directional manner as Dicer moves along and cleaves the dsRNA products of RdRP, giving rise to an antisense strand bias. This suggests that the polarity of Dicer processing defines the polarity of the siRNA strand loaded onto the Argonaute protein.

Argonaute proteins have diversified over evolutionary timescales, evolving a range of functions[8,12]. These findings about small RNA sorting imply that the diversification of the Argonaute proteins is a consequence of which small RNA they recruit. It is possible that the conformation of the Argonaute protein dictates which small RNAs it partners, but the structures of eukaryotic Argonaute proteins will need to be determined before this can be assessed.

Safeguards in silencing pathways

During RNA silencing, a single non-sequence-specific RNA-binding protein (Argonaute) is loaded with small guide RNAs with a variety of sequences, resulting in effector complexes (RISCs). Thus, this system requires gatekeepers to ensure that Argonaute can bind to small guide RNAs but not to degraded small RNAs, thereby avoiding 'off-target' silencing. Such gatekeeper systems seem to depend mainly on structural features specific for small guide RNAs.

As described earlier, Dicer helps to load siRNAs into the RISC, preventing siRNAs from diffusing freely in the cytoplasm after their production. This function of Dicer probably also aids in the discrimination of genuine siRNAs from various RNA-degradation products in the cell. Processing by RNaseIII enzymes (such as Dicer) characteristically yields small RNAs with 5′ monophosphates and 3′ two-nucleotide overhangs. The PAZ domain of Argonaute proteins might, as a first step, distinguish degraded RNAs (derived from unrelated pathways) from these small RNAs by binding to the characteristic 3′ overhangs of the small RNAs[8,12]. In addition, to become incorporated into the RISC and mediate cleavage of the target mRNA, the guide strand of an siRNA must have a phosphate group at the 5′ end[78]. In humans, the 5′ end of siRNAs is phosphorylated by the enzyme CLP1 (ref. 79), which also has roles in splicing transfer RNAs and forming the 3′ ends of mRNAs. Interestingly, both tRNA splicing and mRNA 3′-end formation occur in the nucleus[80,81], suggesting that siRNA duplexes with a 5′ hydroxyl group are transported to, or diffuse into, the nucleus and, after phosphorylation by CLP1, are exported to the cytoplasm and assembled into the RISC.

Amplification of the silencing signal needs to be balanced against the dangers of amplifying off-target silencing. For example, the slicer-mediated ping-pong mechanism for piRNA production does not lead to 'transitive' RNA silencing (in which RdRPs synthesize siRNAs complementary to sequences upstream or downstream of the initial trigger region in the target mRNA). Instead, it leads to conservative amplification of functional primary piRNA sequences (those inherited by germline transmission). However, it is conceivable that any off-target events mediated by RdRPs could lead to a chain reaction or transitive effect of silencing with deleterious consequences. Thus, there must be safeguards to prevent the pervasive use of RdRPs. A striking aspect of RdRP-based trigger amplification is that amplification occurs only when a target has been engaged, so amplification of the silencing signal is limited to cases in which there is a real target[51,52]. In *C. elegans*, the processing of the trigger dsRNA and the loading of primary siRNAs into the RDE-1-containing complex seem to be inherently inefficient, limiting the first round of target recognition by RDE-1-containing complexes and minimizing the risk of amplifying off-target silencing reactions[50]. In addition, each secondary siRNA seems to be generated by non-processive self-termination by RdRP, thereby restricting transitive effects[51–53]. Furthermore, secondary siRNAs associate with SAGOs, which lack catalytic residues for cleaving mRNAs, suggesting that these complexes cannot generate cleaved substrates for further amplification, which in turn would prevent them from inducing the exponential generation of secondary siRNAs[50] (but see also ref. 53 for a conflicting viewpoint). SAGOs are also present in limited supply and thus have a restricted capacity to support multiple simultaneous silencing reactions.

Another factor is that in *C. elegans* and *S. pombe* the RNAi machinery is negatively regulated by a conserved siRNA nuclease called enhanced RNAi (ERI-1 and Eri1, respectively)[82,83]. In *S. pombe*, transgene silencing is linked to a protein complex resembling the TRAMP complex of *Saccharomyces cerevisiae* (budding yeast), which carries out surveillance in the nucleus, targeting aberrant transcripts for degradation by the exosome[84]. Thus, RNAi in *S. pombe* is actively restricted from exerting its effects throughout the genome and seems to be subject to competition from RNA quality-control machinery.

Target-sensing modes and effector modes of the RISC

When the RISC is loaded with the guide strand of a small RNA, how does it find its target mRNA? Most of the binding energy that tethers a RISC to a target mRNA is from nucleotides in the seed region of the small RNA[85]. It seems that the accessibility of the target site can be sensed by the intrinsic, nonspecific affinity of RISC for ssRNA, which follows the initial specific association between the RISC and the target (through the 5′ seed region of the small RNA)[86]. But the accessibility of the target site correlates directly with the efficiency of cleavage, indicating that the RISC cannot unfold structured RNA.

Target mRNAs are present in the cell in complex with ribonucleoproteins (RNPs)[87], so target accessibility is also controlled by several RNA-binding proteins that either mask the target binding site or facilitate unfolding of the target. Therefore, the function of a RISC seems to be context-dependent, with its effector mode influenced not only by the structures of the small-RNA-binding sites on the target but also by the particular proteins associated with each Argonaute protein. For example, animal miRNAs silence gene expression by at least three independent mechanisms through binding sites that are mostly in the 3′ untranslated region of target mRNAs: by cleaving mRNAs, by repressing their translation and/or by promoting mRNA degradation[88,89]. However, the contribution of translational repression or mRNA degradation to gene silencing seems to differ for each miRNA–mRNA pair. Thus, the final outcome of miRNA regulation is probably affected by other proteins interacting with the targeted mRNA or RISC and counteracting the effects of the miRNA, resulting in differential regulation depending on the proteins present in each tissue[90].

Regulation of silencing pathways

So far, the pictures of RNA silencing pathways that we have built up (shown in Figs 1 and 2) are static. To gain further insight into silencing processes, it is important to incorporate information about how these pathways are regulated. It is already clear that competition between different silencing pathways (for example, competition between endo-siRNAs and miRNAs for LOQS in *D. melanogaster*) is a key step in how each stage of the RNAi mechanism is regulated. Many plant and animal viruses are known to encode suppressor proteins that block

host RNAi, and therefore silencing, at various stages[91]. Cellular proteins can also regulate RNAi. For example, processing to form the human miRNA let-7, which is a tumour suppressor and cell-cycle regulator, is post-transcriptionally inhibited in embryonic cells by the pluripotency factor LIN28, which seems to block the microprocessor-complex-mediated cleavage of pri-let-7 and the Dicer-mediated processing of pre-let-7 in series[92,93]. By contrast, in humans, signalling mediated by the transforming growth factor-β (TGF-β) and bone morphogenetic protein (BMP) family of growth factors rapidly increases the production of mature miR-21 (which is oncogenic), by promoting the processing of pri-miR-21 into pre-miR-21 by DROSHA[94]. More specifically, TGF-β- and BMP-specific signal transducers of the SMAD family are recruited to pri-miR-21 in complex with the RNA helicase p68, a component of the microprocessor complex, facilitating the accumulation of pre-miRNA. In addition, heterogeneous nuclear RNP A1 (hnRNP A1), a well-known regulator of precursor mRNA splicing, also assists DROSHA to crop and release pre-miR-18 efficiently, perhaps by refolding the hairpin or by creating a cleavage site for DROSHA through direct binding to the pri-miRNA[95]. This implies that some hairpins within pri-miRNAs might form and be processed only after the binding of a protein with RNA chaperone activity.

The activity of the RISC can also be regulated. In *A. thaliana*, the non-protein-coding gene *IPS1* (*INDUCED BY PHOSPHATE STARVATION 1*) contains a motif with sequence complementarity to the phosphate-starvation-induced miRNA miR-399, but the pairing is interrupted by a mismatched loop at the expected miRNA cleavage site[96]. *IPS1* mRNA is not cleaved but, instead, sequesters miR-399. Thus, *IPS1* overexpression results in increased accumulation of the target of miR-399, *PHO2* mRNA. The idea of target mimicry introduces unanticipated complexity into the network of RNA-regulatory interactions and raises the possibility that a large number of mRNA-like non-coding RNAs recently identified in humans[97] could be attenuators of the regulation of small-RNA–Argonaute complexes.

Perspective

Recent studies hint that human cells contain a large number of small RNAs similar to miRNAs or siRNAs, with the potential to regulate the expression of almost all human genes. The future challenges in this field are clear. Many questions remain to be answered. How many types of small RNA are there? How are these small RNAs generated? What are their biological functions? How are these pathways regulated? One potential problem is that because many types of small RNA are modified at their 5′ and 3′ ends[98], it is unclear whether the current sequencing technologies are sampling the entire range of small RNAs present in cells. But next-generation sequencing technologies[13] should soon help to uncover the full range of small RNA molecules.

One major challenge will be to identify how specific RNA-binding proteins affect the final outcome of gene regulation by small RNAs, given that RNAs in a cell are usually associated with multiple proteins that regulate many aspects of gene expression. For example, genome-wide *in vivo* approaches using a combination of immunoprecipitation and high-throughput sequencing will be required to establish protein–mRNA interactions or RNP complex occupancy at certain regions of mRNA, where expression is suppressed.

Finally, changes in the activity and specificity of silencing pathways could create quantitative and qualitative genetic variation in gene expression, thereby generating new gene-expression networks. Such changes might have contributed to many processes, including human evolution[16]. Given that all vertebrates have almost exactly the same number of protein-coding genes and therefore cannot readily be distinguished in this way, it might be prophetic that the first small guide RNA to be identified, the *C. elegans* miRNA lin-4, has been found to regulate a gene involved in the timing of development[99,100]. In humans, unlike other mammals, the brain tissue of newborns continues to grow at a similar rate to that of the fetus. This is a good example of a change in developmental timing, and there is much speculation about whether changes in this rate contributed to the evolution of humans as a new species. ■

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A three-dimensional view of the molecular machinery of RNA interference

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In eukaryotes, small non-coding RNAs regulate gene expression, helping to control cellular metabolism, growth and differentiation, to maintain genome integrity, and to combat viruses and mobile genetic elements. These pathways involve two specialized ribonucleases that control the production and function of small regulatory RNAs. The enzyme Dicer cleaves double-stranded RNA precursors, generating short interfering RNAs and microRNAs in the cytoplasm. These small RNAs are transferred to Argonaute proteins, which guide the sequence-specific silencing of messenger RNAs that contain complementary sequences by either enzymatically cleaving the mRNA or repressing its translation. The molecular structures of Dicer and the Argonaute proteins, free and bound to small RNAs, have offered exciting insights into the molecular mechanisms that are central to RNA silencing pathways.

The discovery that RNA interference (RNAi) and related small-RNA-mediated pathways have central roles in the silencing of gene expression in eukaryotic cells has profoundly altered the understanding of gene regulation. At least 30% of human genes are thought to be regulated by microRNAs, one of the classes of small RNA[1]. In a wide range of organisms, including petunias, nematodes, fruitflies, zebrafish and mice, mutations in the genetic regions encoding the protein and/or RNA components of RNAi result in severe and sometimes lethal defects in cell growth and development[2]. These findings have raised many questions about how and why this widespread RNA-mediated regulation of genes evolved and how these mechanisms enable gene expression to be precisely tuned in response to internal and external stimuli.

RNAi and related gene-silencing pathways are initiated by the production of small RNAs (~20–30 nucleotides) with sequences that are complementary to portions of the transcripts that they regulate. There are three main classes of small regulatory RNA: short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) (see page 396 for a review of the currently recognized classes of small regulatory RNA). The biogenesis and mechanism of action of the main types of small RNA are described in Box 1. In brief, siRNAs and miRNAs are generated from double-stranded RNA (dsRNA) precursors that are produced in, or introduced into, cells, and their generation depends on the ribonuclease (RNase) Dicer[3]. These small RNAs subsequently associate with members of the Argonaute family of proteins, which function as the core components of a diverse set of protein–RNA complexes called RNA-induced silencing complexes (RISCs)[4]. RISCs use the small RNAs as guides for the sequence-specific silencing of messenger RNAs that contain complementary sequence through inducing the degradation of the mRNAs or repressing their translation. In addition, in certain organisms, a specialized nuclear Argonaute-containing complex, known as the RNA-induced transcriptional silencing complex (RITS), mediates transcriptional gene silencing by inducing heterochromatin formation[5] (see page 413). The biogenesis of the piRNA class of small RNAs also involves proteins belonging to the Argonaute family but differs markedly from that of siRNAs and miRNAs[6] (Box 1).

Biochemical and structural biology studies have provided fundamental insights into the molecular details of RNAi and its possible evolutionary underpinnings. Structural aspects of how viruses inhibit host-cell RNAi pathways are described in refs 7 and 8. In this Review, we focus on two specialized RNases: Dicer, which functions as a molecular ruler in siRNA and miRNA biogenesis; and Argonaute, a versatile RNA-guided molecular machine that cleaves, or otherwise represses, target RNAs. We highlight the ways in which recently obtained molecular structures of these two proteins underlie the current mechanistic understanding of RNA silencing.

Structural aspects of small RNA biogenesis

The production of siRNAs and miRNAs relies on the endonucleolytic processing of dsRNA precursors. In a cell, long dsRNAs can arise from the replication of RNA viruses, from the transcription of convergent cellular genes or mobile genetic elements, and from self-annealing cellular transcripts. Dicer, an endonuclease belonging to the RNaseIII family, processes these long dsRNA molecules to yield siRNA duplexes of ~21–25 nucleotides[3]. By contrast, miRNAs are generated from endogenous transcripts (known as primary miRNAs, pri-miRNAs) that form stem–loop structures (Box 1). The hairpin region is excised from the pri-miRNA in the nucleus by the endonuclease Drosha[9], another RNaseIII-family enzyme. After its export to the cytoplasm, the hairpin (known as a precursor miRNA, pre-miRNA) undergoes another endonucleolytic cleavage, which is catalysed by Dicer, generating a miRNA–miRNA* duplex (where miRNA is the antisense, or guide, strand and miRNA* is the sense, or passenger, strand) of ~21–25 nucleotides.

Two features of siRNAs and miRNAs ensure that they are efficiently incorporated into the RISC: the length of the duplex; and characteristic 5′ and 3′ ends, carrying a monophosphate group and a dinucleotide overhang, respectively[10–12]. Recent structural studies of a prokaryotic RNaseIII and a eukaryotic Dicer have provided insights into how these crucial features of siRNAs and miRNAs are generated during their biogenesis by Drosha and/or Dicer.

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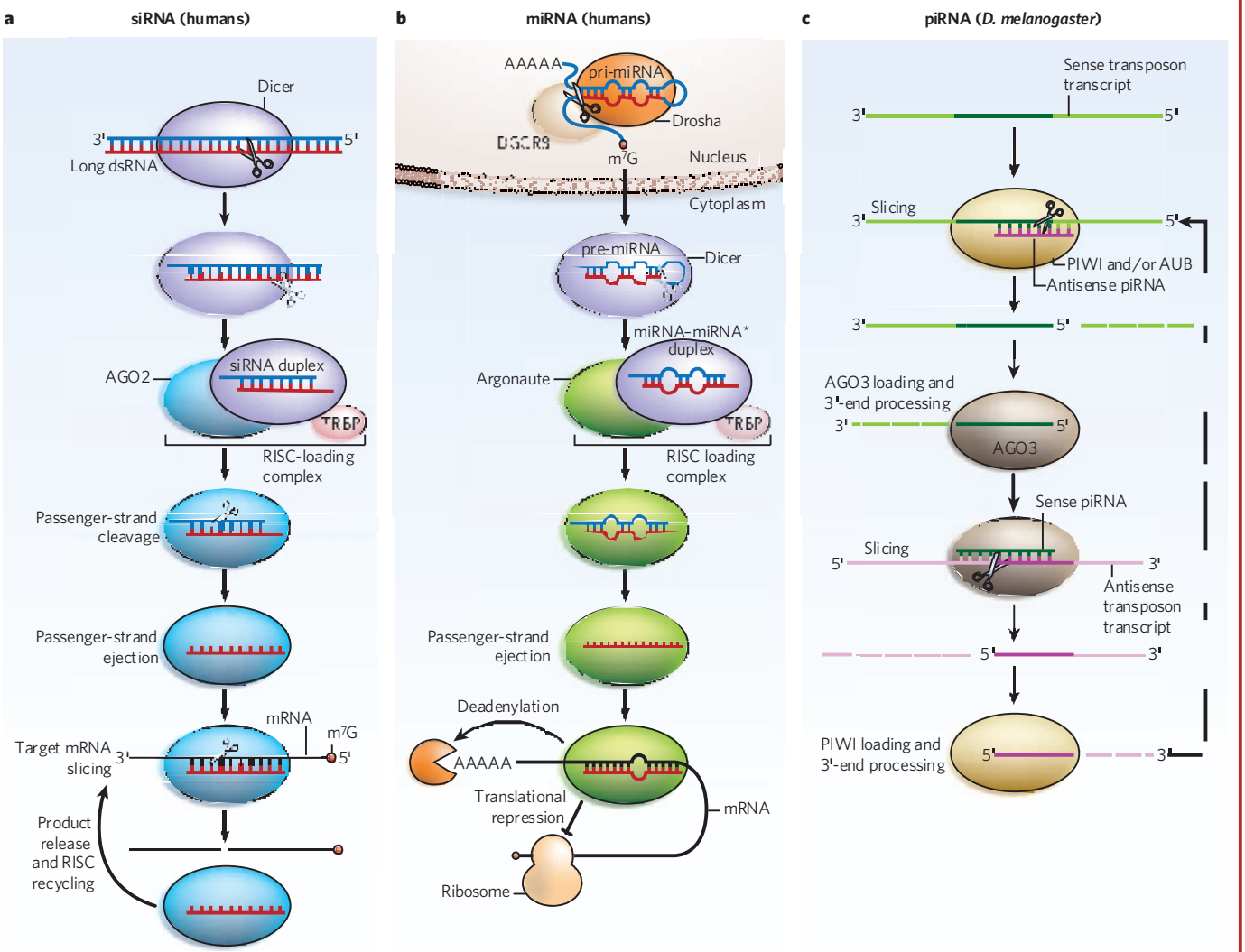
Box 1 | Biogenesis and mechanism of action of the main classes of small regulatory RNA



Small regulatory RNAs are non-coding RNA molecules that silence target RNAs in a sequence-specific manner. The main classes of small RNA are short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs).

The first class, siRNAs, mediate RNAi by downregulating target RNAs through slicing; that is, by endonucleolytic cleavage (see figure, panel **a**; note that RNAs are not drawn to the same scale in each panel, and m^{7}G denotes 7-methylguanosine). These small RNAs are derived from long double-stranded RNA (dsRNA) molecules that result from RNA virus replication, convergent transcription of cellular genes or mobile genetic elements, self-annealing transcripts or experimental transfection. The endonuclease Dicer functions as a molecular ruler to cleave the dsRNA at ~21–25-nucleotide intervals. After Dicer-mediated cleavage, one strand of the siRNA duplex (the guide strand) is loaded onto an Argonaute protein at the core of an RNA-induced silencing complex (RISC). (For simplicity, in the figure, the RISC is represented just by an Argonaute protein.) Argonaute loading takes place in the RISC-loading complex, a ternary complex that consists of an Argonaute protein, Dicer and a dsRNA-binding protein (known as TRBP in humans). During loading, the non-guide (passenger) strand is cleaved by an Argonaute protein and ejected. The Argonaute protein then uses the guide siRNA to associate with target RNAs that contain perfectly complementary sequence and then catalyses the slicing of these targets. After slicing, the cleaved target RNA is released, and the RISC is recycled for another round of slicing.

The second class, miRNAs, are encoded in the genome. Whereas plant miRNAs direct the slicing of target messenger RNAs, much like siRNAs, animal miRNAs silence target mRNAs without slicing (panel **b**). These small RNAs are transcribed from endogenous miRNA genes as primary transcripts (pri-miRNAs), containing ~65–70-nucleotide stem–loop structures. The hairpin structure is excised in the nucleus by the Drosha–DGCR8 complex to yield a precursor miRNA (pre-miRNA). In the cytoplasm, Dicer cleaves the pre-miRNA, producing a miRNA–miRNA* duplex (where miRNA denotes the guide strand and miRNA* the passenger strand). The guide strand is loaded onto an Argonaute protein. Typically, animal miRNAs are only partly complementary to sequences in the 3′ untranslated regions of their target mRNAs, and this lack of complementarity prevents the target from being sliced by the Argonaute protein. In addition, some Argonaute proteins involved in the miRNA pathway lack the catalytic residues needed for slicer activity. The mechanism of miRNA-mediated silencing is unresolved but is thought to occur by repression of target mRNA translation and removal of mRNA poly(A) tails (that is, deadenylation), which leads to mRNA degradation.

The third class, piRNAs, are ~24–31 nucleotides, and they silence transposons (mobile genetic elements) in animal germ cells. The biogenesis and mechanism of action of piRNAs is poorly understood. A model for these processes in *Drosophila melanogaster* is shown in panel **c**. The precursors of piRNAs are single-stranded RNAs, because piRNA biogenesis does not require Dicer. The small RNAs induce reciprocal slicer-dependent cleavages of sense and antisense transposon transcripts. This process is mediated by the PIWI clade of the Argonaute family, which includes the proteins PIWI, Aubergine (AUB) and Argonaute 3 (AGO3) in *D. melanogaster*. PIWI- or AUB-mediated slicing of sense transcripts generates sense piRNAs, which associate with AGO3 and direct the slicing of antisense transposon transcripts. The slicing products give rise to antisense piRNAs, which in turn bind to PIWI and AUB and guide the slicing of sense transposon transcripts to generate sense piRNAs.

