Illuminating the silence: understanding the structure and function of small RNAs

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Abstract | RNA interference (RNAi) is triggered by double-stranded RNA helices that have been introduced exogenously into cells as small interfering (si)RNAs or that have been produced endogenously from small non-coding RNAs known as microRNAs (miRNAs). RNAi has become a standard experimental tool and its therapeutic potential is being aggressively harnessed. Understanding the structure and function of small RNAs, such as siRNAs and miRNAs, that trigger RNAi has shed light on the RNAi machinery. In particular, it has highlighted the assembly and function of the RNA-induced silencing complex (RISC), and has provided guidelines to efficiently silence genes for biological research and therapeutic applications of RNAi.

RNA interference (RNAi) is a potent and highly specific gene-silencing phenomenon that is initiated or triggered by double-stranded (ds)RNA. The discovery that dsRNA is a potent trigger of gene silencing in the nematode *Caenorhabditis elegans*[1] presented two fundamental questions for future research. First, can the RNAi mechanism be used to silence specific genes in other organisms? And second, what are the cellular functions of the gene-silencing machinery that are exploited by RNA triggers? Addressing these two questions has resulted in a surge of scientific information in various disciplines over the past few years and has launched new research areas to better understand the mechanisms of RNAi, its biological and therapeutic applications, and the functional implications of the RNAi machinery in cells and organisms.

RNAi is a widespread and natural phenomenon found in fungi, plants and animals. In all of these kingdoms, long dsRNAs are used as triggers of RNAi. When this powerful genetic tool was used in mammals, it was found that long dsRNAs elicit an innate antiviral immune response by inducing interferon-linked pathways. Long sequences of dsRNA induce the activation of PKR, a cellular dsRNA-dependent protein kinase that causes non-specific destruction of RNA and the inhibition of protein synthesis[2]. Fundamental insights into mammalian RNAi came from biochemical studies that showed that essential components of the RNAi machinery were conserved across species[3,4] and that target mRNA degradation was guided by 21–23-nucleotide (nt) RNA fragments from long dsRNAs[5,6]. These findings indicated that short RNA triggers induce RNAi in mammalian cells without eliciting a PKR response or inducing antiviral pathways. Indeed, two groups reported that short dsRNA (~21–23-nt) triggers could be transfected into mammalian cells to efficiently induce sequence-specific gene silencing[7,8]. These small interfering (si)RNA triggers initiate gene silencing by binding to their target-mRNA sequences and cleaving the target. Shortly after these initial reports, the use of siRNA (21-nt) triggers to study gene functions in mammalian systems became a standard laboratory procedure.

Endogenous, small (19–25-nt), non-coding RNAs that are known as microRNAs (miRNAs) have important roles in widespread functions including development, proliferation, haematopoiesis and apoptosis[9]. Unlike siRNAs, miRNAs usually do not cleave the mRNA of a target gene, but instead suppress mRNA translation[10,11]. Recently, miRNAs have been shown to enhance mRNA degradation[12]. This example elegantly showed that at least two different mechanisms are used by small RNAs to initiate gene silencing. A third, less well understood, pathway of RNAi involves heterochromatin silencing by small RNAs (reviewed in REF. 13). Despite the importance of RNAi in biology and medicine, its underlying molecular and cellular mechanisms are not fully understood. To clarify the mechanisms by which RNA molecules silence genes, the structural and functional relationships of various RNA triggers must be determined. This article reviews the current knowledge regarding the structural

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Box 1 | **Classes of small interfering RNA**

In addition to endogenous microRNAs (miRNAs) and exogenous small interfering (si)RNAs, which are discussed in the main text, endogenous siRNAs have been discovered in various organisms and fall into at least four classes: *trans*-acting siRNAs (tasiRNAs), repeat-associated siRNAs (rasiRNAs), small-scan (scn)RNAs and Piwi-interacting (pi)RNAs . tasiRNAs are small (~21-nucleotide (nt)) RNAs that have been reported in plants, and they are encoded in intergenic regions that correspond to both the sense and antisense strands[110,111]. In *Arabidopsis thaliana*, tasiRNAs require components of the miRNA machinery and cleave their target mRNAs in *trans*[110,111]. rasiRNAs that match sense and antisense sequences could be involved in transcriptional gene silencing in *Schizosaccharomyces pombe* and *A. thaliana*[25,112–114]. scnRNAs are ~28-nt RNAs that have been found in *Tetrahymena thermophila* and that might be involved in scanning DNA sequences in order to induce genome rearrangement[115]. piRNAs are different from miRNAs and are possibly important in mammalian gametogenesis[116–120]. They are small (~26–31-nt) RNAs that bind to MILI and MIWI proteins, a subgroup of Argonaute proteins that belong to the Piwi family and that are essential for spermatogenesis in mice[121].

requirements of various RNA triggers for gene silencing and discusses the effect of this knowledge on our understanding of RNAi pathways.

Triggers of RNAi

RNAi can be triggered by various sources of RNA molecules, including RNA viruses, transposons, exogenously introduced dsRNAs known as siRNAs and endogenous small non-coding miRNAs. In addition to miRNAs and exogenous siRNAs, endogenous siRNAs have been discovered in various organisms (BOX 1).