The RNaseIII family

Cleavage of dsRNA by enzymes of the RNaseIII family, including Drosha and Dicer, yields products with characteristic termini, with a monophosphate group at the 5′ ends, and a two-nucleotide overhang at the 3′ ends[13]. The simplest RNaseIII enzymes are found in prokaryotes and certain fungi. These enzymes consist of an RNaseIII domain, which has the catalytic activity, and (generally) a dsRNA-binding domain (dsRBD) (Fig. 1a), and they function as homodimers[14]. The two RNaseIII domains of the dimer associate to form a single processing centre, with each catalytic domain responsible for the hydrolysis of one strand in the duplex[15]. By contrast, both Drosha and Dicer are monomeric and contain two tandemly arranged RNaseIII domains and a single dsRBD.

From the crystal structure of RNaseIII from the prokaryote *Aquifex aeolicus* in complex with a cleaved dsRNA product[16] (Fig. 1b), it can be seen that homodimerization of the catalytic domains creates a shallow surface cleft separating the two active sites. A cluster of conserved acidic amino-acid residues in each catalytic centre coordinates a single magnesium ion. On substrate binding, the enzyme rearranges such that the dsRBDs clamp the dsRNA substrate over the surface of the catalytic domain dimer.

Dicer

The endonuclease Dicer processes dsRNA substrates (long dsRNAs and pre-miRNAs) into short dsRNA fragments (siRNAs and miRNAs) of defined length, typically ~21–25 nucleotides[3]. In addition to two copies of the conserved RNaseIII domain and a dsRBD in the carboxyl terminus, Dicer enzymes usually have an amino-terminal DEXD/H-box domain, followed by a small domain of unknown function (the DUF283 domain) and a PAZ domain (Fig. 1a). The PAZ domain, which is also found in Argonaute proteins, binds specifically to the 3′ end of single-stranded RNA (ssRNA)[17–19].

The crystal structure of Dicer from the unicellular eukaryote *Giardia intestinalis* revealed that the ability of Dicer enzymes to produce dsRNA fragments of specific length originates from a unique spatial arrangement of the PAZ domain and the RNaseIII domains[20]. *G. intestinalis* Dicer is a naturally 'trimmed-down' version of the enzyme, consisting only of the PAZ domain and the tandem RNaseIII domains (RNaseIIIa and RNaseIIIb) (Fig. 1a). Its structure resembles an axe, with the two RNaseIII catalytic domains forming the blade and the PAZ domain making up the base of the handle (Fig. 1c). The RNaseIIIa domain and the PAZ domain are connected by a long helix running the length of the handle. This connector helix is buttressed by a platform domain formed by the N-terminal segment of the protein. The RNaseIII domains form an intramolecular dimer that closely resembles the homodimeric structure of prokaryotic RNaseIII[16] (Fig. 1b). In Dicer, four conserved acidic amino-acid residues in the active site of each RNaseIII domain coordinate two metal cations, suggesting that Dicer uses a two-metal-ion mechanism to catalyse RNA cleavage. The 17.5 Å distance between the two metal-ion pairs in the two active sites matches the width of the major groove in a dsRNA duplex. Modelling the binding of a dsRNA substrate to the enzyme reveals that the duplex runs along a flat surface formed by the platform domain and makes electrostatic interactions with a number of positively charged residues. Mutation of the sequence encoding these residues impairs the catalytic activity of Dicer, underscoring the importance of these positively charged residues for substrate binding[21].

The PAZ domain of Dicer has the same fold and 3′-overhang-binding residues as the PAZ domain of Argonaute proteins (discussed in the next section)[22,23]. The distance between the 3′-overhang-binding pocket of the PAZ domain and the active site of the RNaseIIIa domain is 65 Å, which corresponds to the length of a 25-nucleotide RNA duplex (Fig. 1c). This is in good agreement with the size of siRNA fragments produced by *G. intestinalis* Dicer *in vitro*. The domain architecture of Dicer thus suggests that it functions as a molecular ruler, generating products of defined length by anchoring the 3′ dinucleotide of the substrate RNA duplex (generated by an initial nonspecific cleavage) in the PAZ domain and cleaving at a fixed distance from that end. This is supported by the observation that a truncated *G. intestinalis* Dicer protein that lacks the PAZ domain yields RNA products of variable length *in vitro*[21]. The structure of *G. intestinalis* Dicer suggests that the length of the non-conserved connector helix is the main determinant of product size, providing a possible explanation for the variation in product size across species.

The structure of a fragment of mouse Dicer comprising solely the RNaseIIIb domain and dsRBD was recently solved and indicates that on substrate binding, the dsRBD undergoes a conformational change analogous to that observed in prokaryotic RNaseIII enzymes[24]. In addition, a truncated human Dicer protein lacking the dsRBD has a significantly decreased rate of RNA substrate cleavage *in vitro*, but its substrate affinity remains unaffected[25]. Most Dicer enzymes contain a DEXD/H-box

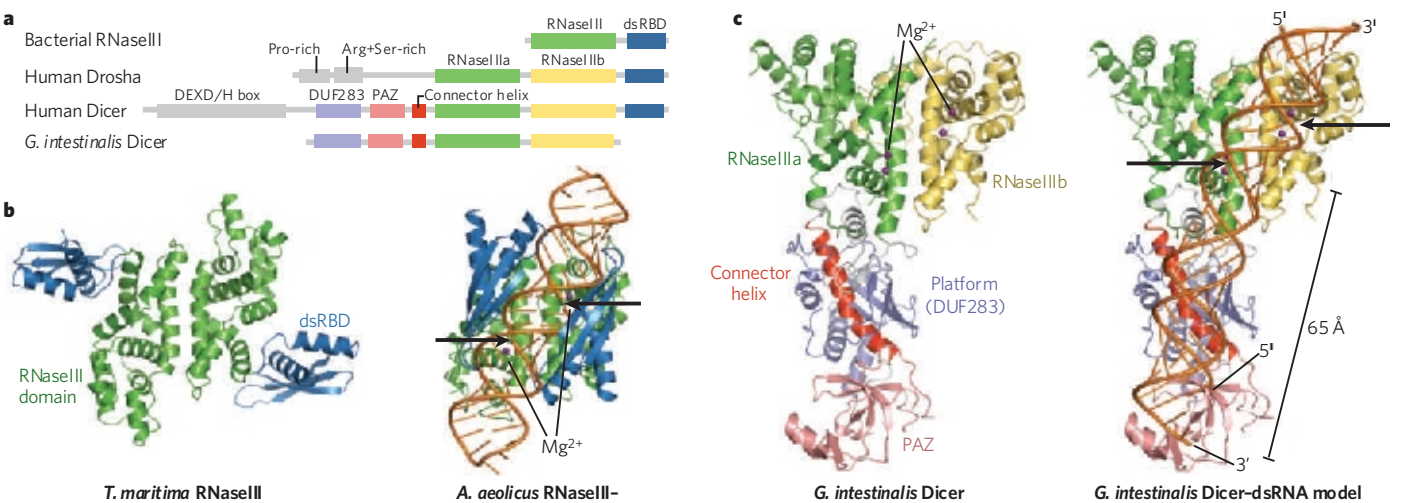


Figure 1 | Structure of RNaseIII-family enzymes. a, A schematic representation of the domain structure of RNaseIII-family enzymes is shown. **b**, The induced fit during dsRNA binding to prokaryotic RNaseIII enzymes, which are homodimeric class I RNaseIII enzymes, is shown. The crystal structure of RNaseIII from the bacterium *Thermotoga maritima* in the RNA-free state (Protein Data Bank (PDB) identity 1O0W) is shown (left). RNaseIII from the bacterium *Aquifex aeolicus* is shown bound to a cleaved dsRNA product (PDB identity 2EZ6) (right). The colours of the protein domains match those in panel **a**; the RNA is shown in gold. Magnesium ions, which are present at the active sites, are shown as purple spheres. Cleavage sites are indicated by arrows. From these structures, it is clear that the dsRNA-binding domains (dsRBDs; blue) undergo a marked rotation on substrate binding. **c**, The molecular mechanism of dsRNA cleavage by *Giardia intestinalis* Dicer is shown. The crystal structure of Dicer (PDB identity 2FFL) is shown (left), together with a model of a dsRNA substrate bound to Dicer (right). In this model, docking of the 3′ overhang of the RNA substrate in the PAZ domain leads to cleavage 65 Å from the 3′ end. Images were generated from files from the PDB using PyMol (http://www.pymol.org).

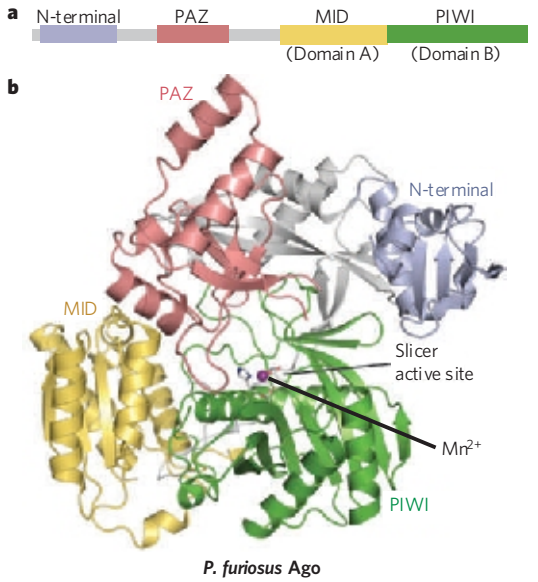


Figure 2 | Modular architecture of Argonaute proteins. a, Eukaryotic Argonaute proteins have four domains: the N-terminal, PAZ, MID and PIWI domains. In some cases, notably for the structures of *Archaeoglobus fulgidus* Piwi protein, the MID domain and PIWI domain have been referred to as domain A and domain B, respectively. **b**, A crystal structure of *Pyrococcus furiosus* Argonaute (Ago) soaked with Mn^{2+} (PDB identity 1Z25) is shown. The protein adopts a bilobate architecture, with the N-terminal and PAZ domains forming one lobe and the MID and PIWI domains forming the other. The metal ion in the active site is shown as a purple sphere. The amino-acid residues involved in metal-ion binding in the slicer catalytic site are shown in stick format.

domain. These domains are found in a diverse group of proteins that are involved in the ATP-dependent binding and remodelling of nucleic acids[26]. Although some invertebrate Dicer proteins (such as *Drosophila melanogaster* DCR-2) seem to require ATP for processive dicing of long dsRNAs (that is, multiple rounds of cleavage without dissociation from the dsRNA substrate), mammalian Dicer proteins seem to be ATP independent[10,25,27,28]. Kinetic analysis of wild-type and mutant human Dicer proteins showed that the DEXD/H-box domain might have an autoinhibitory function, because removal of this domain increases the cleavage rate[25]. This finding suggests that the DEXD/H-box domain imposes on the protein a non-productive conformation that must be rearranged before catalysis can occur. Further structural and biochemical studies of full-length Dicer proteins and their substrate complexes will be necessary to establish whether the DEXD/H-box domain participates in the binding and unwinding of RNA duplexes during the loading of small RNAs into the RISC.

Drosha and the microprocessor complex

The RNaseIII-family member Drosha catalyses the initial processing of pri-miRNAs, yielding pre-miRNAs, which are hairpins with phosphorylated 5′ ends and 3′ dinucleotide overhangs[9]. Drosha is a nuclear protein, and its domain structure consists of a proline-rich region and an arginine- and serine-rich region at the N terminus, followed by two RNaseIII domains and a dsRBD (Fig. 1a). Purified Drosha cleaves dsRNA nonspecifically; specific cleavage of pri-miRNAs requires association with a protein known as DGCR8 (also known as PASHA in invertebrates) in a complex called the microprocessor[29,30]. DGCR8 binds to the base of the pri-miRNA hairpin, positioning Drosha to cleave the pri-miRNA stem at a distance of 11 base pairs from the junction between the duplex stem and the flanking ssRNA regions[31]. Thus, DGCR8 seems to be a *trans*-acting specificity determinant, analogous to the PAZ domain of Dicer, which acts *in cis*. The molecular architecture of Drosha is unknown, but the structure of a core region of human DGCR8, composed of a tandem pair of dsRBDs, was determined recently[32]. The canonical RNA-binding surfaces of the two dsRBDs are non-contiguous, suggesting that the protein binds to two discontinuous segments of the pri-miRNA stem–loop structure.

Structural insights into Argonaute function

The common feature of RNAi and all related small-RNA-mediated silencing pathways is the association of a small silencing RNA (also known as a guide RNA in this context) with a protein of the Argonaute family[4]. The resultant protein–RNA complex forms the minimal core of the effector complex known as the RISC. Within the RISC, the small RNA functions as a sequence-specific guide that recruits an Argonaute protein to complementary target transcripts through base-pairing interactions. The target transcripts, typically mRNAs, are then either cleaved or prevented from being translated by ribosomes, leading to their degradation.

Throughout evolution, the Argonaute family has diverged into specialized clades (or subfamilies) that recognize different small RNA types and confer the specific effects of the various small-RNA silencing pathways[33]. Both siRNAs and miRNAs associate with members of the AGO clade of Argonaute proteins, whereas piRNAs bind to those of the PIWI clade. In classic RNAi, which is elicited by siRNAs, Argonaute proteins silence targeted mRNAs by catalysing their endonucleolytic cleavage, a process known as slicing. The PIWI clade of the Argonaute protein family is thought to use slicing in piRNA-mediated silencing of mobile genetic elements in the germ line[34,35]. During slicing, the target RNA is cleaved at the scissile phosphate group, which is opposite the phosphate group between the tenth and eleventh nucleotides of the guide RNA strand, as measured from the 5′ end of the guide strand[11,12]. Slicing requires perfect complementarity between the guide strand and the target around the cleavage site[36–38]. Argonaute proteins can also silence transcripts independently of slicer activity. In the animal miRNA pathway, Argonaute proteins repress target mRNAs by inhibiting their translation and inducing deadenylation and subsequent mRNA decay. However, the precise mechanism of miRNA-mediated silencing is not fully understood[39].

To function as an effector of small-RNA-mediated silencing, the Argonaute protein must bind to the guide RNA strand, eject the non-guide (passenger) strand of the siRNA or miRNA–miRNA* duplex (where miRNA* is the passenger strand) during loading, and subsequently recognize the target RNA (Box 1). In silencing pathways that rely on RNA slicing, the Argonaute protein carries out multiple cycles of target binding, cleavage and product release, while the guide strand remains bound to the protein. In the metazoan miRNA pathway, in which silencing is achieved in a slicer-independent manner, the Argonaute protein must remain tightly associated with the targeted mRNA to keep its translation repressed.

Functional domains

Argonaute proteins are multidomain proteins that contain an N-terminal domain, and PAZ, middle (MID) and PIWI domains (Fig. 2a). Crystal structures of prokaryotic Argonaute proteins have revealed a bilobate architecture, with the MID and PIWI domains forming one lobe, and the N-terminal and PAZ domains constituting the other (Fig. 2b). The two signature domains of the Argonaute family — the PAZ domain and the C-terminal PIWI domain — were originally identified by phylogenetic sequence analysis[40]. Three-dimensional structures of isolated PAZ domains from *D. melanogaster* Argonaute proteins revealed a fold similar to those of the oligosaccharide/oligonucleotide-binding (OB)-fold domain and Sm-fold domain[17–19]. The first crystal structure of a full-length Argonaute protein, from the archaeal species *Pyrococcus furiosus*, showed that the sequence motif originally defined as the PIWI domain by Lorenzo Cerutti *et al.*[40] consists of two structural domains, termed MID and PIWI, joined by way of an extensive conserved interface that is centred on the buried C terminus of the protein[41] (Fig. 2b). The MID domain resembles the sugar-binding domain of the *lac* repressor. The PIWI domain adopts a fold similar to that in RNaseH, an endoribonuclease that cleaves RNA–DNA hybrids[42]. Crystal structures of a

PIWI-like protein (called Piwi) from the archaeal species *Archaeoglobus fulgidus* (consisting of domain A and domain B, which are equivalent to the MID domain and the PIWI domain, respectively) and of a full-length Argonaute protein from the bacterium *A. aeolicus* also showed the presence of an RNaseH-like fold in their PIWI domains[43,44]. Biochemical studies suggest that, similarly to RNaseH, prokaryotic Argonaute proteins function as DNA-guided ribonucleases[44,45], unlike their eukaryotic counterparts, which have evolved to use RNA molecules as guides.

Slicer activity

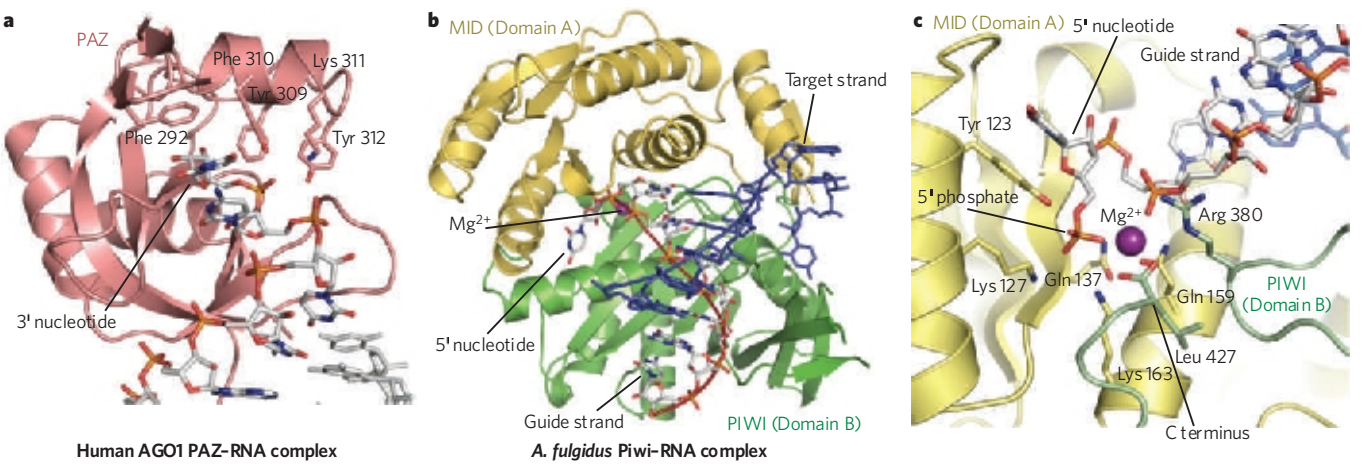
The finding that the PIWI domain of an Argonaute protein adopts an RNaseH fold suggested immediately that Argonaute proteins are responsible for the 'slicer' activity of the RISC[41]. Similarly to the requirements for RNaseH activity, RISC-catalysed RNA cleavage requires divalent metal ions and yields a 5′ product, which has a free 3′ hydroxyl group, and a 3′ product, which carries a 5′ phosphate group[46–48]. The active site of RNaseH contains an Asp-Asp-Glu/Asp motif, which coordinates the two divalent cations required for catalysis[42]. Similarly, in the crystal structure of *P. furiosus* Argonaute (Ago) soaked with manganese ions, a conserved Asp-Asp-His motif was observed to coordinate a single manganese ion[49] (Fig. 2b). Mutagenesis of this motif in human AGO2 confirmed its requirement for slicer activity *in vivo* and *in vitro*, thus establishing the Argonaute protein as the catalytic component of RISCs[46,49]. Similar analysis of *Schizosaccharomyces pombe* (fission yeast) Ago1, a component of the RITS, showed that its slicer activity is required for transcriptional silencing[50]. Of the four human Argonaute proteins (AGO1, AGO2, AGO3 and AGO4), only AGO2 has demonstrable slicer activity[46,51]. The motifs in AGO1 and AGO4 do not precisely conform to the consensus sequence, but AGO3 has a complete Asp-Asp-His sequence and yet is inactive *in vitro*. Moreover, it has recently been shown that *D. melanogaster* PIWI (one of the three members of the PIWI clade of Argonaute proteins in *D. melanogaster*), which has an Asp-Asp-Lys motif, is catalytically active[52], in accordance with the postulated requirement for slicing in the piRNA pathway[34,35]. *D. melanogaster* AGO1 and AGO2 have complete Asp-Asp-His motifs, and both are capable of slicing. But AGO1 is a much less efficient enzyme than AGO2 because it releases products at a slower rate, resulting in a slower turnover[53]. These findings indicate that factors in addition to the conservation of catalytic residues might determine whether a given Argonaute protein is an efficient slicer *in vivo*.

Recognition of RNA termini

The incorporation of siRNAs and miRNAs into the RISC requires the presence of 5′ phosphate groups and 3′ dinucleotide overhangs at the termini[10–12]. The discovery that the PAZ domain is an RNA-binding domain that specifically recognizes the 3′ ends of ssRNAs suggested immediately that it might function as a module for anchoring the 3′ end of the guide RNA strand within the RISC[17–19]. Insights into the mechanism of RNA recognition came from the crystal structure of the PAZ domain of human AGO1 in complex with an siRNA-like duplex[22] and from nuclear magnetic resonance structures of the PAZ domain of *D. melanogaster* AGO2 in complex with ssRNA oligonucleotides[23]. In these structures, the 3′ dinucleotide inserts into a preformed hydrophobic pocket that is lined with conserved aromatic residues (Fig. 3a). Although there are no sequence-specific contacts, the base of the terminal nucleotide stacks against the aromatic ring of a conserved phenylalanine residue (Fig. 3a).

The 5′ phosphate groups of siRNAs and miRNAs, which result from their mechanism of biogenesis, are crucial for the efficient assembly of these small RNAs into the RISC[10,12,38,46]. Moreover, the 5′ phosphate group is essential for slicing fidelity, because the position of the cleavage site in the target RNA strand is determined by its distance from the 5′ phosphate group of the guide RNA strand[12,49,54]. Crystal structures of the *A. fulgidus* Argonaute protein Piwi bound to a short dsRNA duplex (which mimics the interaction between the guide strand and the target strand within the RISC) showed that the 5′ nucleotide of the guide strand is distorted and does not base-pair with the target strand of the RNA duplex[45,55] (Fig. 3b). This is consistent with the observation that a base mismatch at this position is tolerated and can increase slicer activity[56]. The 5′ phosphate group of the guide RNA strand is buried in a deep pocket at the interface between the MID domain and the PIWI domain and is bound to a magnesium ion that is, in turn, coordinated to the protein's C terminus (Fig. 3c). Mutation of any of the four residues involved in metal-ion coordination and 5′-phosphate binding, which are among the most highly conserved residues in the Argonaute protein family, impairs slicing activity[45]. This underscores the functional importance of the 5′-phosphate-binding pocket in anchoring the guide RNA.