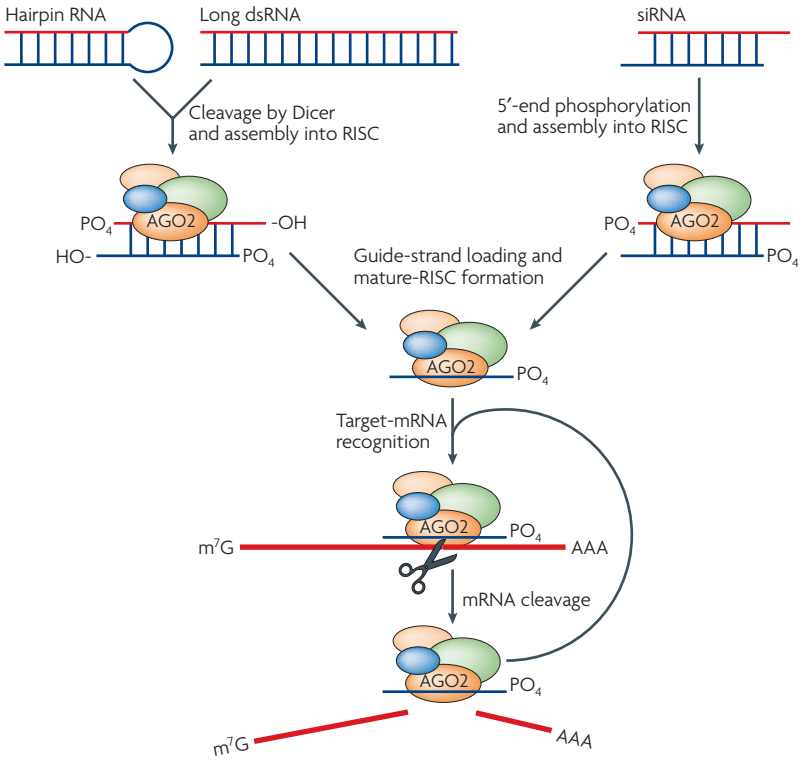

Figure 1 | **Steps in human siRISC function.** Long double-stranded (ds)RNA or hairpin RNA substrates are cut by Dicer into smaller (~21-nucleotide (nt)) small interfering (si)RNAs with 2-nt overhangs at the 3′ ends and phosphate groups at the 5′ ends. Alternatively, siRNA duplexes (19–23 nt) can be introduced into cells, where they are phosphorylated at the 5′ ends by cellular kinases. These small dsRNAs assemble into the RNA-induced silencing complex (RISC), which contains AGO2, Dicer and other cellular factors. For simplicity, only AGO2 is indicated. siRNA then forms activated RISC (siRISC) that contains an antisense (guide) strand. Activated RISC finds its target mRNA and uses the antisense strand to guide the cleavage of the target mRNA. RISC is recycled and could carry out several cleavage events.

RNA viruses can induce RNAi by at least two mechanisms: viral RNA can itself function as a trigger and enter the siRNA- or miRNA-mediated gene-silencing pathway, or the virus can encode miRNAs that use the host RNAi machinery for specific gene silencing. Transposon-mediated gene silencing involves DNA integration into promoters that create dsRNAs by being in proximity to, or transcribing, an inverted copy of the gene. In plants, dsRNAs that have been created by transgenes that express sense and antisense strands of a gene can silence genes much more efficiently than those created by transgenes that express single strands[14].

siRNAs. Long dsRNA molecules initiate RNAi by being converted to smaller 21–23-nt siRNAs by the RNase III-type enzyme Dicer (FIG. 1). This dsRNA-processing step creates RNAs with 2-nt overhangs at their 3′ ends and phosphate groups at their 5′ ends[15,16]. Dicer can use linear dsRNAs or hairpin-RNA substrates[17]. Therefore, DNA vectors that express hairpin RNAs have been commonly used to induce RNAi[15]. Alternatively, the post-Dicer cleavage products, siRNAs, can be exogenously introduced into cells to induce RNAi (FIG. 1). Recent studies have shown that pre-Dicer-cleavage dsRNA triggers are more potent in silencing genes than are siRNAs (REFS 18–20; C. Chu and T.M.R., unpublished observations). The two strands of siRNA have sequences with configurations that are sense and antisense with respect to the target mRNA (FIG. 1). The antisense strand of siRNA is also known as the guide strand because it serves as the template for sequence-specific gene silencing by the RNAi machinery. The sense strand is known as the passenger strand.

siRNA assembles into an RNA-induced silencing complex (RISC) to form an RNA–protein complex, known as siRISC, that incorporates the guide strand into the RISC (BOX 2). The guide-strand-containing RISC binds the target RNA and silences gene expression by cleaving the target RNA. The 5′ end of the guide strand sets the ruler for target-RNA cleavage, because cleavage occurs between nt 10 and 11 upstream of the 5′ end. When perfect complementarity exists between the guide and target strand near the cleavage site, the target RNA is cleaved and degraded. The hallmark of RNAi is its remarkable specificity in silencing genes, apparently governed by the complementarity between the guide strand and the target mRNA.

miRNAs. As mentioned above, the RNAi machinery can also be programmed for gene silencing by endogenous, small non-coding RNAs, or miRNAs. miRNAs have important roles in the post-transcriptional regulation of genes that code for diverse biological functions in most metazoan organisms, from nematodes to mammals[21–23]. RISC assembled with miRNA is known as miRISC. Although RNAi has commonly been associated with siRNAs, this process is also mediated by miRNAs in plants[24,25], and examples of miRNA-mediated gene silencing have been found in mammals and viruses (reviewed in REFS 22,23).

Biogenesis of miRNA is initiated in the nucleus (FIG. 2). miRNA genes are found in intergenic regions and in defined transcription units in both the sense and antisense orientations[26–28]. miRNAs are transcribed by RNA polymerase II to form primary miRNA (pri-miRNA) transcripts that contain a 5′-end cap structure and a polyA-tail sequence. A representative metazoan pri-miRNA contains a stem of ~33 nt, a terminal loop and flanking single-stranded (ss)RNA sequences. These stem–loop pri-miRNAs are cropped to 70-nt pre-miRNAs by the Drosha–DGCR8 (DiGeorge syndrome critical region gene-8) complex. Drosha is a nuclear RNase III that interacts with its cofactor, DGCR8, and produces RNA products that contain 5′ phosphate groups and 2-nt overhangs at their 3′ ends (reviewed in REF. 29). DGCR8, known as Pasha (partner of Drosha) in *Drosophila melanogaster* and *C. elegans*[30–33], was recently shown by Kim and colleagues to specifically recognize and bind pri-mRNA[34]. Moreover, the DGCR8–RNA interaction was found to depend not on the terminal loop structure of pri-miRNA but on its flanking ssRNA[34]. Interestingly, the Drosha cleavage site was shown to be ~11 base pairs (bp) from the stem–ssRNA junction[34]. These findings indicate a new model of microRNA processing, in which the DGCR8–RNA interaction sets the ruler for Drosha to cleave the RNA and produce pre-miRNA. Therefore, this DGCR8–RNA ruler could be used to discover new miRNAs that are correctly processed to carry out specific RNAi events.