In the plant *Arabidopsis thaliana*, distinct types of small non-coding RNA associate with different Argonaute proteins on the basis of the identity of the 5′ nucleotide[57,58]. *A. thaliana* AGO1 binds mainly to RNAs with a uridine at their 5′ end, whereas AGO2 recruits RNAs with



Human AGO1 PAZ–RNA complex

A. fulgidus Piwi–RNA complex

Figure 3| Recognition of the termini of small RNAs by the PAZ and MID domains of Argonaute proteins. a, The crystal structure of the PAZ domain of human AGO1 (ribbon format) is shown in complex with an siRNA-like duplex (stick format) (PDB identity 1SI3). Conserved residues in contact with the 3′ nucleotide are shown in stick format and labelled. The 3′ end of the siRNA inserts into a preformed hydrophobic pocket, with the base of the 3′ nucleotide stacking against an invariant aromatic residue (the phenylalanine at position 292 in the protein, Phe 292). **b**, The crystal structure of *Archaeoglobus fulgidus* Piwi bound to an RNA duplex (PDB identity 2BGG), which mimics the guide–target interaction, is shown. *A. fulgidus* Piwi is composed of a MID domain and a PIWI domain (also referred to as domain A and domain B, respectively). The 5′ nucleotide of the guide strand (backbone shown in red) binds at the MID–PIWI domain interface and does not base-pair with the target strand (blue). **c**, Shown is a detailed view of the pocket in *A. fulgidus* Piwi that binds to the 5′ end of small RNAs. A magnesium ion, coordinated by the C terminus of the protein (Leu 427) and the 5′ phosphate group, is shown as a purple sphere. Conserved residues that are involved in metal-ion coordination and 5′-phosphate binding are shown in stick format and labelled.

a 5′ adenosine. Domain-swap experiments involving chimaeric proteins showed that the specificity of the 5′ nucleotide is conferred by the MID and PIWI domains of the Argonaute proteins[57]. Similarly, the PIWI clade of the Argonaute protein family associates with piRNAs, which are ~24–31-nucleotide RNA species that are 2′-O-methylated and have a bias for uridine as the first nucleotide[59,60]. It is thus probable that the PAZ and MID domains of Argonaute proteins that bind to different small RNA species underwent an evolutionary adaptation that allows small RNAs to be sorted on the basis of particular 3′ and 5′ ends.

RISC loading

In the siRNA- and miRNA-mediated pathways of gene silencing, the loading of a guide RNA onto an Argonaute protein is closely linked with its biogenesis (Box 1). Starting from an siRNA duplex or a miRNA–miRNA* generated by Dicer-mediated cleavage, the strand with its 5′ end at the less thermodynamically stable end of the duplex is selected as the guide strand[61]. The observation, made from the structure of *A. fulgidus* Piwi bound to an siRNA-like duplex, that the first nucleotide of the guide strand is unpaired and buried in the 5′-phosphate-binding pocket suggests that this mode of guide-strand binding contributes to the apparent guide-strand selectivity[45,55]. Argonaute proteins also promote guide-strand selection by subsequently slicing the passenger strand, thereby facilitating its release[62–65]. However, for duplexes with multiple mismatches, as is often the case in the miRNA pathway, slicing is not required during loading[63]. Thus, Argonaute proteins lacking slicer activity (such as human AGO1, AGO3 and AGO4) can still be loaded with miRNAs.

RISC loading takes place in the context of the RISC-loading complex. In human cells, the RISC-loading complex consists of an Argonaute protein, Dicer and the dsRBD-containing protein TRBP[66–68]. *In vitro*, this ternary complex can process a pre-miRNA, load the correct guide strand and cleave a target RNA, all in the absence of ATP[67]. What are the roles of dsRBD-containing proteins in substrate selection and RISC loading? In *D. melanogaster*, DCR-1 associates with the dsRBD-containing protein Loquacious (LOQS) to convert pre-miRNAs into miRNA–miRNA* duplexes[69–71]. By contrast, a complex of DCR-2 with its dsRBD-containing protein partner, R2D2, is required for the efficient production of siRNAs from long dsRNA precursors and for their subsequent loading onto AGO2 (refs 63, 72–74). Recent studies indicate that the structure and the extent of base-pairing (that is, the presence of mismatches) within siRNA and miRNA precursor duplexes determine whether the guide strands associate with AGO1 or AGO2 in *D. melanogaster*[53,75]. The affinity of DCR-2–R2D2 is higher for perfectly matched duplexes than for 'bulged' duplexes (such as pre-miRNA hairpins), and this seems to be the main source of small RNA selectivity during AGO2 loading[75].

Target recognition

In the *A. fulgidus* Piwi–RNA complex structure, the guide–target duplex is positioned over a conserved basic channel that spans the surfaces of both the MID domain and the PIWI domain[45,55] (Fig. 3c). Importantly, these structures show that the bases of nucleotides 2–6 of the guide strand (starting from the 5′ end) are exposed and free to base-pair with a target mRNA. This agrees well with numerous computational and biochemical studies indicating that nucleotides 2–8 of the guide strand (known as the 'seed' region) are crucial determinants of the specificity of target recognition by miRNAs[76–78]. Modelling the trajectory of a full-length siRNA–target duplex places the scissile phosphate group of the target RNA in the putative slicer catalytic site, thus providing a rationale for the specificity of slicer cleavage at a fixed distance from the 5′ end of the guide strand[11,12]. Perfect complementarity around the cleavage site in the guide–target duplex is a prerequisite for slicing[36–38]. Presumably, base-pairing around the 10–11-base step ensures correct orientation of the scissile phosphate group in the active site.

In the context of the bilobate architecture of full-length Argonaute proteins, the guide–target duplex has been proposed to bind in a positively charged cleft between the PAZ–N-terminal and MID–PIWI lobes[41,44,49]. Recent crystal structures of full-length *Thermus thermophilus* Argonaute protein bound to guide DNA strands have identified the molecular details of guide-strand recognition[79]. The structure of *T. thermophilus* Ago in complex with a 5′-phosphorylated 21-nucleotide DNA shows the guide strand bound with its 5′ phosphate group in the MID domain and with its 3′ end in the PAZ domain[79] (Fig. 4a). Through interactions with conserved arginine residues, nucleotides 2–10 of the guide strand adopt a stacked helical conformation. Thus, the seed region of the guide strand is pre-organized to initiate base-pairing with the target strand (Fig. 4a). In the absence of a target strand, the guide strand is kinked at the 10–11-base step, indicating that a further structural rearrangement occurs on target-RNA recognition.

The most recent insights into the mechanism of target-RNA recognition come from the crystal structure of a ternary complex consisting of *T. thermophilus* Ago, a 21-nucleotide guide DNA strand and a 20-nucleotide target RNA with mismatched bases introduced

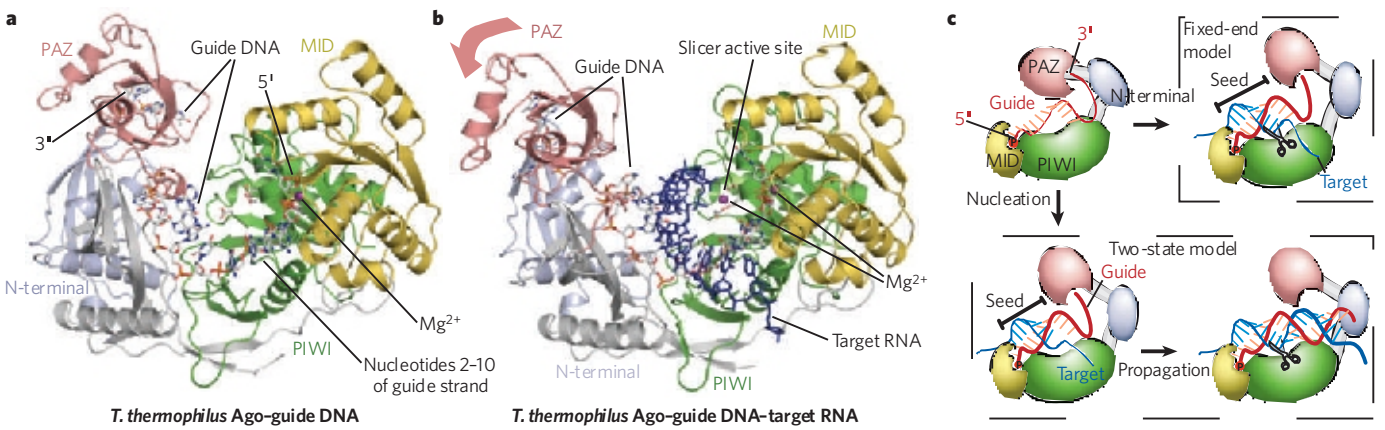


Figure 4 | Mechanism of guide- and target-strand recognition by Argonaute proteins. a, The crystal structure of *Thermus thermophilus* Ago (ribbon format) bound to a 5′-phosphorylated 21-nucleotide guide DNA strand (stick format) (PDB identity 3DLH) is shown. Nucleotides 1–11 and 18–21 of the guide DNA are visible in the structure and shown in stick format. The 5′ phosphate group of the guide strand binds to the MID domain, whereas the 3′ end is anchored in the PAZ domain. **b**, The crystal structure of a ternary complex of *T. thermophilus* Ago with a 5′-phosphorylated 21-nucleotide guide DNA strand and a 20-nucleotide target RNA (PDB identity 3F73) is shown. The complex is shown in the same orientation as the binary complex in panel **a**, after superposition of the PIWI domains. The target RNA (blue) and guide DNA (grey backbone) are shown in stick format. The arrow indicates the conformational change undergone by the lobe containing the PAZ and N-terminal domains on target-RNA binding. **c**, Putative models for target-RNA recognition by Argonaute proteins are illustrated. The fixed-end model postulates that both the 5′ and 3′ ends of the guide strand remain docked in their binding pockets during slicing. The two-state model postulates that the seed region of the guide occurs in two steps: first, nucleation of base-pairing with the target RNA; and second, propagation of the guide–target duplex, leading to the release of the 3′ end of the guide strand from the PAZ domain.

at the 10–11-base step[80] (Fig. 4b). Both termini of the guide strand are anchored in their respective binding pockets, as is the case for the *T. thermophilus* Ago–guide DNA complex. The seed region of the guide strand (nucleotides 2–8) engages in Watson–Crick base-pairing interactions with the target RNA, assuming an A-form helical conformation. To accommodate the target RNA strand in the central channel, Ago undergoes a pronounced conformational change to a more open conformation, achieved by rotating the N-terminal and PAZ domains away from the lobe containing the MID and PIWI domains.

Two models for target recognition and cleavage by Argonaute proteins have been proposed[81,82]. The fixed-end model postulates that both ends of the guide RNA remain bound to the Argonaute protein during slicing. This presents a topological constraint on the guide–target interaction that would limit the extent of base-pairing to less than one helical turn (11 base pairs) and might be the factor that limits the seed region to nucleotides 2–8 of the guide RNA. By contrast, the two-state model proposes that the target binds to the seed region of the guide strand, and then propagation of base-pairing towards the 3′ end of the guide strand results in this end of the guide strand dissociating from the PAZ domain. At present, it is unclear whether the structure of the *T. thermophilus* Ago ternary complex represents a cleavage-competent complex as envisaged by the fixed-end model or whether it depicts a trapped intermediate as postulated by the two-state model. Further structural studies of catalytically inactive Argonaute proteins in complexes with perfectly matched guide–target duplexes will be necessary to clarify this issue. Nonetheless, the mode of target-RNA recognition observed in the *T. thermophilus* Ago ternary complex might be representative of target mRNA recognition during miRNA-mediated silencing in metazoans, in which slicing is typically prevented by a base mismatch between the miRNA and the target at around the 10–11-base step.

Slicer-independent functions

In metazoans, gene silencing by miRNAs occurs by the mere anchoring of a miRNA-containing RISC to the target mRNA, in the absence of slicer-dependent cleavage of the target mRNA[83]. The mechanism of miRNA-mediated repression is unclear, but it must involve interactions between the RISC and the cellular machineries that are responsible for translation and mRNA decay. Recent studies using *in vitro* systems indicate that miRNAs might affect the initiation of translation by interfering with the function of the EIF4F, a complex that binds to the mRNA cap (7-methylguanosine)[84]. The MID domain of human AGO2 shows limited sequence homology to the cap-binding motif of the translation initiation factor EIF4E, a subunit of EIF4F, suggesting that human AGO2 competes with EIF4E for binding to the mRNA cap structure[85]. However, the MID domain of Argonaute proteins lacks any discernible structural homology to EIF4E. Thus, the AGO2–cap interaction, if indeed direct, might be a non-canonical mode of mRNA cap recognition.

Argonaute proteins, miRNAs and their mRNA targets co-localize in P bodies, which are cytoplasmic foci where mRNA decay is thought to occur. In mammalian cells and in *D. melanogaster*, the interaction between Argonaute proteins and the P-body protein GW182 is required for miRNA-mediated repression of mRNAs, as well as for Argonaute localization[86–89]. It was shown recently that the glycine- and tryptophan-rich (GW) motifs of GW182 directly interact with the MID–PIWI region of human Argonaute proteins and that a minimal fragment (termed the Ago hook), encompassing two tandem GW motifs, is sufficient to mediate this interaction[90]. Interestingly, mutations that disrupt the Argonaute–GW182 interaction have been mapped mainly to the 5′-phosphate-binding pocket located at the interface between the MID domain and PIWI domain[89,90].

Future prospects

Structural studies and structure-based biochemical studies will continue to improve our understanding of the mechanisms and evolutionary relationships of RNAi pathways. An important future goal will be to determine the structures of some of the proteins and protein complexes that operate in eukaryotic cells. Elucidating the molecular architecture of the RISC-loading complex will bring important insights into the coupling of small RNA biogenesis, Argonaute loading and guide-strand selection. Recent proteomic studies have uncovered a multitude of Argonaute-interacting proteins (for example GW182, MOV10 and FXR1) that might link Argonaute proteins to downstream effector events[91,92]. Obtaining structural and structure-based functional insights into these interactions will help to uncover the molecular mechanisms that underlie the functions of Argonaute proteins in small-RNA-mediated gene silencing. Results from these studies will not only provide new mechanistic details but also lay the foundation for the informed engineering of RNAi as a therapeutic tool. ■

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Small RNAs in transcriptional gene silencing and genome defence

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Small RNA molecules of about 20–30 nucleotides have emerged as powerful regulators of gene expression and genome stability. Studies in fission yeast and multicellular organisms suggest that effector complexes, directed by small RNAs, target nascent chromatin-bound non-coding RNAs and recruit chromatin-modifying complexes. Interactions between small RNAs and nascent non-coding transcripts thus reveal a new mechanism for targeting chromatin-modifying complexes to specific chromosome regions and suggest possibilities for how the resultant chromatin states may be inherited during the process of chromosome duplication.

RNA interference (RNAi) originally referred to the ability of exogenously introduced double-stranded RNA (dsRNA) molecules to silence the expression of homologous sequences in the nematode *Caenorhabditis elegans*[1]. It has become clear over the past decade that RNAi is mechanistically related to a number of other conserved RNA silencing pathways, which are involved in the cellular control of gene expression and in protection of the genome against mobile repetitive DNA sequences, retroelements and transposons[2–4]. These RNA silencing pathways are all associated with small (~20–30 nucleotide) RNAs that function as specificity factors for inactivating homologous sequences by a variety of mechanisms. At least three classes of small RNA have been identified so far (Table 1). The first two classes, short interfering RNAs (siRNAs) and microRNAs (miRNAs), are ~21–25 nucleotides and are generated from longer dsRNA precursors by Dicer, a ribonuclease III (RNaseIII) enzyme. They are loaded into the RNA-induced silencing complex (RISC) or a nuclear form of RISC, called the RNA-induced transcriptional silencing complex (RITS)[5–10]. RISC and RITS are effector complexes that are targeted to homologous sequences by base-pairing interactions involving the guide strand of the small RNA. The core component of each complex is a highly conserved PAZ- and PIWI-domain-containing protein called Argonaute, which binds to the guide small RNA by means of interactions that involve its PAZ domain, as well as the PIWI and middle (MID) domains, and cleaves the target RNA by means of its RNaseH-like PIWI domain (see page 405 for further information about the structural biology of RNAi proteins).

The Argonaute family of proteins, together with the small RNAs that program them, are the central players in RNA silencing, and seem to participate in all small-RNA silencing pathways thus far described. Phylogenetically, Argonaute-family proteins are divided into the AGO and PIWI clades[11]. The PIWI-clade proteins bind to a third class of small RNAs, called PIWI-interacting RNAs (piRNAs), which have a broader average size (~24–31 nucleotides) than siRNAs and miRNAs and are involved in defence against parasitic DNA elements[12–18]. As discussed later, piRNA-programmed PIWI-clade proteins are also likely to function as RISC- and RITS-like complexes that target the inactivation of homologous sequences (Table 1). With the notable exception of budding yeast, small-RNA-mediated silencing mechanisms and their role in chromatin regulation are conserved throughout eukaryotes, indicating an ancient evolutionary origin.

This Review discusses the roles of diverse small-RNA silencing pathways in the regulation of chromatin structure and transcription in plants, animals and fungi, with particular emphasis on emerging common themes. In addition to their well-known roles in post-transcriptional gene silencing (PTGS), in which silencing is directed at the level of messenger RNA translation or stability, nearly all small-RNA silencing pathways also seem to act at the DNA and chromatin level (Table 1). Studies in *Schizosaccharomyces pombe* (fission yeast) and other organisms suggest that small RNAs access DNA through interactions with nascent RNA transcripts, revealing a close relationship between nuclear and cytoplasmic RNA silencing mechanisms. Moreover, small-RNA silencing pathways seem to be intimately integrated with the RNA surveillance and processing pathways that determine the ultimate fate of RNA transcripts. Together, these studies reveal a broad and previously unsuspected role for RNAi and other RNA-processing mechanisms in the regulation of the structure and expression of eukaryotic genomes. Here, I discuss small-RNA silencing pathways and their role in chromatin regulation, drawing parallels between well-established examples in *S. pombe* and other organisms.

RNA silencing pathways

RNA silencing pathways can be broadly classified into different branches based on their mechanism of action, subcellular location and the origin of the small RNA molecules that they use (Table 1). However, the different branches have common components and intersect in some instances. siRNAs act in both the nucleus and the cytoplasm and are involved in PTGS and chromatin-dependent gene silencing (CDGS). CDGS refers to both transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS)[3]. miRNAs are generated from hairpin precursors by the successive actions of the RNaseIII enzymes Drosha and Dicer, which are located in the nucleus and cytoplasm, respectively (see page 396 for a more detailed discussion of small RNA precursor processing and complex assembly). Although Drosha is absent in plants, the general features of the miRNA pathway are conserved in plants and animals, but not in fungi and other protozoa. Whereas the vast majority of miRNAs seem to act exclusively in the cytoplasm and mediate mRNA degradation or translational arrest[19], some plant miRNAs may act directly in promoting DNA methylation[20]. Furthermore, recent studies describe a role for promoter-directed

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Table 1 | Conservation of small-RNA silencing pathways in eukaryotes

Small RNA	Size (nucleotides)	Mechanism of action	Eukaryotes conserved in
siRNA	~21–25	PTGS (RNA degradation or translational arrest)	Plants, animals, fungi, ciliates
		CDGS	
miRNA	~21–25	PTGS (RNA degradation or translational arrest)	Plants, animals
		CDGS (to a lesser extent)	
piRNA	~24–31*	PTGS (RNA degradation)	Animals
		CDGS (to a lesser extent)	

All three of the major RNA silencing pathways identified thus far seem to act in both post-transcriptional gene silencing (PTGS) and chromatin-dependent gene silencing (CDGS) pathways. CDGS refers to chromatin-dependent silencing events that involve the assembly of small RNA complexes on nascent transcripts and includes both transcriptional gene silencing (TGS) and co-transcriptional gene silencing (CTGS) events. The latter involves the chromatin-dependent processing or degradation of the nascent transcript. **Caenorhabditis elegans* piRNAs are 21 nucleotides.

human miRNAs in facilitating repressive chromatin modifications and TGS[21,22]. siRNAs are generated from long dsRNA precursors, which can be produced from a variety of single-stranded RNA (ssRNA) precursors. These precursors include sense and antisense RNAs transcribed from convergent promoters, which can anneal to form dsRNA, and hairpin RNAs that result from transcription through inverted repeat regions[23–25] (Fig. 1a). In some situations the long dsRNA is produced enzymatically from certain aberrant or non-coding RNA precursors. One example of this pathway involves aberrant RNAs that lack processing signals or are produced by Argonaute slicer activity. These RNAs recruit RNA-dependent RNA polymerase (RdRP) enzymes, which recognize free 3′ ends and synthesize dsRNA[2,26,27] (Fig. 1b, c). Here RdRP enzymes are in competition with the TRAMP polyadenylation pathway, which targets aberrant RNAs for degradation by a 3′→5′ exonuclease complex, called the exosome[28–31](Fig. 1b). The siRNA branch of the pathway seems to be conserved from fungi to mammals (Table 1), although *Drosophila melanogaster* (fruitflies) and mammals lack RdRPs and cannot amplify siRNAs.

piRNAs originate from a diversity of sequences, including repetitive DNA and transposons, and like siRNAs they seem to act at both the post-transcriptional and chromatin levels[12–18]. The mechanism(s) that generates and amplifies piRNAs is not yet fully elucidated but involves the slicer activity of the PIWI-clade proteins themselves[4] (Fig. 1d). This class of small RNAs is present in *D. melanogaster*, *C. elegans* and mammals, but seems to be absent in fungi and plants (Table 1).