To assemble into RISCs and to silence genes, pre-miRNAs have to be moved to the cytoplasm by a nuclear transport receptor complex, exportin-5–RanGTP[35–37]. Ran is a cofactor that binds GTP during the nuclear export of RNA. In the cytoplasm, GTP is hydrolysed to GDP and the pre-miRNAs are released from the export complex. These 70-nt pre-miRNAs are processed by Dicer into ~22-nt mature miRNAs that are subsequently loaded onto RISCs for gene silencing (FIG. 2). The miRISC recognizes its imperfectly matched target and binds in the 3′ untranslated region (3′ UTR) of the target mRNA. Without a perfect match between the two RNA molecules, the target is not cleaved, but its translation is repressed.

Interestingly, one functional component of miRISC is the general translation repressor protein RCK (also known as p54) (REF. 38). Recent studies have shown that certain RISC components localize to P-bodies[38–41] — cytoplasmic foci that contain translationally repressed mRNA–protein complexes[42,43]. Multiple copies of an miRISC that contains RCK/p54 could initiate an oligomerization event that sequesters the whole ribonucleoprotein particle (RNP) and transports it to P-bodies. Once in P-bodies, translationally repressed mRNA could stay in oligomeric structures for storage or could form a complex with decapping enzymes and cap-binding proteins that trigger mRNA decay (FIG. 2). In other words, the miRNA in miRISC could provide the sequence specificity, and RCK/p54 could be the effector molecule that shuttles target mRNA towards a fate of storage or processing in P-bodies.

Why RNA triggers and not DNA triggers?

The finding that only dsRNAs, but not DNA duplexes, are potent triggers of RNAi raised questions about the differences in their structures that might be relevant to triggering RNAi. What is unique or special about the structure of RNA, and why can't DNA trigger RNAi? Let us first analyse the key structural differences between RNA and DNA helices (FIG. 3). Analysis of helical parameters shows that RNA adopts a right-handed A-form helix

P-body
A cytoplasmic structure that is involved in storing and degrading translationally repressed RNA.

Ribonucleoprotein particle (RNP). A complex of proteins and RNA.

A-form helix
A right-handed (clockwise) helix in which base pairs are significantly tilted with respect to the central axis of the helix. The grooves are not as well defined as in the B-form helix. RNA commonly adopts an A-form helical configuration.

DEAD-box RNA-helicase domain
An evolutionarily conserved domain in a family of enzymes that use ATP hydrolysis to unwind RNA duplexes. The domain is named after the DEAD (Asp-Glu-Ala-Asp) motif.

RIII domain
A conserved domain that is present in the RNase III-type enzymes Dicer and Drosha and that is involved in endonuclease reactions that result in cleaving double-stranded RNA substrates.

dsRNA-binding domain (dsRBD)
A conserved protein region that binds double-stranded RNA.

PAZ domain
A conserved domain that is found in Argonaute- and Dicer-family proteins and that specifically binds to small RNA helices.

Box 2 | **The RISC machinery**

Known components of the human RNA-induced silencing complex (RISC) include Dicer, Argonaute proteins, TRBP (HIV-1 transactivation responsive element (TAR) RNA-binding protein) and the double-stranded (ds)RNA-binding protein PACT. Dicer is a highly conserved enzyme in almost all eukaryotes, including *Schizosaccharomyces pombe*, plants and animals. In some species, homologues of Dicer, such as Dicer-1 and Dicer-2, have specific roles in gene silencing (reviewed in REF. 91). In *Drosophila melanogaster*, Dicer-1 and Dicer-2 are involved in pre-microRNA (pre-miRNA) cleavage and in small interfering (si)RNA generation, respectively[122]. Dicer homologues are large (~200-kDa) multidomain proteins[17]. Human Dicer, a 1,922-amino-acid protein, has several domains that include a DEAD-box RNA-helicase domain, DUF283 (of unknown function), an RIII domain, a dsRNA-binding domain (dsRBD) and a PAZ domain[123].

Argonaute proteins, such as AGO1 and AGO2, also contain a PAZ domain[16]. Similarly to Dicer, various Argonaute homologues have been found in different species and have different roles in gene silencing. For example, *D. melanogaster* embryos that lack AGO2 and are defective in siRNA-directed RNA interference showed miRNA-directed target-RNA cleavage[101,124]. By contrast, AGO1 was required for mature-miRNA production, indicating that AGO1 was involved in miRNA biogenesis[101,124]. All Argonaute-family proteins, regardless of their origins, have two signature domains, PAZ and PIWI.

A comprehensive comparison of RISC components across species has been reviewed elsewhere[16]. In human RISC, AGO2 is the catalytic enzyme that cleaves target mRNA[75,76]. TRBP interacts with Dicer and human AGO2 (REFS 18,95,97). A TRBP homologue in *D. melanogaster*, Loquacious (also known as R3D1), interacts with Dicer-1 (REFS 96,125,126). The dsRNA-binding protein PACT associates with Dicer, human AGO2 and TRBP[127].