Small RNAs in DNA and chromatin regulation

An accumulating body of evidence supports an important role for small RNAs in the modulation of chromatin structure and TGS in plants, fungi and animal cells. RNA silencing was first linked to TGS by the discovery that transgene and viral RNAs guide the methylation of homologous DNA sequences in plants[32]. Analysis of the guide RNAs in *Arabidopsis thaliana* revealed that these RNAs were processed into small RNAs of ~25 nucleotides, similar to the size previously described for miRNAs[5,33]. This observation and the realization that exogenously introduced dsRNA in animals is processed into siRNAs[8] established small RNAs as central players in diverse RNA silencing pathways. Later studies in *A. thaliana* indicated that RNA-directed DNA methylation of the *FWA* transgene requires Dicer (DCL3) and Argonaute (AGO4), and is linked to histone H3 lysine 9 (H3K9) methylation, indicating that RNA-directed DNA methylation and RNAi have common molecular mediators[34–36].

Evidence for the role of RNA silencing in mediating changes at the chromatin level also came from studies of silent or heterochromatic DNA domains in unicellular eukaryotes, such as *S. pombe* and the ciliate *Tetrahymena thermophila*. *S. pombe* contains single genes encoding the Argonaute, Dicer and RdRP proteins, called *ago1*, *dcr1* and *rdp1*, respectively. Deletion of any of these genes results in loss of heterochromatic gene silencing, markedly reduced H3K9 methylation at centromeric repeats, and accumulation of non-coding RNAs, which are transcribed from centromeric repeat regions and processed into siRNAs[37,38]. Moreover, RNAi is directly linked to a structural component of heterochromatin through RITS, which in *S. pombe* contains Ago1, the chromodomain protein Chp1, the glycine and tryptophan (GW)-motif-containing protein Tas3 and centromeric siRNAs[10,29,39]. *T. thermophila* cells are binucleate with a germline micronucleus and a somatic macronucleus. Development of a new macronucleus after sexual conjugation and meiosis involves massive DNA elimination of non-genic sequences. This elimination requires TWI1, a *T. thermophila* PIWI-clade protein, and PDD1, a chromodomain protein that binds to both K9- and K27-methylated histone H3 (refs 40–42). In addition, DNA elimination is associated with Dicer-produced small RNAs, called scan RNAs (scnRNAs), giving rise to the idea that a scnRNA RITS-like complex targets sequences destined for elimination into heterochromatin[40]. However, a physical association between chromatin proteins and TWI1 has not yet been reported.

RNAi is also linked to chromatin modifiers in animal cells. In *D. melanogaster*, the introduction of multiple tandem copies of a transgene results in silencing of both the transgene array and the endogenous copies. This repeat-induced gene silencing, which is analogous to RNA-mediated co-suppression in plants[2], requires components of the Polycomb group (PcG) of genes, as well as several RNAi factors, including PIWI and AGO2 (ref. 43). The PcG gene products are chromatin-binding and -modifying repressors that prevent the expression of homeobox (HOX) regulators outside their proper domains of expression[44]. The requirement for both PcG proteins and PIWI in transgene silencing suggested the possibility that in *D. melanogaster*, as in plant cells, RNA silencing could operate at the chromatin level. In fact, later studies showed that RNA silencing factors are also required for the formation of *D. melanogaster* centric heterochromatin, recruitment of heterochromatin protein 1 (HP1) and silencing of transgenes that are inserted in pericentromeric heterochromatin[43,45]. In addition to HP1 and PIWI, efficient silencing requires DCR-1, PIWI, Aubergine (AUB) and the putative helicase HLS (also known as SPN-E)[43]. Moreover, silencing of a mini-*white* gene, which is mediated by a *cis*-acting repeated element from the heterochromatic Y chromosome, requires HP1, SU(VAR)3-9 (the H3K9 methyltransferase), as well as PIWI, AUB, HLS and DCR-1 (ref. 46). Transgene-induced gene silencing in *C. elegans* has also been shown to require RNAi and chromatin modifiers[47,48]. Surprisingly, screens for defects in classical RNAi, mediated by feeding of dsRNA, have also uncovered several chromatin modifiers, suggesting that perhaps the connection between RNAi and chromatin modifiers may not be limited to repeat-induced silencing[49].

In contrast to their apparent requirement for PcG-mediated repeat-induced gene silencing, RNAi components do not seem to be required for PcG-mediated silencing of HOX genes outside their proper domains of expression[50]. Mutations in several RNA silencing factors disrupt the silencing of a tandem mini-*white* gene array and perturb the nuclear clustering of PcG-repressed HOX loci[50]. However, despite their requirement for PcG-mediated repeat-induced silencing, loss of PIWI and RNAi components does not lead to a loss of HOX gene silencing. The simplest explanation for these observations is that RNAi is required for some, but not all, PcG-mediated silencing events.

Linking heterochromatin to RNAi

Heterochromatin is associated with repetitive DNA sequences and transposons, and has important roles in chromosome transmission, maintenance of genomic stability, and regulation of gene expression[51–53]. With the exception of budding yeast, which lacks centromeric DNA repeats, heterochromatin is concentrated at repeats and transposons that surround centromeres, telomeres and other genomic loci (Fig. 2a). Two important defining properties of heterochromatin involve its modes of assembly and inheritance. First, heterochromatin assembly involves nucleation sites, which act as entry points for the recruitment and spreading of repressor proteins. Unlike recruitment, which involves the action of a site-specific DNA-binding protein or RNA molecule,

spreading occurs in a sequence-independent manner and involves changes in chromatin structure that are mediated by histone-modifying enzymes. The second defining property of heterochromatin is its mode of inheritance. Once assembled, heterochromatin is inherited through many cell divisions, at least partly independently of the underlying DNA sequence. The mechanisms of spreading and epigenetic inheritance of heterochromatin are poorly understood, but, in *S. pombe*, require components of the RNAi pathway[3,53,54].

At the molecular level, heterochromatin is characterized by association with hypoacetylated histones and, in organisms ranging from *S. pombe* to humans, by association with H3K9 dimethylation and trimethylation[3,51]. H3K9 is methylated by SU(VAR)3-9 in *D. melanogaster*, SUV39H in humans and Clr4 in *S. pombe*, and creates a binding site for HP1 (Swi6 and Chp2 in *S. pombe*)[55–58]. HP1 proteins contain a chromodomain that binds to methylated H3K9 and a chromoshadow (CSD) domain, which is involved in other protein–protein interactions[54].

Biochemical isolation of *S. pombe* heterochromatin and RNAi complexes has provided direct physical links between heterochromatin and RNAi proteins, leading to models for how RNAi mediates heterochromatin assembly and participates in gene silencing. In addition to HP1 proteins, heterochromatic gene silencing in *S. pombe* requires the chromodomain protein Chp1 (ref. 59). Chp1 is larger than HP1 and, like the Polycomb (Pc) subfamily of chromodomains, contains only a single chromodomain at its amino terminus. Like Swi6 and Chp2, Chp1 is a structural component of heterochromatin and is required for heterochromatic gene silencing[59]. Unlike Swi6 and Chp2, which are not required for H3K9 methylation within centromeric repeat regions[58,60], a lack of Chp1 in cells leads to a marked loss of H3K9 methylation, indicating that Chp1 has a critical role in heterochromatin formation.

Biochemical purification of Chp1 showed that it is associated with Ago1 in RITS[10]. RITS acts as a specificity determinant for the recruitment of other RNAi complexes and chromatin-modifying enzymes to specific DNA regions. RITS also physically associates with and is required for recruitment of the RNA-directed RNA polymerase complex (RDRC) to non-coding RNAs that are transcribed from centromeric repeats[61,62]. RDRC contains the *S. pombe* RNA-directed RNA polymerase, Rdp1, a putative helicase termed Hrr1, and Cid12, a member of the Trf4 and Trf5 family of polyadenylation polymerases[61], which were first identified in the budding yeast *Saccharomyces cerevisiae* and are involved in the degradation of aberrant transcripts[30,31]. The physical association of RITS and RDRC is siRNA- and Clr4-dependent, suggesting that this association occurs on chromatin and requires histone H3K9 methylation[61]. These observations further suggest that RITS and RDRC localize to chromatin-bound nascent RNA by a mechanism that involves tethering the nascent transcript to chromatin via the bivalent complex RITS (Fig. 2). In addition to RITS, *S. pombe* contains a second Ago1-containing complex, named Argonaute siRNA chaperone (ARC) complex[63]. The Ago1 protein in the ARC complex contains duplex, rather

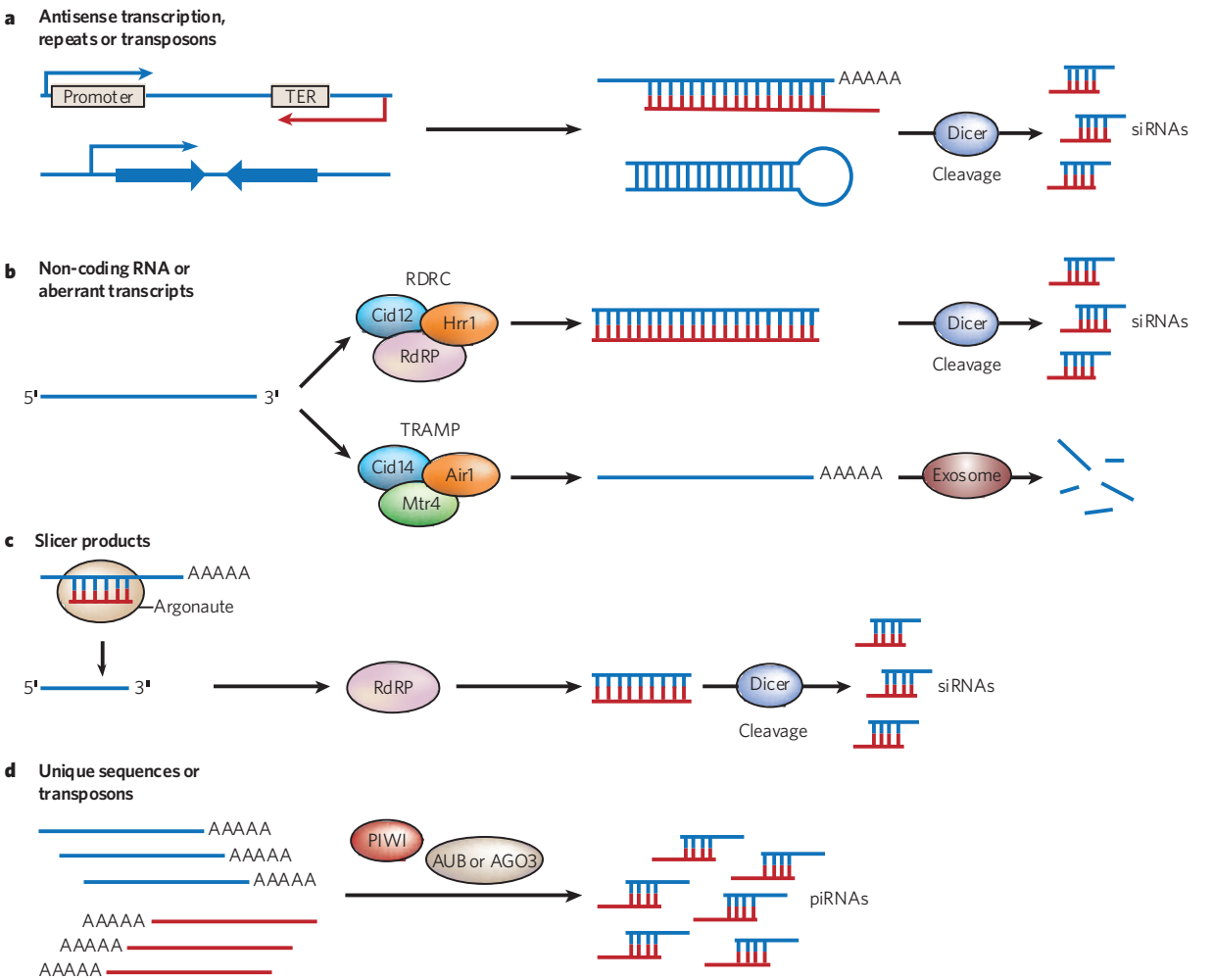

Figure 1 | Pathways of RNA processing and biogenesis of small RNAs. **a**, Generation of endogenous siRNAs from dsRNA resulting from convergent transcription (sense–antisense RNA base-pairing; top) or transcription through inverted repeat sequences (hairpin RNA formation; bottom). TER, transcription termination signal. **b**, Processing of non-coding and aberrant RNAs by the RDRC and TRAMP complexes, containing the Cid12 and Cid14 non-canonical polyadenylation polymerases, respectively; the RDRC/Dicer pathway produces duplex siRNAs, whereas the TRAMP/exosome pathway produces single-stranded degradation products. **c**, Generation of a free 3' end by the slicer activity of an Argonaute protein, which can be processed into dsRNA by RdRP or targeted for degradation by the exosome (not shown). **d**, Pathway for the generation of piRNAs by the PIWI clade of Argonaute proteins: PIWI, AUB and AGO3.

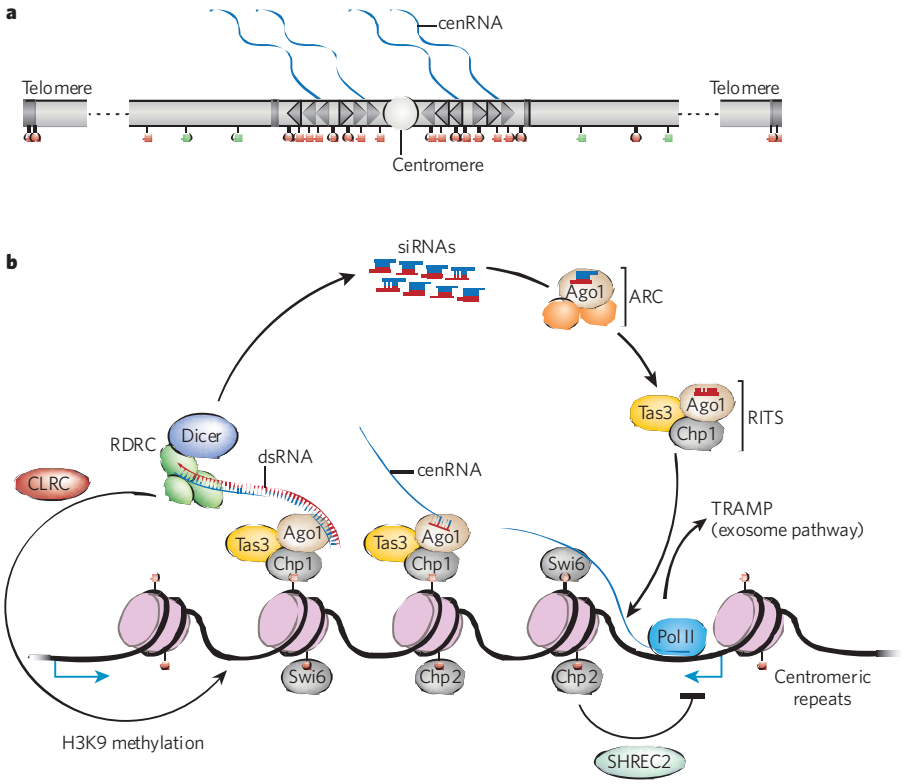

Figure 2 | Chromosome organization and the nascent transcript model for heterochromatic gene-silencing assembly in *Schizosaccharomyces pombe*. a, The structure of *S. pombe* centromeric repeat regions, highlighting the presence of non-coding centromeric transcripts (cenRNA) and association with histone H3 that is dimethylated and trimethylated on lysine 9 (red lollipops) as opposed to histone H3 that is methylated on lysine 4 (green lollipops) in euchromatic regions. **b**, The nascent transcript model for heterochromatin assembly. The RITS is tethered to chromatin through base-paring interactions between siRNAs and nascent non-coding transcripts and interactions with H3K9-methylated nucleosomes, resulting in the recruitment of RDRC–Dicer, dsRNA synthesis and siRNA amplification. This RNAi positive-feedback loop then recruits the CLRC H3K9 methyltransferase. Efficient silencing also requires two HP1 proteins (Swi6 and Chp2), which promote the association of RITS with the non-coding RNA or mediate TGS through recruitment of the SHREC2 deacetylase complex, respectively. Another tier of regulation, involving the degradation of heterochromatic transcripts by the TRAMP/exosome pathway, further ensures full gene silencing. Blue arrows (bottom) highlight convergent transcription resulting in synthesis of sense and antisense RNAs, which may contribute to the production of trigger siRNAs.

than single-stranded, siRNA, indicating that the slicer activity of Ago1 (refs 63, 64), which is required for the release of the siRNA passenger strand, is inhibited in this complex[63].

Nascent transcripts as assembly platforms

In principle, siRNAs in RITS can base-pair with either unwound DNA regions or with nascent non-coding RNAs that are transcribed from their target DNA. The two models are not mutually exclusive and base-pairing with DNA and RNA may contribute to different aspects of the mechanism of siRNA biogenesis and function. However, although a role for siRNA–DNA base-pairing cannot be ruled out at this point, several lines of evidence support siRNA–RNA base-pairing interactions in which the siRNA targets nascent non-coding transcripts (Fig. 2). First, RITS associates with the RDRC, which uses ssRNA as a template to synthesize dsRNA, providing evidence that RITS itself is RNA-associated[61]. Furthermore, the RITS–RDRC interaction requires siRNA and the Clr4 H3K9 methyltransferase, suggesting that it occurs on heterochromatin-bound transcripts[61]. Together with the observation that proteins required for heterochromatin formation — such as Sir2, Swi6, Clr4 and other components of the Clr4 methyltransferase complex (CLRC), as well as RITS and RDRC — are required for siRNA accumulation[10,61,65–67], these studies suggest that siRNA-programmed RITS localizes to nascent chromatin-tethered non-coding transcripts and recruits the RDRC to initiate dsRNA synthesis and siRNA amplification (Fig. 2b). Direct support for this model comes from experiments in which the Tas3 component of RITS was fused to the phage λN (λN) protein and tethered to the transcript of a euchromatic *ura4*$^+$ gene, which was modified with the addition of five λN-binding sites upstream of its transcription termination sequences (*ura4-5BoxB*)[66]. In cells containing *ura4-5BoxB*, the Tas3-λN protein could efficiently initiate *de novo* siRNA generation and heterochromatin formation[66]. Like the situation at centromeres, siRNA generation in this system is H3K9 methylation-dependent, suggesting that Tas3-λN associates with chromatin-bound nascent transcripts and initiates RNAi-mediated heterochromatin assembly. Finally, several splicing factors associate with RDRC[61,68] and are required for RNAi-mediated centromeric gene silencing[68]. These results provide additional support for co-transcriptional processing of non-coding centromeric RNAs, as spliceosomal components are known to associate with nascent RNA transcripts co-transcriptionally. A role for the nascent transcript in acting as a template for the recruitment of chromatin-modifying activities may be conserved throughout eukaryotes. For example, large non-coding RNAs such as XIST, which is involved in X-chromosome inactivation, are thought to be involved in the recruitment of histone and DNA methyltransferase enzymes[69]. However, the mechanism of recruitment of chromatin-modifying activities to XIST may involve site-specific RNA-binding proteins rather than small RNAs.

Chromatin-dependent processing of siRNAs

A remarkable observation in studies of RNAi in *S. pombe* is that the generation of centromeric siRNAs is a heterochromatin-dependent event[61,65]. In the nascent transcript model (Fig. 2b), RITS associates with methylated H3K9 through the chromodomain of its Chp1 component and captures the nascent non-coding transcript through base-pairing interactions involving siRNAs bound to its Ago1 protein. In cells lacking the H3K9 methyltransferase Clr4 or any component of the CLRC, the levels of centromeric siRNAs are greatly diminished[61,67]. Moreover, one of the two HP1 proteins, Swi6, is required for efficient siRNA generation[61,66,70] and the association of RDRC with centromeric DNA repeats[62] and non-coding centromeric RNAs[71]. Furthermore, the crucial chromatin-dependent step in siRNA generation involves dsRNA synthesis by RDRC, as the introduction of a long dsRNA-containing hairpin into *S. pombe* cells circumvents the requirement for both RDRC and Clr4 in siRNA generation[70]. These results suggest that RDRC is only able to synthesize dsRNA on chromatin-bound templates after it has been recruited by RITS, revealing the existence of a chromatin-dependent step in the activation of the dsRNA biogenesis and siRNA amplification pathway in *S. pombe*. The resultant dsRNA is processed into siRNA by Dcr1, which is also physically tethered to RDRC[72]. Heterochromatin regulation of small RNA production may be conserved in metazoans. X-TAS (transposable *P* elements inserted in telomeric-associated sequences on the X chromosome) and *flamenco*, two major piRNA-producing loci that control the transposition of *P* and *gypsy* elements in *D. melanogaster*, respectively, are embedded

in heterochromatin, and their genome defence function requires both PIWI and HP1 (refs 73, 74).

siRNA-mediated initiation of chromatin silencing

An important question regarding the role of RNA in gene silencing is whether small RNAs can initiate *de novo* chromatin modifications. Although small RNAs are important components of some CDGS mechanisms, their ability to initiate chromatin modifications seems to be under strict control by other mechanisms. In *S. pombe*, ectopically produced hairpin siRNAs can initiate H3K9 methylation and gene silencing at only a subset of target loci[70]. siRNA-mediated CDGS correlates with chromosomal location and the occurrence of antisense transcription at the targeted locus, and requires overexpression of the Swi6 (HP1) protein[70]. This may be reflecting the importance of cooperativity in the recruitment of RITS and other Argonaute or PIWI effector complexes to chromatin. In addition to siRNAs, stable association of RITS with chromatin requires the binding of the chromodomain in Chp1 to H3K9-methylated nucleosomes[10,65]. In the absence of H3K9 methylation, the initial binding of RITS to chromatin may be inefficient. Swi6 overproduction may help in initial RITS binding by stabilizing low levels of H3K9 methylation that occur throughout the genome, or alternatively by tethering the nascent transcript at the target locus to chromatin[71] (Fig. 2b). Similar limitations may explain the context-dependent ability of siRNAs to promote DNA methylation in plants[75], as well as the observed variability in siRNA-mediated chromatin modifications in animal cells (for example, see refs 76, 77). The ability of siRNAs to act as initiators is reminiscent of the role of DNA-binding transcription factors in the regulation of transcription, which often involves cooperativity between two or more transcription factors and is sensitive to local chromatin structure.

Small RNAs and epigenetic inheritance

Mechanisms that mediate the *cis*-inheritance of chromatin states and their associated gene-expression patterns remain enigmatic. It has long been known that during DNA replication, old parental histones are randomly distributed onto the two newly synthesized daughter DNA strands[78]. This retention of old histones during DNA replication has given rise to the idea that histone modifications mediate the epigenetic inheritance of chromatin states. Histone modifications, such as H3K9 methylation, create binding sites for proteins such as Chp1, Chp2 and Swi6, as well as the methyltransferase Clr4 (ref. 79) (Fig. 2b). Their retention during DNA replication could in principle serve as a mark for the re-recruitment of new chromatin-modifying activities that re-establish old modification patterns. However, the affinity of modified histones for specific binding proteins may be too low to allow the specific re-establishment of chromatin states, and other inputs into the mechanism are required[44]. The nascent transcript model, described above, provides a possible mechanism for epigenetic inheritance of heterochromatin. As in plants and other systems that contain an RdRP-dependent siRNA amplification mechanism[2,80], the siRNA generation mechanism in *S. pombe* is likely to form a positive-feedback loop[61,62]. Two specific features of this loop may underlie the mechanism that ensures the epigenetic inheritance of histone H3K9 methylation and heterochromatin. First, siRNAs can recruit H3K9 methylation to chromatin, possibly through physical interactions with the CLRC or dsRNA[70,81]. Thus, so long as siRNAs corresponding to a specific chromatin domain are present, they can recruit H3K9 methylation to that domain (Fig. 2b). The second feature involves a requirement for H3K9 methylation and chromatin localization in activating the siRNA positive-feedback loop[61,62,65]. This ensures that siRNAs are *cis*-restricted and is central to the role of siRNAs as epigenetic maintenance factors: siRNAs act only on those daughter DNA strands that have inherited old parental histone H3 molecules containing H3K9 methylation. Such cooperativity-based mechanisms involving the dual recognition of histone marks and other specificity factors (siRNAs or DNA-binding proteins) are likely to underlie all epigenetic *cis*-inheritance mechanisms.

RNAi and exosome-mediated RNA degradation

It may seem paradoxical that RNAi, which requires transcription, is required for assembling heterochromatin, a state that is associated with gene inactivation and TGS[3,51]. However, multiple mechanisms seem to ensure that transcription in heterochromatin does not result in the production of mature transcripts, thereby keeping heterochromatic genes off, despite transcription. First, heterochromatic transcripts are degraded or processed into siRNAs by the RNAi machinery itself through a process that has been referred to as CTGS or *cis*-PTGS[65,66] (Fig. 2b). CTGS requires the tethering of the RNAi machinery to heterochromatin by H3K9 methylation. This mechanism makes a major contribution to the silencing of some promoters in centromeric DNA repeats, although TGS is also an important contributing mechanism[66,71,82]. Second, an RNAi-independent RNA surveillance mechanism involving the TRAMP polyadenylation complex, which contains Cid14 (a Trf4/5 homologue), Air1, and Mtr1 in *S. pombe*, also targets heterochromatic transcripts for degradation[28]. In *S. cerevisiae*, TRAMP recognizes aberrant transcripts that lack polyadenylation signals and targets them for degradation by the exosome, a 3′→5′ exonuclease complex[30,31,83]. The presence of another member of the Trf4 polyadenylation polymerase family, Cid12, in the RDRC[61] suggests that RDRC and TRAMP may compete for access to heterochromatic transcripts (Fig. 1b). Furthermore, TRAMP and RDRC may compete more broadly for RNA substrates, because in *cid14* deletion cells new classes of RNAs become RNAi targets and are processed into siRNAs[29]. The involvement of members of the Trf4 family in RNAi processes in *C. elegans* and *T. thermophila* suggests a conserved role for members of this family in the coordination of exosome-mediated RNA surveillance with RNAi[84,85]. Finally, transcription in heterochromatin is cell-cycle regulated and is largely restricted to the S phase of the cell cycle[82,86]. This transcription is associated with high levels of siRNAs during the S phase, which may be important for epigenetic re-establishment of histone H3K9 methylation by the RITS–RDRC–CLRC complexes. However, it remains to be determined whether the increase in centromeric transcription and siRNA levels in S phase is merely a reflection of cell-cycle-associated changes in chromatin structure or has an important role in RNAi-mediated heterochromatin assembly.

Nearly all co-transcriptional RNA-processing events studied so far, including pre-mRNA capping, splicing and 3′-end processing, involve association between components of the processing machinery and RNA polymerase II (Pol II). Association with the polymerase is thought to help ensure that processing occurs in an orderly fashion and couples mRNA maturation with mRNA export. In addition, these associations serve to couple RNA quality control with transcription, ensuring that only true mRNAs are exported from the nucleus for translation. There is evidence that RNAi-mediated co-transcriptional heterochromatin assembly also involves interactions with Pol II[87,88]. Point mutations in two different Pol II subunits in *S. pombe*, Rpb2 and Rpb7, have been isolated in screens for defects in centromeric heterochromatin assembly. Neither mutation is associated with a growth defect or general perturbation of transcription[87,88], suggesting that the mutations may affect specific interactions with components of the RNAi machinery or the CLRC. Such interactions may contribute to efficient siRNA generation or H3K9 methylation by stabilizing the association of RITS–RDRC with nascent transcripts. Interestingly, in *A. thaliana*, RNA-dependent DNA methylation involves interactions between an Argonaute protein and RNA Pol IV, a plant-specific DNA-dependent RNA polymerase[89] (discussed below).

Conservation of small-RNA-mediated silencing

As discussed above, RNA silencing mechanisms have critical roles in endogenous chromatin-mediated processes in plants, *C. elegans*, *D. melanogaster*, ciliates and fungi. The role of small RNAs in chromatin silencing can also be extended to mammalian cells, although the mechanisms and physiological pathways are not yet clear. Reports from several laboratories provide evidence for the occurrence of DNA and histone modifications, which are promoted by the introduction

of siRNAs or hairpin RNAs into mammalian cell lines[76,77,90,91]. In these studies, siRNAs are directed to the promoter regions of target genes and induce the recruitment of repressive histone marks such as H3K9 and H3K27 methylation[91], but silencing is not always associated with CpG methylation[76]. In addition to chromatin-modifying complexes, siRNA-mediated TGS in mammalian cells requires AGO1 and AGO2 when the gene encoding progesterone is targeted[77], and AGO1 when the gene encoding the human immunodeficiency virus 1 co-receptor (CCR5) is targeted[91]. The recent identification of endogenous siRNAs in *D. melanogaster* and mammalian cells, which map to intergenic regions and are produced from dsRNA resulting from antisense transcription or long-hairpin structures, raises the intriguing possibility that some of these siRNAs modulate chromatin structure[23–25].

The PIWI-clade proteins and their associated piRNAs have important roles in the control of transposons in the germline — and possibly somatic cells — of *D. melanogaster* and mammals[4]. The mouse MIWI2 member of this family is required for silencing the long interspersed nuclear element 1 (*LINE-1*) and intracisternal A particle (*IAP*) transposable elements in the testis, and in *Miwi2* mutants both *LINE-1* and *IAP* DNA is demethylated[92], suggesting that piRNAs, directly or indirectly, mediates changes in DNA methylation. It remains unclear how the role of PIWI proteins in transposon silencing in the germline may be related to their function in repeat-induced and heterochromatic gene silencing in somatic cells described in *D. melanogaster*[45,46].

Although the mechanisms that link RNA to chromatin and the biochemical nature of the relevant complexes have not been defined yet, the available evidence allows us to draw some parallels between the nascent transcript model in *S. pombe* and other systems. The common denominator in the RNA silencing pathways operating in genome regulation is the linkage of Argonaute or PIWI proteins to chromatin- or DNA-associated molecules (Fig. 3). Argonaute proteins associate with adaptor proteins containing the conserved GW motif, which binds to their PIWI domain and is required for miRNA-mediated silencing[93] (Fig. 3a). In *S. pombe*, the GW-motif-containing protein Tas3 links Ago1 to Chp1 (refs 10, 94, 95). The binding of Chp1 to a methylated nucleosome then serves to tether nascent non-coding RNA, which is base-paired with siRNA in Ago1, to chromatin (Fig. 3b). This tethering seems to be crucial in that it links RNAi to chromatin and 'activates' the Ago1-bound nascent transcript complex to mediate chromatin modifications[61,65]. A similar Argonaute tethering situation seems to exist in *A. thaliana*, where, in addition to AGO4 and DCL3, RNA-directed DNA methylation requires the plant-specific Pol IV[96–99]. Pol IV exists as Pol IVA and Pol IVB complexes, which differ in their largest component, NRPD1A and NRPD1B, respectively. Pol IVB and AGO4 are thought to act downstream of Pol IVA and DCL3, which are required for siRNA generation, to trigger DNA methylation. NRPD1B contains a GW motif at its carboxyl terminus[89]. This GW-motif-containing domain links Pol IVB to AGO4, providing a parallel with the function of other GW-domain-containing proteins, such as the Tas3 component of RITS in *S. pombe*[89,96] (Fig. 3e). Thus, in plants, the strategy for coupling RNAi to chromatin involves a physical interaction between a repeat- or heterochromatin-specific RNA polymerase and an Argonaute protein. Once an siRNA-programmed AGO4 localizes to a nascent transcript synthesized by Pol IVB, it may trigger histone H3K9 and DNA methylation by recruiting the appropriate methyltransferase enzymes (Fig. 3e).

The role of the *D. melanogaster* PIWI protein in repeat-induced gene silencing and heterochromatin assembly seems to involve a direct association between PIWI and HP1 (ref. 100) (Fig. 3c). PIWI–HP1 may function as a RITS that targets nascent transcripts in repeat DNA elements and tethers these transcripts to chromatin by means of base-pairing interaction between piRNAs in PIWI and the association of PIWI with HP1 (Fig. 3c). Unlike the case with RITS and AGO4, this tethering does not seem to involve a GW-domain-containing protein and is mediated by the HP1 CSD and a conserved CSD-binding PXVXL motif (where X

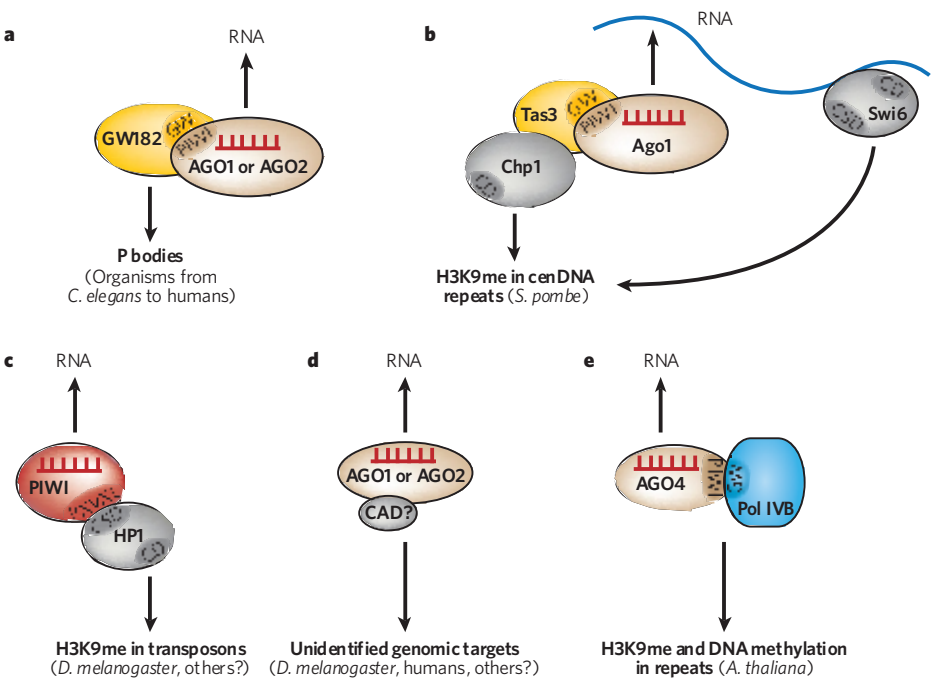

Figure 3 | Argonaute complexes that link RNA silencing to chromatin modifications. Argonaute proteins in different silencing pathways, including miRNA- and siRNA-mediated PTGS, are associated with conserved GW-motif-containing adaptor proteins, which help direct them to different targets. **a**, In many organisms, GW182 (a GW-motif-containing protein) or one of its homologues associates with the AGO1 and AGO2 proteins and directs them to P bodies. **b**, In *S. pombe*, Ago1 in the RITS is linked to heterochromatin through its association with the GW protein Tas3, which also binds to Chp1. Chp1 in turn associates with H3K9 methylated nucleosomes (H3K9me) through its chromodomain (CD). Swi6 (a homologue of HP1) acts as an accessory factor that helps tether the non-coding RNA to heterochromatin. The chromoshadow domain (CSD) is involved in protein–protein interactions. cenDNA, centromeric repeat DNA. **c**, In *D. melanogaster*, PIWI is targeted to heterochromatin through direct interactions with HP1; the association of PIWI with HP1 is mediated through the PXVXL motif, present in many HP1-binding proteins, rather than through a GW motif. **d**, In *D. melanogaster* and possibly other organisms, AGO1 and AGO2 have been implicated in mediating chromatin modifications, but the putative chromatin adaptor (CAD) protein has not been identified. **e**, In *A. thaliana*, AGO4 is linked to Pol IVB, which contains a GW motif at its carboxyl terminus and is specifically required for DNA methylation and silencing of heterochromatic repeats.

is any amino acid) present in *D. melanogaster* PIWI[100]. The PIWI–HP1 complex may be required for the recruitment and spreading of H3K9 methylation or possibly for the co-transcriptional degradation of RNAs that may escape heterochromatic TGS. It remains to be determined whether this PIWI–HP1 complex acts more broadly in piRNA-mediated silencing of transposons in the germline. Similarly, the possible role of AGO1 and AGO2 in siRNA-dependent gene silencing may be mediated by interactions with unidentified chromatin adaptors (Fig. 3d).

Future prospects

RNAi and related RNA silencing pathways have emerged as new mechanisms for the regulation of the structure and activity of genes and genomes. Our understanding of the mechanisms that allow some small RNAs to act at the DNA and chromatin level, and restrict other small RNAs to mRNA regulation in the cytoplasm, is still at an early stage. Although accumulating evidence suggests that nuclear small-RNA pathways are conserved, the endogenous pathways that may use small RNAs for genome regulation in animal cells remain for the most part unknown. Another gap in our knowledge of nuclear small-RNA pathways in animal cells involves the biochemical identification of the molecular networks that link different types of small RNA to chromatin proteins. Whereas Argonaute and PIWI proteins, as well as small RNAs, have been implicated in mediating chromatin or DNA modifications, it remains unclear how specific chromosome regions are targeted and how modifying enzymes are recruited. Future studies are likely to provide new and surprising insights about the way in which small and large non-coding RNAs regulate chromatin structure and how this ability is, in turn, regulated. ■

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Viral and cellular messenger RNA targets of viral microRNAs

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Given the propensity of viruses to co-opt cellular pathways and activities for their benefit, it is perhaps not surprising that several viruses have now been shown to reshape the cellular environment by reprogramming the host's RNA-interference machinery. In particular, microRNAs are produced by the various members of the herpesvirus family during both the latent stage of the viral life cycle and the lytic (or productive) stage. Emerging data suggest that viral microRNAs are particularly important for regulating the transition from latent to lytic replication and for attenuating antiviral immune responses.

MicroRNAs (miRNAs) are a class of small (~21–25 nucleotides) single-stranded RNAs that can inhibit the expression of specific messenger RNAs by binding to complementary target sequences within the mRNAs. Viruses were first reported to express miRNAs in 2004 (ref. 1), when Thomas Tuschl and colleagues described five miRNAs that were produced in human B cells after infection with the γ-herpesvirus Epstein–Barr virus (EBV). Subsequently, miRNAs have been found to be expressed by all of the herpesviruses examined. EBV is now known to encode at least 23 miRNAs[2,3], and the distantly related human γ-herpesvirus Kaposi's sarcoma-associated herpesvirus (KSHV) encodes 12 (refs 3–5). Similarly, the β-herpesvirus human cytomegalovirus (HCMV) encodes 11 miRNAs[5,6], and the human α-herpesvirus herpes simplex virus 1 (HSV-1) encodes at least 6 miRNAs[7,8]. (These numbers refer to the known pre-miRNA precursors encoded by each virus. A pre-miRNA can give rise to a single mature miRNA or to two miRNAs, one of which will be more abundant.) Several miRNAs have also been identified in herpesviruses that infect other species, including the simian γ-herpesviruses rhesus rhadinovirus[9] and rhesus lymphocryptovirus[2], murine γ-herpesvirus 68 (MHV68)[5], murine cytomegalovirus[10,11], and the avian α-herpesviruses Marek's disease virus types 1 and 2 (refs 12, 13).

Unlike herpesviruses (which are a family of DNA viruses), other, unrelated, DNA viruses seem to encode either one or two miRNAs (for example, primate polyoma viruses and human adenoviruses) or none at all[5,14–17]. Viruses that have an RNA genome, including retroviruses and flaviviruses, have been reported to lack miRNAs[5,17], although this result remains somewhat controversial for human immunodeficiency virus 1 (HIV-1)[18]. The absence of viral miRNAs in the RNA viruses examined so far might be partly explained by the fact that if the viral genome contained an appropriate precursor, this might be excised by the miRNA-processing enzyme of the host cell (that is, by Drosha)[19], resulting in degradation of the viral genome. Moreover, most RNA viruses — as well as DNA viruses belonging to the poxvirus family — replicate in the cytoplasm, away from the nucleus, where the Drosha-containing microprocessor complex is located. Therefore, even if the genomes of these cytoplasmic viruses encode a miRNA, it is not apparent how they could be processed to yield a mature miRNA.

It is less clear why miRNAs seem to be rare in nuclear-replicating DNA viruses that are not members of the herpesvirus family. It seems possible that the presence of miRNAs in herpesviruses is associated with the characteristic ability of herpesviruses to establish long-term latent infections. Avoiding the host immune response is particularly important during latent infection, and viral miRNAs not only have the advantage of not being recognized by the host immune system but also might be an ideal tool for attenuating immune responses by downregulating the expression of key cellular genes. Moreover, miRNAs might provide a way to regulate the entry of herpesviruses to the latent stage of the life cycle and/or their exit from this stage[20]. Other DNA virus families usually establish productive infections that often result in the infected cell's dying rapidly as a result of pathogenic factors produced by the virus or cytotoxic responses induced in the host. On the one hand, given that miRNAs operate at the level of the mRNA, they might not be as useful during a productive (lytic) replication cycle, because the proteins encoded by the targeted mRNAs might have a half-life that approaches, or even exceeds, the duration of the viral life cycle. On the other hand, viral miRNAs could be effective inhibitors of cellular mRNAs that are produced *de novo* during infection and that might encode proteins with antiviral activities. It will be interesting to see whether additional viral miRNAs, encoded by DNA viruses other than those of the herpesvirus family, will be uncovered in the future. In this Review, I briefly discuss what is known about the biogenesis and function of the known viral miRNAs, focusing on the limited number of viral and cellular mRNA targets that have been identified for these viral miRNAs so far.

Viral miRNA generation

The genomic regions encoding cellular miRNAs are generally transcribed by RNA polymerase II. The initial product is a capped, polyadenylated transcript that includes one or more stem–loop structures, each of which contains a mature miRNA sequence as part of one arm (Fig. 1). This precursor is known as a primary miRNA (pri-miRNA)[19] (see page 396 for further details about miRNA biogenesis). The nuclease Drosha cleaves the pri-miRNA stem, excising hairpin intermediates of ~65–70 nucleotides known as precursor miRNAs (pre-miRNAs). These are exported to the cytoplasm and processed by another nuclease, Dicer, generating mature miRNAs of ~22 nucleotides. The miRNAs are loaded into a protein complex known as the RNA-induced silencing complex (RISC), which they then guide to the target mRNA to exert their effector function. Binding of the RISC to an mRNA bearing extensive sequence complementarity to the miRNA generally results in mRNA cleavage and degradation, whereas binding to mRNAs bearing partial complementarity results mainly in translational arrest.

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At present, there is no evidence that any vertebrate virus encodes novel miRNA-processing factors or RISC components. So it seems that, in general, viral miRNAs are transcribed and processed in the same way as cellular miRNAs and, moreover, that RISCs programmed by viral miRNAs are functionally equivalent to those programmed by cellular miRNAs (Fig. 1). One exception is miRNAs encoded by MHV68 and by human adenoviruses, because the regions of the viral genome encoding these miRNAs are initially transcribed by RNA polymerase III (refs 5, 16). Although the processing steps involved in the biogenesis of MHV68 miRNAs remain to be fully elucidated, mature MHV68 and human adenovirus miRNAs are probably excised by Dicer and loaded into the RISC normally.

A key characteristic of DNA viruses is that gene expression during productive replication is temporally regulated: viral proteins can be subdivided into immediate early, early, and late species. Immediate early gene products are usually regulatory proteins. Early proteins are more diverse and are often involved in viral genome replication or in host immune response regulation. Late proteins are usually structural. The γ-herpesviruses, in particular, also produce viral proteins during latency, and these proteins have roles in episome maintenance, cell growth regulation and immune evasion. It is important therefore to consider whether the expression of viral miRNAs is also temporally regulated. For most herpesviruses, this is unclear at present, because the known viral miRNAs have been cloned either exclusively from latently infected cells or from cells at a relatively late stage in productive replication. However, there is evidence indicating that some viral miRNAs are active during latency, whereas others are more important during productive replication. For example, in the case of KSHV, all 12 viral miRNAs are derived from a cluster that is transcribed as a single pri-miRNA during latent infection. During the transition to productive replication, a viral lytic promoter is activated, and this promoter lies 3′ to the genomic sequences encoding ten of the KSHV miRNAs but 5′ to the genomic sequences encoding two of them. Consequently, the expression of ten of the KSHV miRNAs is largely unaffected when productive replication is activated, whereas the expression of two of the miRNAs is substantially induced[5,21].