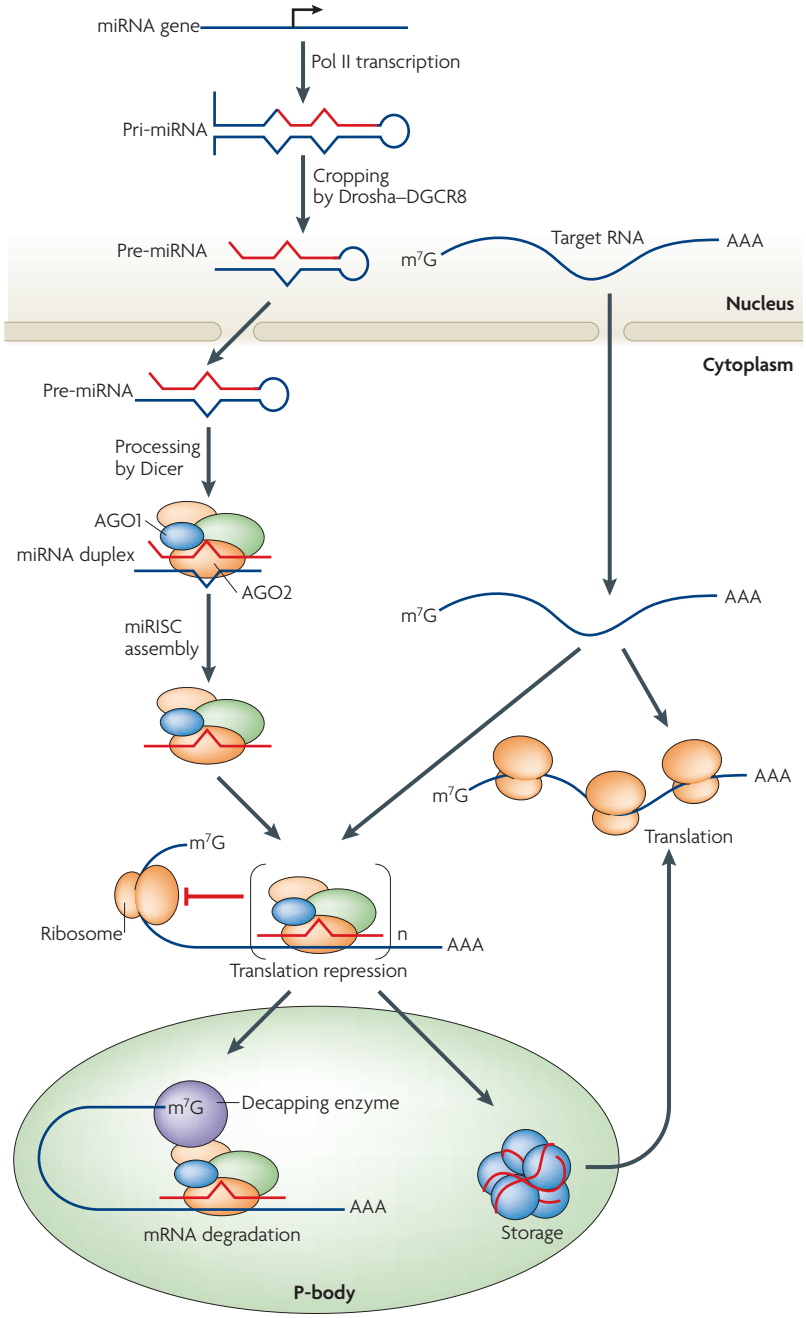

Figure 2 | **A model for human miRISC function.** MicroRNA (miRNA) genes are transcribed by RNA polymerase II (Pol II) into long (60–100-nucleotide (nt)) primary miRNA (pri-miRNA) sequences that fold into stem–loop structures. Pri-miRNAs are cleaved by the Drosha–DGCR8 (DiGeorge syndrome critical region gene-8) complex to form ~70-nt pre-miRNA structures that contain 2-nt overhangs at their 3′ ends[29]. Pre-miRNAs are transported to the cytoplasm by binding to exportin-5–RanGTP and perhaps to other adaptor proteins. In the cytoplasm, pre-miRNAs are processed by Dicer into small (~22- nt) miRNAs that assemble into the RNA-induced silencing complex (RISC) for gene silencing. RISC contains AGO2 (orange), AGO1 (blue) and other proteins, including known (for example, Dicer, TRBP (HIV-1 transactivation responsive element (TAR) RNA-binding protein) and RCK (also known as p54)) and unidentified proteins. Several RISC complexes that are loaded with miRNA (miRISC) bind to target mRNA by forming a bulge sequence in the middle that is not suitable for RNA cleavage, accumulate in P-bodies and repress translation by exploiting the translational machinery[38]. Translationally repressed mRNA is either stored in P-bodies or enters the mRNA-decay pathway for destruction. Depending on cellular conditions and stimuli, stored mRNA can re-enter either the translation pathway or the mRNA-decay pathway.

with 11 bp per helical turn and a diameter of 23 Å. DNA is a right-handed B-form helix with 10 bp per helical turn and a diameter of 20 Å, which is smaller than that of the A-form helix. Sugar pucker analysis shows that the ribose in the A-form helix has a C3′-endo configuration, whereas that of the B-form helix has a C2′-endo configuration. This variation in sugar pucker affects the helical structure in two ways. First, the intrastrand phosphate–phosphate distance in the A-form helix is 5.9 Å, whereas this distance in the B-form helix is 7.0 Å. Second, the helical pitch of the A-form helix is 28 Å and that of the B-form helix is 34 Å. Even though the 11-bp A-form helix is longer than the 10-bp B-form helix, its helical pitch is 6 Å smaller than that of the B-form helix. These structural differences create an A-form helix that is more tightly packaged and more stable than the B-form helix, resulting in a narrow and deep major groove in which functional groups are inaccessible for protein interactions. Therefore, helical geometry has been postulated as the major determinant for RNA being a specific trigger of gene silencing[44,45].

So, which step (or steps) of the RNAi pathway requires an A-form helix? Long dsRNA is processed by Dicer (that Dicer is RNA specific and does not process dsDNA contributes to the preference for RNA triggers) and associated proteins into short siRNAs. These associated proteins have to be loaded into RISC so that the guide strand can recognize the target mRNA and lead to its cleavage (FIG. 2). The A-form helix structure could be important in the RNAi pathway at two checkpoints: first, at RISC formation and the loading of the guide strand, and second, at target binding and cleavage.

The role of the trigger's helical structure in RNAi was elucidated in experiments with siRNA duplexes that contained internal bulge structures or mutations in the RNA helices[44,46,47]. The A-form RNA helix has a deep, narrow major groove and a shallow, wide minor groove (FIG. 3). More than one nucleotide bulge has been shown to distort the helical structure of RNA, widening the major groove and enhancing accessibility to its functional groups to simulate the signature feature of the B-form helix of DNA[48,49]. Mutant siRNAs were synthesized in which the A-form helices were distorted because of the introduction of two extra nucleotides into the passenger (sense) or guide (antisense) strands of siRNA duplexes, thereby creating 2-nt bulges. Combining these mutant siRNA strands with wild-type siRNA sequences produced three siRNA duplexes with internal bulge structures: the first bulged only in the sense strand (called the ss-bulge–as), the second bulged only in the antisense strand (called the ss–as-bulge), and the third bulged in both strands (called the ss-bulge–as-bulge)[44]. Interestingly, the siRNA duplex that contained a bulge in its sense strand retained most of its RNAi activity, indicating that an A-form siRNA helix is not essential for effective RNAi *in vivo*[44]. However, bulges in the antisense strand or in both strands of duplex siRNA completely abolished RNAi activities, indicating that effective siRNA-mediated gene silencing *in vivo* absolutely requires A-form helix formation between the target mRNA and its guiding antisense strand[44,46,47].

B-form helix
The Watson–Crick double helix of DNA, a right-handed (clockwise) helix in which 10 base pairs complete a single 360° rotation (helical turn). Grooves are prominent and well defined.

Sugar puckers
Ribose and deoxyribose sugar rings in RNA and DNA, respectively, are made of five atoms (one oxygen and four carbons (C1′–4′)), one of which lies out of the plane of the others. These sugar rings are flexible and dynamic structures that twist C2′ or C3′ atoms out of the plane, resulting in twisted forms or puckers.

Endo configuration
A sugar-ring configuration in which a specific carbon atom (C2′ or C3′) twists up from the plane with respect to the other four atoms of the ring.

Helical pitch
The length of one complete helical turn.

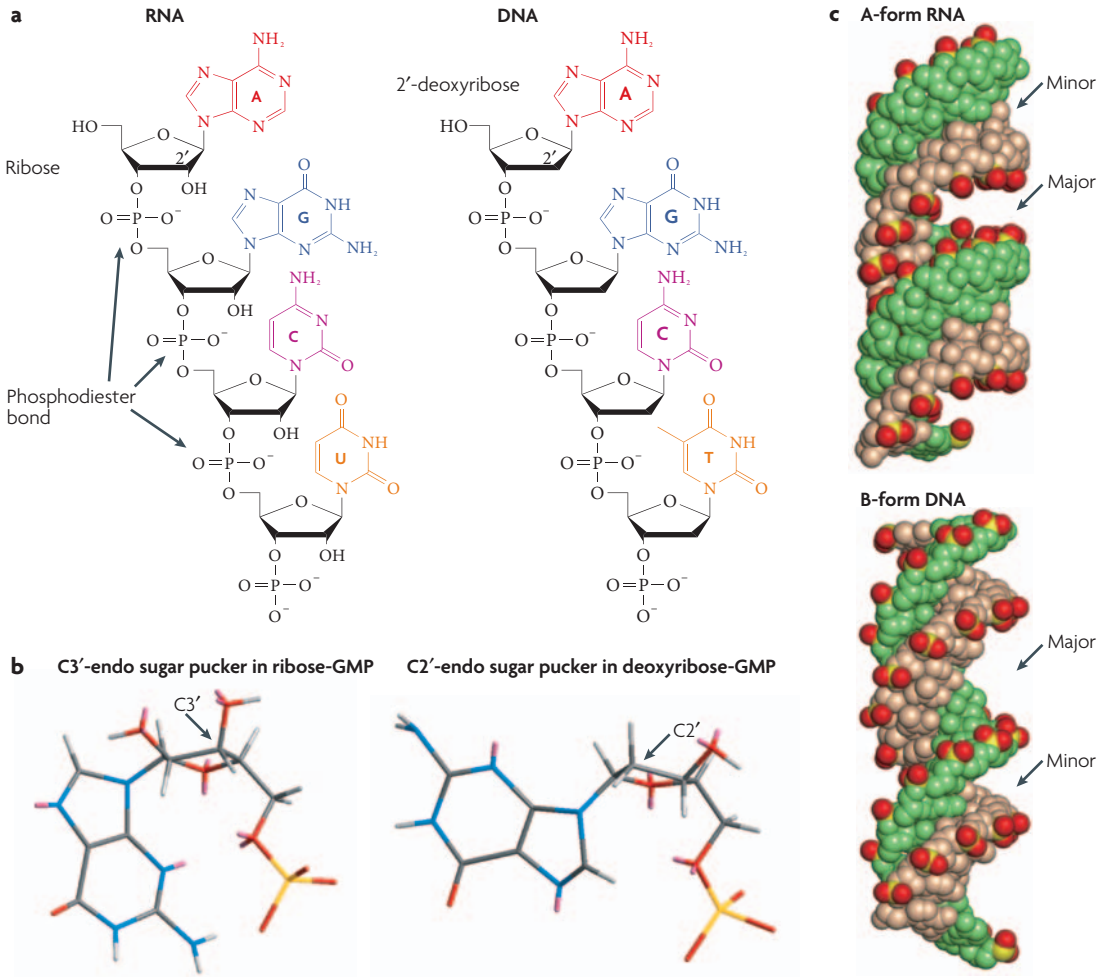

Figure 3 | **Chemical and structural features of RNA and DNA helices. a** | Chemical composition of RNA (left) and DNA (right). The 5′ end of the RNA strand starts at the top with an A base followed by a G, a C and a U. The ribose moiety of RNA has a 2′-OH group. All the riboses are linked by phosphodiester bonds. DNA has a 2′-deoxyribose sugar ring and the T base has a methyl group at the C5 position. **b** | Two main forms of sugar pucker found in nucleic acids. C3′-endo sugar puckers in ribose-GMP (left) and C2′-endo sugar puckers in deoxyribose-GMP (right) are found in A- and B-form helices, respectively. Heterocyclic nitrogen, oxygen and phosphorus are shown in blue, red and yellow, respectively. **c** | Space-filling models of A- and B-form helices. Due to sugar puckers, the distance between two phosphate groups in the C3′-endo configuration is ~5.9 Å, but this distance in the C2′-endo configuration is ~7.0 Å. Consequently, 11–12 base pairs (bp) are found in one helical turn of RNA, whereas DNA has 10.5 bp per helix. The A-form helix is more tightly packed than the B-form. The major groove in the B-form helix is wide, making functional groups accessible for protein interactions. The A-form helix has a deep and narrow major groove, making functional groups inaccessible for interactions with other macromolecules. However, functional groups in the minor groove of the A-helix are accessible for protein recognition. In addition, A-form helices are more thermodynamically stable than B-form helices.