In the case of HCMV, the 11 known viral miRNAs were all identified in productively infected cells[5,6], and most of these viral miRNAs seem to be produced with 'early' kinetics (that is, gene expression depends on viral immediate early transcription factors). It remains unclear which HCMV miRNAs are produced during latency. By contrast, in cells infected with HSV-1, four viral miRNAs seem to be expressed mainly during latency, one exclusively during productive replication and one during both stages[7,8]. Finally, in cells infected with simian virus 40 (SV40), which is a polyoma virus, viral miRNAs are expressed with late kinetics[14]. A full appreciation of the functions of viral miRNAs will certainly require a more detailed understanding of how their expression is regulated during the viral life cycle.

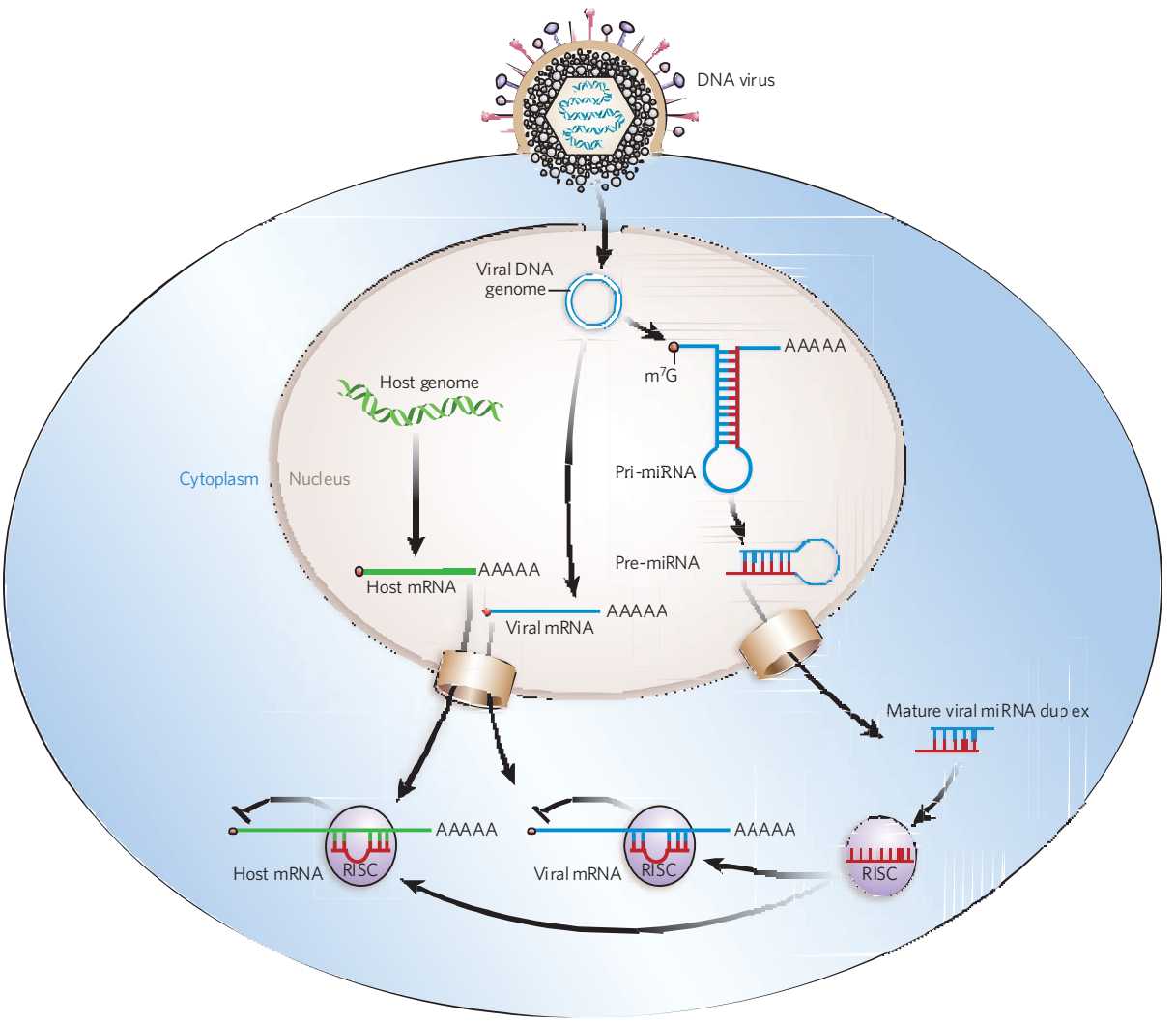

Figure 1 | How DNA virus miRNAs target host and viral mRNAs. After a host cell is infected by a DNA virus, the viral genome is transcribed in the nucleus to yield both pri-miRNAs and mRNAs. The pri-miRNA is processed by host factors in the nucleus to yield the pre-miRNA intermediate, which is then exported to the cytoplasm, where the mature viral miRNA is generated and incorporated into the RISC. RISCs that are programmed by viral miRNAs in this way can then inhibit expression of viral and/or host mRNAs in the infected cell's cytoplasm. m^7G, 7-methylguanosine.

Table 1 | Viral mRNA targets of viral miRNAs

Virus	Viral miRNA	Viral mRNA target	Function of viral protein
EBV	miR-BART2	*BALF5*	DNA polymerase
	miR-BART1-5p miR-BART16 miR-BART17-5p	*LMP1*	Signalling molecule
HvAV	miR-1	*ORF1*	DNA polymerase
SV40	miR-S1	T antigens	Early proteins
HSV-1	miR-H2-3p	*ICP0*	Immediate early protein
	miR-H6	*ICP4*	Immediate early protein
HSV-2	miR-I	*ICP34.5*	Pathogenicity factor
HCMV	miR-UL112-1	*IE* (*IE72*, *UL123*)	Immediate early protein

EBV, Epstein–Barr virus; HCMV, human cytomegalovirus; HSV, herpes simplex virus; HvAV, *Heliothis virescens* ascovirus; SV40, simian virus 40.

Although many viral miRNAs have now been identified, knowledge about their functions remains scarce. There are no published reports examining the *in vivo* phenotypes of viral mutants specifically lacking individual viral miRNAs, and only a small number of mRNA targets have been described (Tables 1 and 2). It can be envisaged that viral miRNAs evolved to downregulate cellular mRNAs and/or viral mRNAs (Fig. 1). Cellular mRNA targets might include transcripts encoding proteins involved in host innate or adaptive immune responses or, more generally, involved in cell-cycle regulation or signal transduction. Viral mRNA targets might include transcripts involved in regulating the transition from latency to productive replication (or vice versa) or products of immediate early genes that need to be eliminated at later stages in the viral life cycle as a result of toxicity or because they are targets for host cytotoxic T cells[14,20]. Although the current understanding is limited, the known targets of viral miRNAs have been found to belong to almost all of these categories.

Viral mRNA targets of viral miRNAs

The first paper to describe viral miRNAs also provided the first indication of a viral mRNA target for a viral miRNA. Specifically, one of the five EBV miRNAs described by Tuschl and colleagues[1], miR-BART2, was found to lie antisense to the mRNA encoding the EBV DNA polymerase, also called *BALF5*, and was proposed to inhibit the production of DNA polymerase by inducing cleavage of this mRNA. Although there is evidence supporting partial inhibition of EBV DNA polymerase expression by miR-BART2 (ref. 22), the functional significance of this inhibition is unknown. However, inhibiting EBV DNA polymerase expression might promote entry of the virus to latency by reducing viral genome amplification early after infection. Recently, it was reported[23] that a miRNA encoded by the insect DNA virus *Heliothis virescens* ascovirus (HvAV) also downregulates expression of the viral DNA polymerase. Unlike miR-BART2, the HvAV miRNA does not lie antisense to the viral DNA polymerase mRNA and has only moderate homology to the proposed mRNA target, but a reduction in the DNA polymerase mRNA level was nevertheless observed. The fact that two miRNAs, expressed by two unrelated viral species, both reduce the level of mRNAs encoding the cognate viral DNA polymerase might indicate convergent evolution.

Another example of a viral miRNA that is transcribed antisense to a viral mRNA, and induces degradation of that mRNA, occurs in the polyoma virus SV40. SV40 encodes a single pre-miRNA stem–loop structure that is expressed exclusively as a late gene product[14]. The viral miRNAs derived from this stem–loop structure lie antisense to the early viral mRNAs that encode the SV40 T antigens, which are viral transcription factors that induce the expression of late viral genes. These SV40 miRNAs, which show perfect complementarity to the T antigen mRNAs, induce the cleavage and degradation of the mRNAs and reduce T-antigen expression late in the SV40 life cycle. Epitopes derived from SV40 T antigens are recognized by cytotoxic T cells, and the effect of these viral miRNAs is therefore to partly protect SV40-infected cells from being killed by T cells[14].

Additional cases of viral miRNAs regulating mRNAs to which they are antisense have been reported in HSV-1 and the related virus HSV-2 (refs 8, 24). During latency, HSV-1 generates a set of five miRNAs: miR-H2-3p (3p denoting derivation from the 3′ side of the pre-miRNA stem), miR-H3, miR-H4, miR-H5 and miR-H6. One of these miRNAs, miR-H2-3p, lies antisense to the mRNA encoding the viral immediate early protein ICP0 and has been shown to downregulate ICP0 production[8]. Surprisingly, miR-H2-3p does not, however, induce *ICP0* mRNA degradation, despite being fully complementary. Although the molecular basis for this phenomenon is unclear, other research groups have also reported examples of miRNAs or short interfering RNAs (a related class of small RNA) that reduce the expression of mRNAs bearing perfectly complementary targets mainly by inhibiting their translation[25,26].

In addition to miR-H2-3p lying antisense to *ICP0* transcripts, HSV-1 miR-H3 and miR-H4 lie antisense to the mRNAs encoding the pathogenicity factor ICP34.5 and, on the basis of genetic data, were proposed to inhibit ICP34.5 expression in latently infected neurons[8]. This hypothesis has now been validated for the related virus HSV-2, which encodes a miRNA similar to miR-H3 (called miR-I)[24]. When overexpressed in HSV-2-infected cells in culture, miR-I reduces the amount of *ICP34.5* mRNA expressed and the amount of protein produced. A final example of an HSV-1 miRNA that targets a viral mRNA is provided by miR-H6, which was shown to downregulate production of the HSV-1 protein ICP4 (ref. 8). This miRNA does not lie antisense to the *ICP4* gene in the HSV-1 genome, but it does show extensive complementarity to *ICP4* mRNA, including the entire miRNA sequence extending from position 2 to position 8 (the miRNA 'seed' region). Full mRNA complementarity to the miRNA seed region is generally, but not always, required for inhibition of translation[19].

Overall, it seems that four of the six known HSV-1 miRNAs function to downregulate viral mRNAs in latently infected cells. The combined action of miR-H2-3p and miR-H6, which downregulate the production of the HSV-1 immediate early proteins ICP0 and ICP4, respectively, might increase the likelihood of HSV-1 entering latency and/or inhibit the transition from latency to productive replication[8]. The inhibition of ICP34.5 expression by miR-H3 and, potentially, miR-H4 is more difficult to explain, because ICP34.5 is a pathogenicity factor that blocks activation of the host antiviral factor PKR (double-stranded-RNA-activated protein kinase) and inhibits autophagy (an innate immune response in which cells are induced to degrade the bulk of their contents, including any newly formed virion particles)[27,28]. Inhibiting ICP34.5 expression might shield latently infected neurons from the severe cytopathic effects induced by a full-blown HSV-1 productive replication cycle, and this idea is supported by the finding that HSV-1 mutants lacking the *ICP34.5* gene are much less neurotoxic[28].

Another example of a viral miRNA that downregulates a crucial viral immediate early protein is the HCMV miRNA known as miR-UL112-1, which downregulates production of the viral protein IE1 (also known as IE72 and UL123) by targeting two partly complementary sites located in the 3′ untranslated region (UTR) of *IE1* mRNAs[20,29]. This observation prompted the proposal that herpesviruses in general might use miRNAs to regulate the expression of viral proteins that can trigger the transition from latency to productive replication[6]. This hypothesis is far from proven, but two observations are consistent with the idea. First, miRNAs produced in the latent stage of HSV-1 infection downregulate production

Table 2 | Cellular mRNA targets of viral miRNAs

Virus	Viral miRNA	Host mRNA target	Function of host protein
KSHV	miR-K12-11	*BACH1* (and others)	Transcriptional suppressor
	miR-K12-6-3p (and others)	*THBS1*	Adhesion molecule, angiogenesis inhibitor
HCMV	miR-UL112-1	*MICB*	Natural-killer-cell ligand
EBV	miR-BART5	*PUMA*	Pro-apoptotic factor
	miR-BHRF1-3	*CXCL11*	Chemokine, T-cell attractant

BACH1, BTB and CNC homology 1; *CXCL11*, CXC-chemokine ligand 11; KSHV, Kaposi's sarcoma-associated herpesvirus; *MICB*, major histocompatibility complex class I polypeptide-related sequence B; *PUMA*, p53-upregulated modulator of apoptosis; *THBS1*, thrombospondin 1.

of the immediate early proteins ICP0 and ICP4 (ref. 8), as noted earlier. Second, recent data show that viral miRNAs generated during KSHV latency downregulate the production of the KSHV immediate early proteins Rta and Mta, which are known to have a key role in the activation of productive KSHV replication (P. Konstantinova and B.R.C., unpublished observations).

A final example of a viral gene product that is downregulated by viral miRNAs is the EBV protein LMP-1, which has been reported to be suppressed by three EBV miRNAs, miR-BART1-5p, miR-BART16 and miR-BART17-5p[30]. LMP-1 is a cytoplasmic signalling molecule that is produced during EBV latency and can induce cell growth and transformation. However, overexpression of LMP-1 can result in growth inhibition and increased apoptosis[30]. So the role of these miRNAs might be to ensure an optimal level of LMP-1 expression during EBV latency.

Cellular mRNA targets of viral miRNAs

In principle, viral mRNA targets for viral miRNAs should be easier to identify than cellular mRNA targets. If a viral miRNA is antisense to a viral mRNA, then this suggests an obvious potential target, although not all viral mRNAs lying antisense to a viral miRNA are downregulated by that miRNA[31]. Even if the viral miRNA interacts with a partly complementary viral mRNA, this should be an easier target to identify than a cellular mRNA, given that viral genomes are much smaller than host cell genomes. It could be envisaged that viral miRNAs evolved to efficiently degrade host cell mRNAs that encode particularly 'troublesome' host defence factors; however, no fully complementary cellular mRNA targets for viral miRNAs have been identified so far. Instead, viral miRNAs seem to inhibit the translation of cellular mRNAs bearing partly complementary sites: that is, viral miRNAs seem to function just like cellular miRNAs[19] (Table 2).

An extreme example of this is the KSHV miRNA miR-K12-11, which has a seed region identical to the human cellular miRNA miR-155 and seems to downregulate an identical, or nearly identical, set of target mRNAs[32,33]. The most fully characterized of these is *BACH1* (BTB and CNC homology 1) mRNA, which contains several targets for both miR-K12-11 and miR-155 in its 3′ UTR. BACH1 is a transcriptional suppressor, and the significance of this downregulation for KSHV replication remains unclear. Even though several human genes downregulated by both miR-K12-11 and miR-155 have been identified[32,33], it is unclear why miR-K12-11 evolved to phenocopy miR-155. Overexpression of miR-155 is, however, associated with B-cell transformation, so miR-K12-11 might contribute to the transformation of B cells by KSHV[33]. Interestingly, the avian α-herpesvirus Marek's disease virus type 1 encodes a miRNA that also functions as an orthologue of miR-155 (ref. 34), and EBV (although it does not itself encode a miR-155 equivalent) induces endogenous miR-155 production in infected B cells[35]. It therefore seems that downregulation of specific cellular genes by either miR-155 itself, or by viral orthologues of miR-155, might facilitate the replication of a range of different herpesviruses.

Another cellular gene that is downregulated by KSHV miRNAs is thrombospondin 1 (*THBS1*). *THBS1* encodes a protein that is involved in facilitating cell-to-cell adhesion and has been reported to have anti-proliferative and anti-angiogenic activities[36]. *THBS1* expression is downregulated in Kaposi's sarcoma tumours, in keeping with the fact that tumour survival, particularly in highly vascularized Kaposi's sarcoma tumours, requires angiogenesis. Rolf Renne and colleagues[36] observed that *THBS1* mRNA was downregulated in cells engineered to produce KSHV miRNAs and also showed that translation of *THBS1* mRNA is inhibited by several KSHV miRNAs, in particular by miR-K12-6-3p, which shows miRNA seed-region complementarity to two sites in the *THBS1* mRNA 3′ UTR. It therefore seems possible that downregulation of *THBS1* by KSHV miRNAs contributes to the development of Kaposi's sarcoma *in vivo*.

An obvious prediction is that viral miRNAs might downregulate cellular mRNAs encoding antiviral factors, and three such cellular targets have been uncovered. First, the HCMV miRNA miR-UL112-1 has been reported to target mRNAs encoding MICB (major histocompatibility complex class I polypeptide-related sequence B). MICB is a ligand for a cell-surface receptor of natural killer (NK) cells, which are innate immune cells that provide one of the early lines of defence against viral infection. The MICB–receptor interaction is a key regulator of NK-cell activity and hence of NK-cell killing of virus-infected cells[37]. The proposed target for miR-UL112-1 in the 3′ UTR of *MICB* mRNA is unusual in that it does not have complete complementarity to the seed region of miR-UL112-1, and in this case extensive complementarity to the central and 3′ regions of the miRNA might compensate[37]. Despite this lack of complete seed-region complementarity, cells producing miR-UL112-1 were found to display less cell-surface MICB and to be resistant to NK-cell killing *in vitro*. Conversely, cells infected with a mutant form of HCMV lacking miR-UL112-1 had more cell-surface MICB and were killed more effectively by NK cells than were cells infected with wild-type HCMV. Interestingly, the function of MICB is also inhibited by the HCMV protein UL16, suggesting that UL16 and miR-UL112-1 might be functioning synergistically to protect infected cells against the NK-cell arm of the human immune system[37]. Recently, it was reported that cellular miRNAs, including miR-93, also target the 3′ UTR of the *MICB* mRNA at sites that partly overlap with, but are distinct from, the site targeted by miR-UL112-1 (ref. 38). Although these cellular miRNAs are not similar in sequence to the viral miRNA, it seems that miR-UL112-1 is mimicking the function of a subset of cellular miRNAs and thereby exerting a similar protective effect against NK-cell killing. As discussed earlier, miR-UL112-1 has also been reported to downregulate production of the viral immediate early protein IE1 in HCMV-infected cells (Table 1), thus providing the first example of a viral miRNA that targets both viral mRNAs and cellular mRNAs.

A second example of a viral miRNA that downregulates an antiviral factor is EBV miR-BART5, which inhibits production of the pro-apoptotic protein PUMA (p53-upregulated modulator of apoptosis)[39]. Depletion of miR-BART5 from EBV-infected nasopharyngeal carcinoma cells was found to trigger higher levels of PUMA-mediated apoptosis, suggesting that miR-BART5 might shield EBV-infected epithelial cells, as well as EBV-transformed cells, from elimination by apoptosis.

The third antiviral gene product known to be downregulated by a viral miRNA is CXC-chemokine ligand 11 (CXCL11), an interferon-inducible T-cell chemoattractant. *CXCL11* mRNA is downregulated by EBV miR-BHRF1-3, which is present in large amounts in many EBV-induced B-cell tumours[40]. CXCL11 has also been shown to have anti-tumour activity in animal studies, so this finding raises the possibility that, by downregulating CXCL11 production, miR-BHRF1-3 might shield EBV-infected B cells from cytotoxic T cells *in vivo*.

Conservation of viral miRNAs

The specificity of a miRNA can be altered by changing just one or two bases, especially in the seed region[19], so the genomic sequences encoding viral miRNAs might therefore be subject to rapid evolutionary drift. But if the presence of a particular viral miRNA results in a significant increase in viral replication, then the gene encoding this miRNA might be expected to be conserved. Furthermore, if a viral miRNA targets a viral mRNA, co-evolution might be expected to occur. By contrast, if a viral miRNA targets a cellular mRNA, then the evolution of the viral miRNA gene might be expected to be restricted.

In fact, analysis of miRNAs encoded by different members of the herpesvirus and polyoma virus families has so far uncovered little sequence conservation. One exception occurs in EBV and its simian relative rhesus lymphocryptovirus: 7 of the 16 miRNAs encoded by rhesus lymphocryptovirus are markedly similar to EBV miRNAs[2]. Because EBV and rhesus lymphocryptovirus are thought to have diverged ~13 million years ago, this suggests a strong evolutionary pressure for retaining the same miRNA sequences, especially as genomic sequences adjacent to the regions encoding the mature miRNAs (for example, those encoding the terminal loop of the pre-miRNA) were found to have diverged significantly[2].

By contrast, other related viruses (for example KSHV and rhesus rhadinovirus or Marek's disease virus types 1 and 2) show no

miRNA sequence conservation[9,12], although the genomic location of the miRNAs encoded by these viruses is conserved. Conservation of genomic location, but lack of sequence similarity, is also observed for the simian polyoma virus SV40 and its human relatives the BK virus and JC virus, all of which express miRNAs that are antisense to, and degrade, viral T-antigen mRNAs[14,15]. This finding might imply that the sole function of the miRNAs expressed by these viruses is to target these particular viral mRNAs[15]. However, viral miRNAs with no known viral mRNA targets also tend to be transcribed from the same genomic location, even when their nucleotide sequences have diverged[2,9,12]. So it might simply be easier for favourable sequence changes to be selected in genomic sequences that encode a pre-existing miRNA stem–loop structure than for a novel stem–loop structure to be generated *de novo*. It therefore remains possible that these diverse polyoma virus miRNAs also target cellular mRNAs for downregulation. Moreover, the fact that two viral miRNAs have divergent sequences does not necessarily imply that they have different functions. Two distinct miRNAs, encoded by two different viruses, could, for example, target two distinct regions in a single mRNA 3′ UTR, or they could target two gene products that function at different steps in the same host metabolic pathway, resulting in a similar phenotype. Until the mRNA targets for viral miRNAs are better understood, and until there is some idea of their *in vivo* functions, the conservation (or lack of conservation) of viral miRNAs is not readily interpretable.

Outlook

Despite our still limited knowledge of viral miRNA functions, the large number of miRNAs that are encoded by diverse members of the herpesvirus family, and their high-level expression during latent infections, suggests that these small non-coding RNAs have a key role in regulating viral pathogenesis *in vivo*. In particular, it will be important to test the hypothesis that herpesvirus miRNAs that are produced during latency help to maintain the latent state[8,20], which could be examined by using viral mutants and/or antisense reagents. It certainly seems possible that antisense reagents specific for particular viral miRNAs could significantly attenuate herpesvirus-induced diseases in humans, if they could be delivered effectively to infected cells *in vivo*. ■

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The promises and pitfalls of RNA-interference-based therapeutics

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The discovery that gene expression can be controlled by the Watson–Crick base-pairing of small RNAs with messenger RNAs containing complementary sequence — a process known as RNA interference — has markedly advanced our understanding of eukaryotic gene regulation and function. The ability of short RNA sequences to modulate gene expression has provided a powerful tool with which to study gene function and is set to revolutionize the treatment of disease. Remarkably, despite being just one decade from its discovery, the phenomenon is already being used therapeutically in human clinical trials, and biotechnology companies that focus on RNA-interference-based therapeutics are already publicly traded.

Before 1980, RNA was generally considered to be no more than a passive intermediate carrying information between DNA and protein synthesis. The discovery of catalytic RNAs in the early 1980s merited a shared Nobel prize to Tom Cech and Sidney Altman, and in 1986 the concept of 'the RNA world', an idiom created by Walter Gilbert, was proposed. Today, this is a common expression, and RNA has claimed a pivotal place in cellular biology.

Just ten years ago, RNA's functional repertoire was expanded further with the discovery in the nematode *Caenorhabditis elegans*[1] that double-stranded RNAs (dsRNAs) can trigger silencing of complementary messenger RNA sequences, and the term 'RNA interference' (RNAi) was born. Shortly thereafter, short dsRNAs — or short interfering RNAs (siRNAs) (reviewed in ref. 1) — were generated artificially and used to demonstrate that this process also occurs in mammalian cells, usually, but not always, without triggering the innate immune system, which normally recognizes RNAs as part of an antiviral defence mechanism (see page 421). The knowledge that small RNAs can affect gene expression has had a tremendous impact on basic and applied research, and RNAi is currently one of the most promising new approaches for disease therapy.

That RNAi could be triggered *in vivo* in mammals was first shown in animals infected with hepatitis B virus[2]. This was followed by the first therapeutic application of siRNAs: siRNAs were targeted to *Fas* mRNA in a mouse model of autoimmune hepatitis, resulting in protection of the treated animals against liver fibrosis[3]. In 2004, only six years after the discovery of RNAi, the first siRNA-based human therapeutics — developed as treatments for wet age-related macular degeneration — entered phase I clinical trials. RNAi is one of the fastest advancing fields in biology, and the flow of discoveries gives true meaning to the expression 'from the bench to the bedside'.

Although much is known about the mechanisms of RNAi, there are a number of challenges that applications of this gene-silencing technology need to overcome. For one, RNAi is a fundamentally important regulatory mechanism in the cell, and tapping into it in the interests of therapeutic benefit could result in side effects. Exogenously introduced dsRNA sequences can sequester components that make up the cellular machinery involved in gene silencing (see page 396), thereby reducing the accessibility of the machinery to a class of small RNAs known as microRNAs (miRNAs) that are entering the natural cellular pathway[4,5]. In addition, some synthetic siRNAs contain sequence motifs that can induce type I interferon responses and stimulate the production of pro-inflammatory cytokines[6–8].

During the past few years, many scientists have searched for solutions to overcome these limitations and to increase the safety of potential RNAi-based therapeutics. This article explores recent strategies to minimize undesirable secondary effects, describes new approaches to delivery and discusses RNAi therapies that are being tested. As it is anticipated that this technology will be applied to an increasing range of diseases, the potential problems and solutions that could one day transform RNAi into a conventional treatment for human diseases warrant careful attention.

Endogenous gene silencing

The effector RNA molecules of RNAi consist of ~20–30 nucleotides[9]. They are complexed with the protein components of the RNA-induced silencing complex (RISC). Its catalytic core in plants and animals (with the exception of single-celled organisms) is AGO2, a member of the highly conserved Argonaute protein family[10]. These small RNAs can silence gene expression by two mechanisms: post-transcriptional gene silencing (PTGS)[11], and transcriptional gene silencing (TGS)[12,13] (Fig. 1). PTGS can, in turn, be divided into two main mechanisms: direct sequence-specific cleavage, and translational repression and RNA degradation. Direct sequence-specific cleavage occurs when the targeted mRNA is perfectly complementary to the siRNA and is degraded after site-specific cleavage by the RISC. Translational repression and RNA degradation occur when the small RNA guide sequence has only limited complementarity to the target in the 'seed' region (nucleotides 2 to 8 from the 5′ end of the guide strand), with base-pairing usually occurring in the 3′ untranslated region (UTR). The latter mechanism is used by miRNAs.

TGS has been demonstrated in *Schizosaccharomyces pombe* (fission yeast), plants and, most recently, mammalian cells[14–17]. In *S. pombe*, the process is mediated by the RNA-induced transcriptional silencing complex (RITS), which contains Ago1, the chromodomain protein Chp1 and the glycine and tryptophan (GW)-repeat-containing protein Tas3 (ref. 18) (see page 413). Although in mammalian cells the mechanism by which small-RNA-directed silencing occurs is still hotly debated, both AGO1 and AGO2 have been shown to be integral to the overall

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process[19,20]. Most recently, a miRNA (miR-320) has been shown to regulate transcription of the POLR3D subunit of RNA polymerase III (Pol III)[21] in human cell culture.

Endogenous small RNAs have been found in various organisms, including humans, mice, the fruitfly *Drosophila melanogaster* and *C. elegans*. Many of these originate from transposons, viruses and repetitive sequences and are characterized by their interactions with the PIWI subfamily (or PIWI clade) of Argonaute proteins[22–25] — these are thus named PIWI-interacting RNAs (piRNAs). The identification of piRNAs has been restricted to germline cells. Recently, a new class of endogenous siRNAs (endo-siRNAs or esiRNAs) has been identified in the gonads and somatic tissues of *D. melanogaster*[26–29] and in mouse oocytes[30,31]. In mice, endo-siRNAs have been proposed to regulate retrotransposon movement[30,31]. Several families of small RNAs, including repeat-associated siRNAs (ra-siRNAs), tiny non-coding RNAs (tncRNAs), *trans*-acting siRNAs (ta-siRNAs) and scan RNAs (scnRNAs) (Table 1) are found in fungi, plants and animals, but so far none of these has been observed in mammals. The evidence suggests that piRNAs act through different cellular pathways from siRNAs and miRNAs and so could offer alternative targeting strategies for therapeutic targets.

Superior designs for small molecules

Cellular genes can be targeted by exogenous introduction of siRNAs, which then take advantage of the endogenous PTGS mechanism. The siRNAs can be either transfected into cells, where they enter the RISC directly, or generated within cells through gene expression by the use of vectors containing Pol II or Pol III promoters. These RNAi triggers can be expressed in animals and plants, but not in *S. pombe*, in the form of miRNAs or as short hairpin RNAs (shRNAs), which are cleaved into small (~21–25-nucleotide) RNAs by the enzymes Drosha and/or Dicer. In both cases, if the two strands of the RNA trigger are completely complementary, the passenger strand is cleaved by AGO2 (refs 32, 33), leaving behind a single-stranded guide sequence, which acts as the template for recognition of the targeted gene sequence by the RISC (Fig. 1).

Most of the impending therapeutic applications based on RNAi propose using direct introduction of synthetic siRNAs. The advantage of

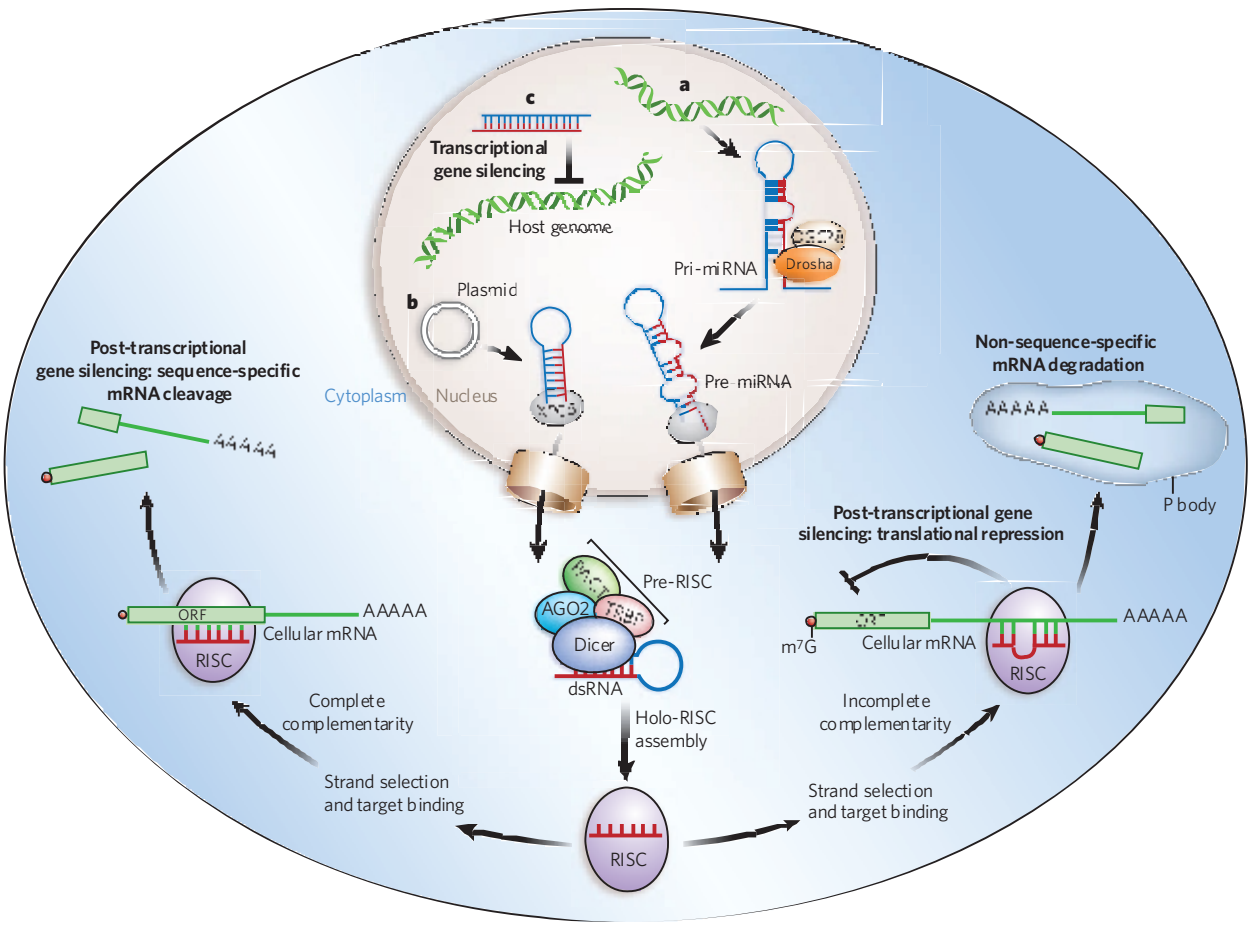

Figure 1 | Mechanisms of cellular gene silencing. **a**, Primary microRNAs (pri-miRNAs) are, in plants and animals, processed by Drosha and its partner DGCR8 into precursor miRNAs (pre-miRNAs) and then transported to the cytoplasm by exportin 5 (XPO5). In the cytoplasm, they are bound by a Dicer-containing pre-RISC and processed to yield the guide sequence that is loaded into the holo-RISC, which contains all the components required for gene silencing. AGO2 is the catalytic core of the RISC (present but not shown in the schematically drawn holo-RISC). The guide sequence binds to the corresponding target sequences in the 3′ UTRs of cellular mRNAs. If the miRNA guide sequence is fully complementary to its target site (left pathway), it triggers site-specific cleavage and degradation of the mRNA through the catalytic domain of AGO2. If the base-pairing is incomplete (right pathway) but includes pairing of the seed region (nucleotides 2–8 of the miRNA) with the target, translational inhibition occurs, and this can be accompanied by non-sequence-specific degradation of the mRNA in P bodies. **b**, Similarly to miRNAs, artificially transcribed shRNAs (in this case from a plasmid) are transported to the cytoplasm by XPO5. The dsRNA in the cytoplasm is recognized and processed by Dicer into ~21–25-nucleotide siRNA fragments that are loaded into the RISC. The siRNAs can target complementary sequences of cellular mRNAs and trigger their degradation through AGO2-mediated cleavage. **c**, When siRNAs are present in the nucleus and are complementary to promoter regions, they can trigger chromatin remodelling and histone modifications that result in transcriptional gene silencing. In mammalian cells, the details of this mechanism are still under investigation but are known to include Argonaute-family proteins. Accessory proteins indicated in the figure are TRBP (HIV *tar*-RNA-binding protein; also known as TRBP2P) and PACT (activator of protein kinase PKR; also known as PRKRA). m^7G, 7-methylguanosine.

Table 1 | Cellular small RNAs involved in gene silencing

Class	Size (nucleotides)	Functions	Mechanisms	Origin	Organisms found in
siRNAs	21–25	Regulating gene expression, providing antiviral response, restricting transposons	RNA degradation, transposon restriction	Intergenic regions, exons, introns	*Caenorhabditis elegans*, *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana*, *Oryza sativa* (rice)
endo-siRNAs	21–25	Restricting transposons, regulating mRNAs and heterochromatin	RNA degradation	Transposable elements, pseudogenes	*D. melanogaster*, mammals
miRNAs	21–25	Regulating gene expression at the post-transcriptional level	Blocking translation, RNA degradation	Intergenic regions, introns	*C. elegans*, *D. melanogaster*, *S. pombe*, *A. thaliana*, *O. sativa*, mammals
piRNAs	24–31*	Regulating germline development and integrity, silencing selfish DNA	Unknown	Defective transposon sequences and other repeats	*C. elegans*, *D. melanogaster*, *Danio rerio*, mammals
ra-siRNAs	23–28	Remodelling chromatin, transcriptional gene silencing	Unknown	Repeated sequence elements (subset of piRNAs)	*C. elegans*, *D. melanogaster*, *S. pombe*, *Trypanosoma brucei*, *D. rerio*, *A. thaliana*
ta-siRNAs	21–22	*Trans*-acting cleavage of endogenous mRNAs	RNA degradation	Non-coding endogenous transcripts	*D. melanogaster*, *S. pombe*, *A. thaliana*, *O. sativa*
natRNAs	21–22	Regulating gene expression at the post-transcriptional level	RNA degradation	Convergent partly overlapping transcripts	*A. thaliana*
scnRNAs	26–30	Regulating chromatin structure	DNA elimination	Meiotic micronuclei	*Tetrahymena thermophila*, *Paramecium tetraurelia*
tncRNAs	22	Unknown	Unknown	Non-coding regions	*C. elegans*

*C. *elegans* piRNAs are 21 nucleotides. endo-siRNAs, endogenous siRNAs; miRNAs, microRNAs; natRNAs, natural antisense transcript siRNAs; piRNAs, PIWI-interacting RNAs; ra-siRNAs, repeat-associated siRNAs; scnRNAs, scan RNAs; siRNAs, short interfering RNAs; ta-siRNAs, *trans*-acting siRNAs; tncRNAs, tiny non-coding RNAs.

using a chemically synthesized molecule is that chemical modifications can be introduced to increase stability, promote efficacy, block binding to unintended targets that contain sequence mismatches (specific off-target effects), and reduce or abrogate potential immunostimulatory effects (general off-target effects). However, the effects of these molecules are transient, whereas the promoter-expressed shRNAs or miRNAs can potentially mediate long-term silencing with a single application.

Conventional siRNAs are ~22 nucleotides and have 3′ dinucleotide overhangs that mimic Dicer cleavage products. Because not all siRNAs achieve equivalent levels of target knockdown, large-scale siRNA screening is often performed for any given target to find the most potent inhibitors. These have yielded some rules for siRNA design. For example, to facilitate incorporation into the RISC, the 5′ end of the antisense (guide) strand should be designed to have a lower thermodynamic stability than the 5′ end of the sense strand. The proportion of the nucleotides guanosine and cytidine should be around 50% or lower, and targeting of known protein-binding sites in mRNA regulatory regions should be avoided because binding of regulatory proteins may block siRNA–target pairing. For the same reason, intramolecular structures in the target should be avoided. Statistical analyses have also found a preference for certain nucleotides at specific positions within the siRNA[34]. Many computer programs are available for identifying the optimal target sequences for a given gene[34,35]. One of these, an artificial neural network, has been used to develop a genome-wide siRNA library for humans and to identify effective siRNAs for 34 targets[36].

Chemical modifications are often included in the design of synthetic siRNAs. Selective addition of phosphorothioate linkages or substitution of 2′ fluoropyrimidines or a 2′-*O*-methyl for the 2′ ribose at certain positions does not compromise siRNA activity and concomitantly increases resistance to ribonucleases[37], which is important for *in vivo* applications. A single 2′-*O*-methyl group on the passenger strand of an siRNA duplex can abrogate activation of the Toll-like receptors[38] and prevent toxicities due to the activation of type I interferon pathway gene expression. It has recently been demonstrated that fluoro-β-D-arabinonucleic acid (FANA[39] or as 4′-*S*-FANA[40]) or arabinonucleic acid (ANA[41]) modifications can increase both the serum stability and the potency of siRNAs. Some chemical modifications also have the important advantage of decreasing or blocking the activity of the siRNA's sense (passenger) strand, thereby reducing specific off-target effects. Other modifications, such as the addition of lauric acid, lithocholic acids and cholesterol derivatives, can be made to increase cellular uptake[42], which is currently one of the main hurdles of RNAi therapy.

Breaking and entering

Therapeutic applications of siRNAs require effective delivery to the target cells and tissues. The two main strategies are delivery of chemically synthesized siRNAs (non-viral delivery), or delivery of shRNA-encoding genes by engineered viruses that will ultimately generate siRNAs by transcription in the target cells.

Non-viral delivery

Because of their size and negative charge, siRNAs cannot easily cross cell membranes. Delivery has therefore been one of the major challenges for RNAi technology. Various means of delivery have been developed and tested in murine and non-human primate models, ranging from the injection of naked RNAs into a target organ such as the lung or eye to systemic delivery of the RNA in nanoparticles, complexed with polycations, attached to cholesterol groups or conjugated with cell-surface receptors. Some delivery approaches are detailed in Fig. 2.

Two polymers that have been examined for their delivery properties are atelocollagen and chitosan. Chitosans have mucoadhesive properties and have been used for intranasal delivery to bronchiolar epithelial cells[43]. Intranasal delivery has proved an effective means of delivering siRNA in mice[44] and in non-human primates[45] to block respiratory syncytial virus infection of the upper respiratory tract. In fact, the delivery of siRNAs to mucosal membranes seems to be an effective approach in general. For example, intravaginal delivery of lipid-encapsulated siRNAs targeting herpes simplex virus 2 (HSV-2) provided protection against lethal viral infection in more than two-thirds of the siRNA-treated mice[46].

Targeting of anti-apolipoprotein B (APOB) and peroxisome proliferator-activated receptor-α (PPAR-α) siRNAs to the liver has been achieved by means of a 'membrane-active' polymer, which can mask its activity until it reaches the endosome, resulting in the delivery of siRNAs to hepatocytes after a simple intravenous injection[47]. A different siRNA delivery approach used transferrin conjugated to a cyclodextrin-polycation polymer to deliver siRNAs targeting the Ewing's sarcoma *Ews–Fli1* fusion mRNA by means of the transferrin receptor in mice[48], resulting in inhibition of tumour progression. And conjugation of an siRNA to a cholesterol group permitted its delivery to the liver and the jejunum, where it downregulated its target, APOB, leading to consequent lowering of blood cholesterol levels in a murine model system[49].

An important advance for siRNA delivery was the successful application of stable nucleic-acid lipid particles decorated with polyethylene glycol (PEG) polymer chains (termed SNALPs) for the delivery of siRNAs directed against *APOB* mRNA (*APOB*-targeted siRNAs) to the livers of

non-human primates[50]. In this case, the siRNA effect of a single intravenous injection lasted for more than 11 days and resulted in greater than 90% target knockdown and no toxicity[50]. These exciting results have increased confidence in the potential of therapeutic siRNAs for treating liver diseases.

Until recently, most approaches to *in vivo* delivery have targeted a particular organ, primarily the eye, the lungs or the liver. A significant advance in targeting siRNAs to a specific class of leukocytes involved in gut inflammation has now been reported[51]. In this study, a cyclin D1 (*Cyd1*)-targeted siRNA was loaded into stabilized nanoparticles, the surfaces of which incorporated an antibody specific for a receptor expressed by the leukocytes. The targeted siRNA-containing nanoparticles down-regulated the cyclin D1 target, suppressed leukocyte proliferation and reversed experimentally induced colitis in mice[51].

Delivery of siRNAs to the nervous system has been particularly challenging. The brain is notoriously refractory to targeting because of difficulties in crossing the blood–brain barrier. However, delivery of siRNAs to the peripheral nervous system by direct infusion into the brain for the relief of chronic pain[52–54] or anxiety[55] has been demonstrated in rats. Conjugates of liposomes and antibodies or neuropeptides have also been used to deliver siRNAs into the murine brain[56]. Nevertheless, these methods do not target neurons, and a less invasive alternative to direct cranial injection is required to make such therapies more palatable.

A recent study unlocked the possibility of selective delivery of siRNAs to the central nervous system by systemic intravenous injection[57]. The siRNA involved — designed to target Japanese encephalitis virus — was conjugated with a short peptide derived from the rabies virus glycoprotein, which binds to the neuronal cell acetylcholine receptor. After transvascular delivery, 80% of the mice treated with the therapeutic siRNA survived infection with Japanese encephalitis virus, whereas 100% of the untreated controls died from complications of the infection[57].