The role of the A-form helix in RNAi was further examined by chemically modifying various functional groups in siRNA residues and analysing the effects on RNAi in human cells[45,47,50], as well as by kinetic analyses of *D. melanogaster* RISC loaded with various mutant siRNAs[46]. The results of these studies showed that target-RNA cleavage by siRNA-programmed RISC requires a central A-form helical geometry, thereby highlighting the structural requirement for the ribonuclease activity of Argonaute-2 (AGO2), the cleavage enzyme in RISC, in gene silencing (see below). Therefore, chemically modified and stable siRNAs can be designed for robust RNAi as long as the A-form geometry between the guide strand and the target RNA is maintained (TABLE 1).

Endogenous miRISC programmed with miRNA binds imperfectly matched target mRNA and inhibits translation (FIG. 2). In a simplistic model of miRNA-mediated gene silencing, miRISC is unable to cleave its target mRNA because an internal bulge structure is created when the target mRNA is bound. This target-mRNA–RISC interaction distorts the helix, thereby preventing RISC from cleaving the target, and gene silencing occurs by translational suppression. Interestingly, miRISC can cleave a target mRNA when the sequences of the miRNA and its target mRNA are perfectly complementary[11,51,52]. Similarly, siRNAs can function as miRNAs when no central A-form helix is present between the guide strand and the target mRNA[10,53].

Table 1 | **Guidelines for the chemical modification of RNA-interference triggers**

Passenger strand	Guide strand	RNAi function*	References
5′-end capping	3′-end capping	Not affected	44,62,63
3′-end capping	Unmodified	Not affected	44,62,63,130
Unmodified	5′-end capping	Severely affected	44,62,63
Pyrimidines that contain 2′-fluoro ribose	Pyrimidines that contain 2′-fluoro ribose	Not affected	45,70,131,132
2′-deoxyribose	Unmodified	Moderately affected	45
Unmodified	2′-deoxyribose	Severely affected	45
2′-*O*-methyl ribose	Unmodified	Moderately affected	45,47,62,70,71, 131–133
Unmodified	2′-*O*-methyl ribose	Severely affected	45,47,62,70,71, 131–133
Unmodified	2′-*O*-methyl ribose at position 2 from the 5′ end	Reduced off-target RNAi	72
2′-*O*-(2-methoxyethyl)	Unmodified	Not affected	131
Unmodified	2′-*O*-(2-methoxyethyl)	Moderate–severe effect depending on the position of the base	131
Phosphorothioate backbone	Unmodified	Not affected	45,131,132
Unmodified	Phosphorothioate backbone	Moderately affected	45,131,132
Phosphorothioate backbone	Phosphorothioate backbone	Moderately affected	45
3-methyl-U	3-methyl-U	Severely affected	45
Unmodified	C5-halogenated pyrimidine	Not affected	45

*RNA interference (RNAi) function assigned: not affected, >50% gene silencing; moderately affected, 20–50% gene silencing; severely affected, <20% gene silencing.

Although these studies imply that siRNAs and miRNAs are structurally and chemically identical, it remains unclear whether siRISCs and any miRISCs that are capable of cleavage have the same components or exhibit similar enzymatic properties.

Amplification of RNA triggers

One particularly fascinating aspect of RNAi is its extraordinary efficiency. For example, silencing effects in *C. elegans* can spread systemically and be passed through several generations[1,54]. The conversion of long dsRNAs into many 21–23-nt siRNA fragments would itself provide some degree of amplification, which might explain the potency of RNAi. Another plausible explanation is that RISC is a multiple-turnover enzyme, in which a single active centre can catalyse multiple reactions, with catalytic targeting and cleavage activities. The amplification process has also been suggested to involve RNA-dependent RNA polymerase (RdRP), as its gene has been identified as an essential requirement for gene silencing in plants, fungi and worms[55–59].

As primers can randomly bind to target sequences in this mechanism, a random degradative PCR model of amplification has been suggested[59–61], in which siRNA serves as the primer for the RdRP reaction. siRNA-primed RdRP converts target mRNAs into dsRNAs, which can serve as Dicer substrates that are degraded to siRNAs, thereby initiating an RdRP chain reaction. The polarity of the RdRP reaction limits the synthesis of secondary siRNAs to the region upstream of the trigger sequence. Certain structural features of siRNA, including the 3′ hydroxyl and 5′ phosphate groups, are crucial for the RdRP reaction and for RNA ligation. As Dicer processing creates a 5′ phosphate group and a 3′-OH with 2-nt overhangs, this RNA could serve as a template for RdRP reactions. No homologue of RdRP has been identified in available mammalian genomic sequences. Could other enzymes amplify the dsRNA triggers? Or, alternatively, could any enzymes amplify siRNAs?

The role of 3′-OH groups in RNAi activity was determined by using siRNA duplexes that contained a 3′ end that had been blocked with various moieties such as puromycin, biotin or fluorescein instead of 3′-OH groups on the overhanging deoxythymidine[44,50,62,63]. These 3′-end modifications blocked any processing of the siRNA duplex that required a free 3′-OH group. Analysis of RNAi activities indicated that blocking the 3′ end of either the passenger or guide strand of the siRNA duplex had little effect on its RNAi activity[44,50,62,63]. Modifications could be introduced in the 3′ overhangs without affecting siRNA efficacy, indicating that RNAi, at least in mammalian cells, does not occur through an RdRP-dependent, degradative PCR mechanism, which requires a free 3′-OH group. Similarly, RdRP homologues have not been found in the *D. melanogaster* genome, and no fruitfly RNAi-amplification mechanism has been reported[64].

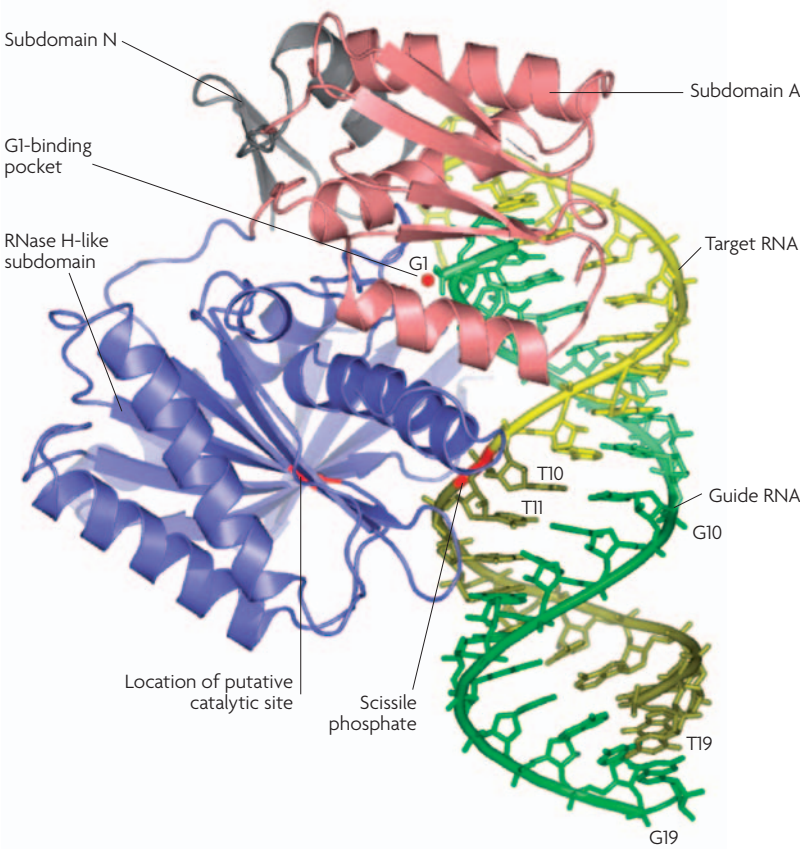

Figure 4 | **Structural model of a 19-nucleotide guide strand and its target RNA bound to the PIWI-domain-containing protein of *Archaeoglobus fulgidus*.** This model is based on the crystal structure of *Archaeoglobus fulgidus* Piwi protein bound to an RNA duplex that mimics the 5′ end of a guide strand and an overhanging target RNA. Guide- and target-strand nucleotides are labelled G1–19 (5′→3′) and T1–19, respectively. RNA adopts an A-form helix and the protein binds to the 5′ end of the guide strand in the G1-binding pocket region[65,66]. The scissile phosphate in the RNA backbone is shown in red between nucleotides T10 and T11 of the target strand. This crystal structure of a dsRNA–Piwi complex suggests a model for RISC function in which the 5′ region of the guide strand provides a nucleation site for AGO2 recognition, and in which interactions between the guide strand and Argonaute set the ruler for its cleavage of the target RNA. Subdomain A (amino acids 44–170) consists of β-strand–α-helix repetitions and forms an α–β sandwich architecture. The N-terminal 40 amino acids of the protein, known as subdomain N, consists of small two-stranded antiparallel β-sheets lying against a single α-helix[128]. This model is consistent with functional studies that demonstrated that the 5′ end of the guide strand should contain phosphate groups or free hydroxyl groups (that are phosphorylated *in vivo*) for effective RNA interference (RNAi), because blocking the 5′ end resulted in a dramatic loss of RNAi function[44,45,62]. Reproduced with permission from REF. 66 (2005) Macmillan Publishers Ltd.

End capping of the RNA helix

As discussed above, the siRNA helix can be capped at its 3′ end without affecting its RNAi function in human cells. Is the same true of the 5′ end of the RNA helix? During the processing of long dsRNA or hairpin RNA by Dicer, siRNAs are generated with phosphate groups at their 5′ ends. Duplex siRNAs with 5′-OH groups are used in standard laboratory procedures. Therefore, given that endogenously produced siRNAs contain phosphate groups at 5′ ends, it is reasonable to propose that in the first step of siRNA-induced RNAi, the hydroxylated 5′ ends of the siRNA duplex are phosphorylated, which is required for the formation of an siRISC (FIG. 1). Phosphorylation at the 5′ end might be important for the binding of AGO2 to the siRNA and for the helicase function of RISC to incorporate the guide strand into siRISC (FIG. 1). Indeed, the importance of these 5′ termini in RNAi in human cells was determined by modifying the 5′ termini of synthetic siRNAs with amino groups containing 3-carbon linkers and by measuring their RNAi activities[44]. Synthetic siRNAs with this modification lacked a hydroxyl group for *in vivo* phosphorylation by kinases. This modification also blocked access to siRNAs by cellular factors that might need to recognize the 5′-OH termini. Analysis of RNAi activities using various siRNA duplexes revealed that only the 5′-end modification of the guide strand completely abolished the RNAi effect[44,62,63], highlighting the importance of a 5′ phosphate group in the guide strand of the siRNA duplex.

Further evidence for the importance of the RNA helix and of the 5′ phosphate end of the siRNA guide strand comes from recent structural studies of an *Archaeoglobus fulgidus* Piwi protein bound to an siRNA duplex[65,66]. These Piwi structures define a short channel for RNA exit and show that base-paired nucleotides adopt an A-form helix, placing the scissile phosphate of the target RNA close to the active site of the enzyme[65,66] (FIG. 4). These structural studies demonstrate the obvious significance of the 5′ end of the guide strand. The 5′ region of the guide strand provides a recognition site for AGO2, and this interaction defines the exact site in the target RNA that is cleaved by the enzyme. Based on biochemical and structural studies of AGO2 and Piwi, one could propose that Dicer and the Drosha–DGCR8 complex use RNA–protein recognition and cleavage mechanisms similar to the one used by AGO2. It is therefore not surprising that the Drosha–DGCR8 complex cleaves pri-miRNA at a distance of ~11 bp, exactly one helical turn of the RNA helix, from the stem–ssRNA junction[34]. These results, combined with those on 3′-end helix capping, have laid the foundation for designing siRNA duplexes that are end-labelled with various molecular probes to study the structure and function of RISC.