Another interesting approach that allows systemic and targeted siRNA delivery uses a protamine–antibody fusion protein[58]. The protamine moiety is linked to the heavy-chain antigen-binding region (Fab) of an antibody to the human immunodeficiency virus 1 (HIV-1) envelope protein gp160. The positively charged protamine binds the negatively charged siRNAs — which are targeted against the HIV gene *gag* — allowing selective delivery to cells expressing the gp160 envelope protein on their surfaces[58]. This results in internalization of the antibody–siRNA complex, release of the siRNAs and down regulation of the HIV Gag-encoding

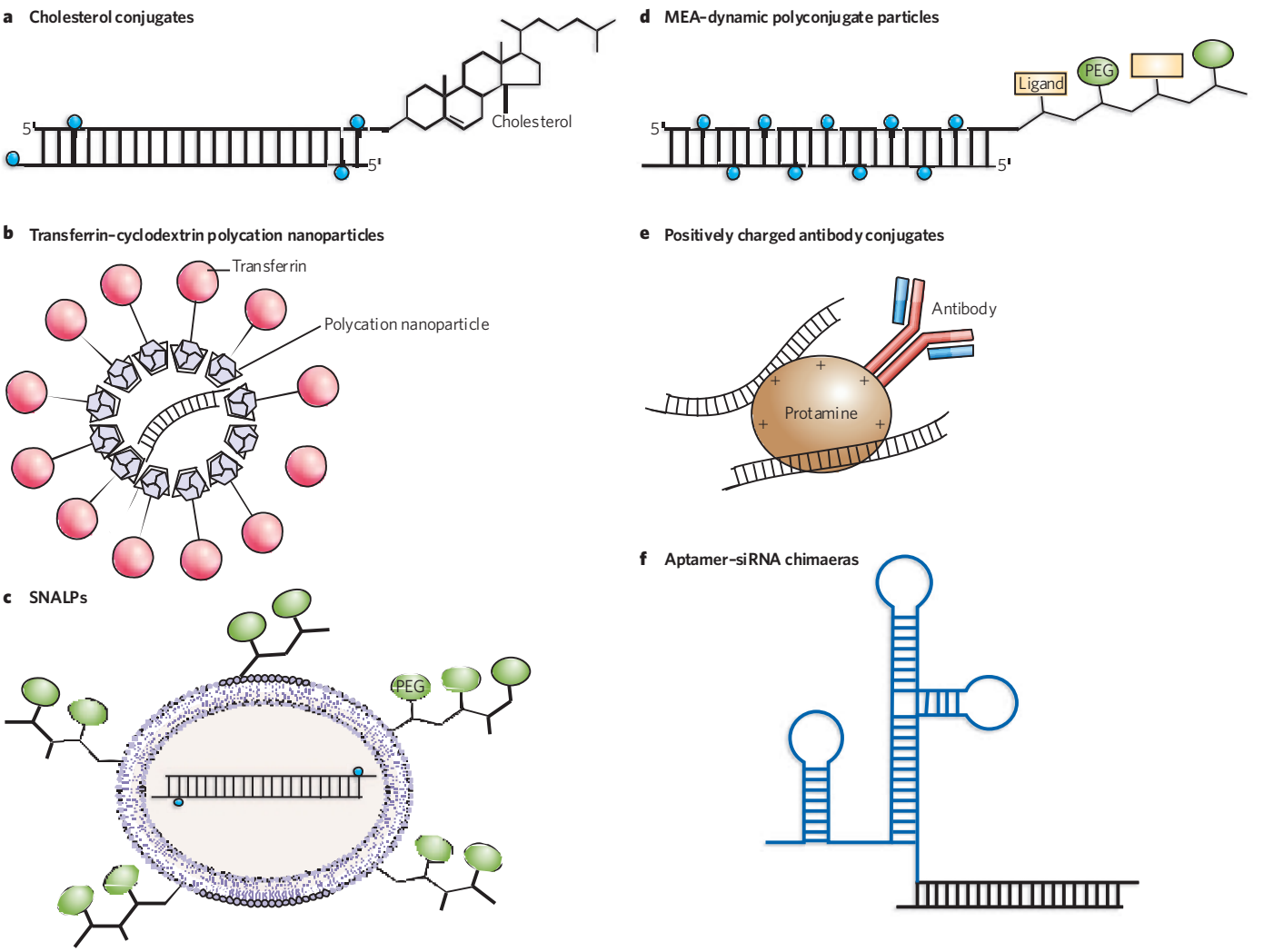

Figure 2 | *In vivo* delivery strategies for therapeutic siRNAs. **a**, Cholesterol groups can be linked to modified siRNAs to enhance their stability before systemic delivery. The most common siRNA modifications are 2'-*O*-methyluridine or 2'-fluorouridine substitutions (blue circles) combined with phosphorothioate linkages. **b**, Polycation nanoparticles can direct delivery of the siRNAs to specific cells through the use of surface ligands (such as transferrin) that bind to receptors on target cells. **c**, SNALPs encapsulate modified siRNAs into cationic or neutral lipid bilayers coated with diffusible PEG–lipid conjugates. SNALPs allow siRNAs to be taken up by cells and released by endosomes. **d**, Masked endosomolytic agent (MEA)–Dynamic PolyConjugates (DPCs) are similar to SNALPS but smaller, and contain a ligand that allows targeted cell delivery. The release of the siRNA from the endosome is also improved by the inclusion of a pH-labile bond in the MEA–DPC particles. **e**, Tagging specific antibodies with protamine or other positive charges allows the delivery of siRNAs to specific cell types via receptor-mediated uptake. **f**, Chemically linking or co-transcribing siRNAs with RNA aptamers allows the targeted delivery of the siRNAs to cells expressing the appropriate receptor.

Table 2 | Current clinical trials of RNAi-based therapeutics

siRNA	Company	Disease	Stage
Bevasiranib	Acuity Pharmaceuticals	Wet age-related macular degeneration	Phase III
		Diabetic macular oedema	Phase II
Sirna-027	Merck–Sirna Therapeutics	Wet age-related macular degeneration	Phase II
RTP801i-14	Quark Pharmaceuticals, and Silence Therapeutics	Wet age-related macular degeneration	Phase I/IIA
ALN-RSV01	Alnylam Pharmaceuticals	Respiratory syncytial virus infection	Phase II
NUC B1000	Nucleonics	Hepatitis B	Phase I
Anti-*tat/rev* shRNA	City of Hope National Medical Center, and Benitec	AIDS	Pilot feasibility study
CALAA-01	Calando Pharmaceuticals	Solid tumours	Phase I
TD101	TransDerm, and the International Pachyonychia Congenita Consortium	Pachyonychia congenita	Phase I

transcripts in a murine model *in vivo*. In this same study, fusion to protamine of an antibody specific for the hormone receptor ERBB2 allowed siRNA targeting of cancer cells expressing that receptor[58].

A similar technology for specific targeted delivery is based on aptamer–RNAi chimaeras[59]. Aptamers are *in vitro*-evolved, synthetically prepared nucleic acids that selectively bind specific ligands. An RNA aptamer designed to bind prostate-specific membrane antigen (PSMA; also known as FOLH1) was linked to a *PLK1*-targeted siRNA, and binding of the aptamer to the PSMA receptor resulted in the selective delivery into prostate cancer cells of siRNAs that target pro-survival genes[59,60]. Intratumoral injection of the PSMA–*Plk1*-targeted siRNA or PSMA–*Bcl2*-targeted siRNA conjugates into a mouse xenograft model resulted in triggering of apoptosis, growth inhibition and tumour regression[59].

A different conjugation of an siRNA to vitamin-A-coupled liposomes succeeded in delivering antifibrotic siRNAs to hepatic stellate cells, which are produced in response to liver damage[61]. In this study, multiple siRNA treatments targeting collagen chaperone-encoding genes reversed liver fibrosis by preventing collagen deposition and increased survival in rats, providing a potential therapeutic approach to treating liver cirrhosis.

Also noteworthy is the recent report of libraries of lipid-like molecules (lipidoids) that can be selected for siRNA delivery to various tissues[62].

Viral delivery

An alternative means of triggering RNAi is through promoter-expressed siRNA sequences processed from shRNAs or miRNA mimics. The genes encoding these hairpin structures are most commonly inserted into the backbones of viral vectors under the control of Pol II or Pol III promoters. A potential advantage of vector delivery is that a single administration triggers long-term expression of the therapeutic RNAi. This is particularly appropriate for chronic viral diseases such as HIV and viral hepatitis.

Lentiviral vectors have been used successfully to deliver shRNA constructs in various mammalian systems. For example, it was shown that downregulation of an activated *Ras* oncogene by a lentiviral-delivered shRNA resulted in suppression of tumour growth in mice[63]. And downregulation of the expression of a mutant form of superoxide dismutase 1 (SOD1) in mouse models of amyotrophic lateral sclerosis delayed the onset of disease[64,65]. More recently, a lentiviral vector was used to deliver a *Smad3*-targeted shRNA for regeneration of satellite cells and repair of old tissue in aged and injured muscle[66]. Viral-vector expression of shRNAs has also been explored in mouse models of neurodegenerative disorders such as Huntington's disease and Alzheimer's disease[67].

To deliver genes to the central nervous system, adenoviral vectors have proved very useful. For instance, direct brain injection of an adenoviral vector expressing a shRNA directed against the mRNA encoding the polyQ-harbouring SCA1-encoding transcript of spinocerebellar ataxia type 1 was shown to be an effective treatment in a mouse model of this disorder[68].

Despite the successes of viral delivery, it is important to bear in mind that although some viruses are non-pathogenic, they are still potentially immunogenic. Another major concern with this technique is the risk of incurring mutations in viral sequences, causing insertional mutagenesis or triggering aberrant gene expression. However, viral vectors can transduce both dividing and non-dividing cells, yield a prolonged expression of the therapeutic gene and need not be delivered in large doses. Ultimately, any therapeutic gene when expressed in large quantities has the potential to cause toxicity and immunogenicity. Critical parameters such as tolerability, long-term expression, efficacy and the ability to regulate expression and targeting should be taken into consideration when choosing a delivery method. There is no ideal delivery system for every application; rather, the delivery method needs to be tailored to the application.

Clinical trials using RNAi to treat human diseases

For a new technology, siRNAs have moved into the clinic at an unprecedented pace. Some examples of the diseases and siRNA-targeting strategies that are currently under investigation are described below.

The first siRNA protocol granted investigational new drug (IND) status and tested in a human clinical trial is the vascular endothelial growth factor (*VEGF*)-targeted siRNA Bevasiranib (Acuity Pharmaceuticals, Philadelphia, Pennsylvania) for the treatment of wet age-related macular degeneration (see Table 2 for a summary of ongoing siRNA clinical trials). This involves the overgrowth of blood vessels behind the retina, and causes severe and irreversible loss of vision; it affects 1.6 million people in the United States alone, and it is predicted that 11 million individuals worldwide will have the disease by 2013. Preclinical studies of Bevasiranib in mice showed reduced neovascularization resulting from downregulation of *Vegf* expression after direct ocular injection of the siRNA[69]. This siRNA, which is now in a phase III trial, is also in a phase II clinical trial for the treatment of diabetic macular oedema. By the conclusion of these trials, several hundred patients will have received the siRNA treatments.

Two other companies are also focusing on siRNA-based treatments against macular degeneration: Merck's Sirna Therapeutics (San Francisco, California) with an siRNA (Sirna-027) that targets the VEGF receptor VEGFR1, and Quark Pharmaceuticals (Fremont, California) in collaboration with Silence Therapeutics (London and Berlin; previously SR Pharma), with one targeted against a hypoxia-inducible gene, *RTP801* (also known as *DDIT4*), that is known to be involved in disease progression. This siRNA, RTP801i-14, has been licensed to Pfizer, UK, which is now running a phase I/IIA clinical trial. Quark Pharmaceuticals has also received IND status for another preclinical trial, in which it is currently enrolling patients. This trial is for an siRNA targeting *TP53* mRNA (which encodes the protein p53), inhibition of which delays the induction of cell-death pathways and thereby reduces acute kidney injury after surgery.

Calando Pharmaceuticals (Pasadena, California), meanwhile, has initiated a phase I clinical trial for solid tumours using an siRNA that targets a subunit of ribonucleotide reductase (RRM2), an enzyme required for the synthesis of DNA building blocks. Importantly, this trial is the first to utilize receptor-mediated delivery of siRNAs, which are encapsulated in cyclodextrin particles decorated with transferrin. This results in uptake by cells expressing the transferrin receptor, which is highly expressed on cancer cell surfaces.

The clinical trials performed by Acuity Pharmaceuticals and Merck's Sirna Therapeutics successfully stabilized patients' conditions against further degeneration and improved their vision without adverse effects. These results engendered great optimism for intravitreal injection of siRNAs, but in a stunning turn of events a report by Kleinman *et al.* demonstrated that the observed decrease in vascularization could be a consequence not of an siRNA-specific effect on angiogenesis, but rather a nonspecific activation of Toll-like receptor 3 (TLR3) and subsequent activation of interferon-γ and interleukin 12, which, in turn, downregulate VEGF[70]. In other words, both the targeted and the control siRNAs mediated nonspecific inhibition of angiogenesis through a direct interaction of the siRNAs with TLR3. Cellular uptake is not necessary for this effect, and because TLR3 is involved in several other cellular pathways the finding has highlighted another level of concern for safe clinical use of siRNAs.

Alnylam Pharmaceuticals (Cambridge, Massachusetts) is a well-established siRNA-therapeutics company whose leading candidate siRNA, ALN-RSV01, is now in a phase II clinical trial. This siRNA targets respiratory syncytial virus — which affects almost 300,000 people every year in the United States alone — by silencing the virus's nucleocapsid 'N' gene, a gene essential to viral replication. ALN-RSV01 was the first antiviral siRNA to enter clinical trials, and trials will soon be expanded to paediatric patients. Thus far it has been shown to be effective and well tolerated. Recently, Alnylam Pharmaceuticals formed an exclusive alliance with Kyowa Hakko Kogyo to develop and commercialize ALN-RSV01 in Japan and other Asian countries.

Also in development at Alnylam Pharmaceuticals are siRNAs directed against genes implicated in hypercholesterolaemia, Huntington's disease (in a joint venture with Medtronic of Minneapolis, Minnesota), hepatitis C (in a joint venture with Isis Pharmaceuticals in Carlsbad, California), progressive multifocal leukoencephalopathy (in a joint venture with Biogen Idec of Cambridge, Massachusetts) and pandemic flu (in a joint venture with the Swiss company Novartis).

The International Pachyonychia Congenita Consortium (IPCC), in collaboration with TransDerm (Santa Cruz, California), has developed an siRNA to allow the correct production of keratin as a treatment for a rare skin disorder called pachyonychia congenita.

The City of Hope National Medical Center in Duarte, California, in collaboration with Benitec (Melbourne, Australia), has started a phase I trial for the treatment of AIDS lymphoma. This trial uses a Pol III promoter-expressed shRNA targeting the HIV *tat* and *rev* shared exons. The shRNA has been incorporated into an HIV-based lentiviral vector, which in turn has been used to insert the shRNA gene (along with two other RNA-based anti-HIV genes) into blood stem cells[71]. The gene-modified stem cells have been infused into HIV-positive patients in a trial that uses autologous bone marrow transplantation to treat AIDS-related lymphomas. Four patients have now been treated in this trial.

As indicated above, partnerships have become quite accepted in the field of siRNA biotechnology. These consortia are considerably increasing the capital available for these efforts and are shortening the time involved in commercializing siRNA-based drugs.

Some companies, such as Regulus Therapeutics (Carlsbad, California), have chosen to focus on miRNAs as therapeutic targets. Santaris Pharma in Hørsholm, Denmark, has recently started the first phase I trial to target a human miRNA (miR-122). In this trial, miR-122 is being targeted for downregulation with a locked nucleic acid (LNA) anti-miRNA (SPC3649). LNA is a backbone modification that enhances the hybridization of the oligonucleotide with its target and protects it from nuclease degradation. The approach is intended to treat hepatitis C virus infection because miR-122 facilitates replication of this virus in the liver[72,73]. Downregulation of miR-122 is also potentially useful in the treatment of hypercholesterolaemia. Targeting miRNAs expressed in the heart, such as miR-208, which regulates cardiac hypertrophy and fibrosis[74], may have an advantage, because in the medical field there is a considerable experience in delivering drugs directly into this organ.

Gain or loss of miRNA function has been linked to the onset and progression of various diseases[75–77]. Protein function can be regulated either directly or indirectly by miRNAs, and alterations in miRNA expression can have profound effects on gene regulation. In instances in which disease results from altered miRNA expression, it is conceivable that normal levels could be achieved, either by targeting the specific miRNA if expression is too high or by delivering a miRNA mimic if expression is too low. However, the specificity and efficacy of delivery systems would need to be improved for this goal to be accomplished. Moreover, correct modulation of the targeted miRNA's expression is not an easy task, and it is not clear whether one miRNA can be specifically targeted without affecting other miRNAs of the same family.

The regulatory complexities of miRNAs should also be taken into consideration when either ablation or restoration of miRNA function is being considered in a therapeutic setting. A single miRNA can regulate the levels of hundreds of proteins[78,79], raising cautionary flags about the consequences of downregulating or ectopically expressing even a single miRNA species.

The safety issue

The application of siRNAs to therapeutics has raised a number of concerns about their safety. After the initial excitement, a number of reports underscored potential drawbacks to this promising technology. The first warning came from a study that recorded the deaths of mice after Pol III promoter-driven expression of shRNAs in the liver[4]. The exact mechanisms leading to mortality are still under investigation, but seem to be due at least in part to saturation of the transport factor, exportin 5, that ferries miRNAs from the nucleus to the cytoplasm. There are now indications that other factors involved in the RNAi process can also be saturated by high-level expression of exogenous siRNAs, which can sequester them from their cognate cellular miRNAs. Because each cellular miRNA can potentially modulate the expression of several hundred genes[78,79], minor alterations in the miRNA pathway can have major consequences.

One strategy to mitigate this problem is to use the lowest possible concentration of siRNAs that provides therapeutic efficacy by designing the exogenous siRNAs to be Dicer substrates (by increasing their length). These RNAs enter the RNAi pathway upstream of the RISC at the step of Dicer cleavage, which facilitates passing the siRNA to AGO2 for selection of the guide strand, often resulting in enhanced RNAi at lower concentrations than can be achieved with the exogenous delivery of cognate 21-base siRNAs[80–83]. Although small amounts of siRNAs are not expected to saturate the RNAi machinery, they can compete with miRNAs for selective incorporation into the RISC[5]. The long-term consequences of such competition are poorly understood.

With the use of microarrays, it has become increasingly obvious that introducing foreign siRNAs into the cell alters the expression of non-target genes, as well as target genes[84,85]; as few as six or seven nucleotides complementary to the seed region could result in a specific off-target effect[86] through a miRNA-like mechanism. Because microarrays only reflect mRNA levels, they do not take into account any genes affected at the translational level, and so at present it is not clear how extensive the problem of off-target effects really is. Given that the application of synthetic siRNAs results in transient inhibition of gene expression, specific off-targeting may not be a major concern for many clinical applications. Nevertheless, appropriate toxicity testing should take into account the potential for a particular siRNA to target 3′ UTRs in non-target genes.

Some strategies can be used in siRNA design to minimize the problem of off-targeting. For instance, it has been shown that 2′-*O*-Me modifications[87] or DNA substitutions[88] in siRNA duplexes can significantly reduce off-target effects. It would also be valuable to improve antisense-strand selectivity by taking into account thermodynamic stability (see 'Superior designs for small molecules') or by blocking the 5′ phosphorylation of the sense strand[89].

RNAi is a widely conserved mechanism that may originally have evolved to combat viral infections. As such, it is perhaps not surprising that in some cases siRNAs can act as agonists of Toll-like receptors[90] and that specific sequence motifs, such as uridine-rich regions and guanosine- and uridine-rich regions, can induce cellular immune responses[6,7].

The ability of an siRNA to stimulate cellular immune responses is based not only on specific sequences but also on structure, the type of delivery system used and the cell type[7,91]. Although the immunostimulatory potential of siRNAs could be advantageous in certain circumstances[92], it is usually an unwanted outcome. The above-mentioned finding of the TLR3 response to non-sequence-specific modulation of VEGF or the VEGF receptor[70], as well as a separate report showing that a macrophage migration inhibitory factor (*Mif*)-targeted siRNA (in a murine model) and a nonspecific control siRNA increased the proliferation of breast cancer cells through activation of dsRNA-activated protein kinase (PKR)[93], raise serious concerns in interpreting the results of *in vivo* siRNA applications.

Although we have yet to reach a universal solution for avoiding all off-target effects, it is foreseeable that these problems will be overcome by the use of appropriate backbone modifications, as well as delivery systems that can mask RNAs from the receptors of the innate immune system[94].

Gazing ahead

Despite the technique's youth, the list of diseases for which RNAi is being tested as a therapeutic agent is extensive, and includes Parkinson's disease, Lou Gehrig's disease, HIV infection, wet age-related macular degeneration, type 2 diabetes, obesity, hypercholesterolaemia, rheumatoid arthritis, respiratory diseases and cancers. It is already a multimillion dollar business, projected to reach US$1 billion by 2010, and intellectual property rights will become an increasingly important concern in the coming years.

However, although much has been accomplished, obstacles remain that will hamper the race to the clinic. The ultimate goal of achieving RNAi-based therapies for life-threatening or debilitating diseases cannot be attained without improving the safety, effectiveness and reliability of RNAi-trigger delivery systems. The use of targeted delivery strategies that permit systemic delivery will be a big step towards fulfilling this difficult task. The development of new, noninvasive imaging methods to monitor the *in vivo* delivery of siRNAs, such as labelling with near-infrared dyes[95], will aid studies of tissue uptake and biodistribution.

Although RNAi is not yet an accepted therapeutic modality, the enormous interest in this phenomenon ensures that we will soon witness fast advances and new applications for RNAi-based therapies. Given the way that RNAi has transformed basic research and the unprecedented speed with which it has reached the clinic, the coming years promise to be exciting. ■

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