Chemical modification of siRNA

Why does natural RNA need to be modified for RNAi? RNA is not a stable biomolecule, because its 2′-OH groups promote RNA hydrolysis under acidic and basic conditions. In addition, nucleases can further reduce the half-life of an RNA *in vivo*. Therefore, siRNA is chemically modified for at least two important reasons. First, enhancing the stability of siRNA in extracellular and intracellular environments is crucial for broad applications of RNAi in reverse genetics and for developing RNAi-based therapeutics. Second, chemical modifications could influence specific steps of RISC function and possibly reduce off-target effects of RNAi that arise from partial complementarity between the guide and target-mRNA strands[67,68]. But is it necessary to modify siRNAs to achieve the two goals outlined above? Fortunately, recent evidence indicates that chemically modified siRNAs are more stable *in vitro*[45,47,62] and in animals[69–71] and that they can indeed reduce off-target effects[72].

Off-target effects
In the context of RNA silencing, this refers to the decreased expression of genes other than the intended target gene.

On the basis of chemical modification studies, guidelines can be derived to design stable RNA triggers for RNAi experiments (TABLE 1).

When designing modified siRNAs, there are a number of rules to keep in mind. First, the 5′ end of the guide strand should have a free hydroxyl or phosphate group. Second, the 3′ ends of the passenger strand and guide strand can be modified with a dye, or another molecular probe, as well as with *in vivo* delivery reagents. Third, the 5′ end of the passenger strand can be capped with a chain-blocking reagent or modified with a dye or another molecular probe. This modification would also inhibit passenger-strand incorporation into RISC. Fourth, a ribose that is modified at the 2′ position with halogen atoms such as fluoride and with a small hydrophobic group such as *O*-methyl can be incorporated into the passenger and guide strands. A bulkier group, such as *O*-(2-methoxyethyl), can be used in the passenger strand, but is not well tolerated in the guide strand. Fifth, the backbone structure of the RNA duplex can be further stabilized by phosphorothioates in either strand. But note that a large number of phosphorothioate-containing biomolecules can be toxic in animals. Sixth, pyrimidines that are halogenated in their heterocyclic ring structures are tolerated in the guide strand. Although no data are available on purine halogenation, it is reasonable to postulate that these modifications would not significantly affect siRNA activities. Last, modifications of functional groups in the major groove of the RNA duplex should be avoided because these modifications severely inhibit RNAi.

In summary, most modifications in the passenger strand do not negatively influence siRNA function as long as the duplex retains its A-form-like helical structure. Future studies will undoubtedly determine how chemical modifications of siRNA modulate RISC function and specificity.

siRISC assembly and mRNA cleavage

As mentioned above, both classes of small RNAs, siRNAs and miRNAs, are assembled into silencing complexes that contain Dicer, Argonaute-family proteins and other proteins[73]. However, they initiate silencing of gene expression by two different pathways (miRNA-mediated gene silencing is discussed in the next section). Upon recognizing complementary mRNA, activated siRISC forms an effector complex with the target mRNA[74]. Antisense siRNA in RISC serves as a guide for AGO2 (REFS 75–77) to catalyse the cleavage of target mRNA at a site that is ~10 nt from the 5′ end of the siRNA[78]. Following cleavage, the target mRNA is degraded. RISC is recycled to cleave additional mRNA targets (FIG. 1).

Kinetic checkpoints in RISC assembly and function. RISC assembly and function can be divided into at least two catalytically controlled steps, and for simplicity only two kinetic checkpoints are considered — RISC loading, and target recognition, cleavage and product release (FIG. 5). As the mechanisms of RISC function become fully understood, each kinetic checkpoint might include several specific steps. For example, target recognition, cleavage and product release can be independently investigated by kinetically controlled steps. Similarly, RISC loading can be divided into three steps, including dsRNA binding, guide-strand incorporation into RISC and passenger-strand destruction.

In the first step, RISC loading, the RISC complex is assembled on a 21-nt substrate, and RISC is loaded with the antisense guide strand of siRNA. The catalytic efficiency of the RISC-loading step can be represented by k_{cat}, which is also known as the turnover number of an enzymatic reaction. k_{cat} defines the number of reactions that occur at each active site of the enzyme per unit of time. The k_{cat} for the formation of RISC is denoted as k_2. The second step involves target recognition and cleavage, with k_{cat} represented by k_4. Therefore, we reason that a catalytically exceptional RISC has to achieve high k_2 and $k_4$values.

Thermodynamics of siRNA duplexes. Analysis of siRNA-duplex sequences shows that the thermodynamic stability of their two RNA ends determines which strand is incorporated into RISC[79,80]. A duplex RNA that is less thermodynamically stable in the 5′ region than in the 3′ region of the guide strand would efficiently initiate the directional unwinding activity of RISC from the 5′ end and would incorporate the guide strand into a functional RISC. During the siRISC-assembly process, the passenger strand is destroyed and the removal of this strand facilitates RISC formation[81–83], but destruction of the sense strand is not crucial for miRISC assembly.

Analysis of siRNA sequences and of their correlation with thermodynamic stability and RISC function has led to the development of algorithms to design effective siRNA duplexes[47,79,80,84–89]. As shown in FIG. 5, k_2 can be enhanced by using thermodynamic rules for siRNAs. If the thermodynamics of siRNA duplexes and their incorporations into RISCs were the only factors for effective RNAi in mammalian cells, then all activated RISCs should cleave their target mRNAs with high efficiencies. In reality, however, not all siRISCs that have been efficiently loaded with antisense strands are equally efficient in silencing target genes. Therefore, we postulated that in addition to a high k_2, k_4 is a crucial parameter in achieving efficient gene silencing under physiological conditions. k_4 could be controlled by at least two factors: first, the accessibility of target sites in mRNA, and second, target-cleavage and product-release steps. Therefore, it is important to understand the mechanism (or mechanisms) that is involved in controlling k_4 to silence gene expression from any target mRNA in order to decrease off-target effects and to define physiological roles for the RISC-enzyme complexes.

Comparative analysis of RISC catalysis. To determine the kinetic parameters and sequence requirements for target-RNA cleavage, a minimal human RISC was used[90]. This minimal RISC was assembled and affinity purified *in vitro* by incubating HeLa cell extracts with siRNAs in which the 3′ ends had been conjugated to biotin groups[90]. Results of these studies indicated that product release and conformational transitions in RISC

Argonaute-family proteins
A group of proteins that are characterized by the presence of two conserved domains, PAZ and PIWI. These proteins are essential for diverse RNA-silencing pathways.

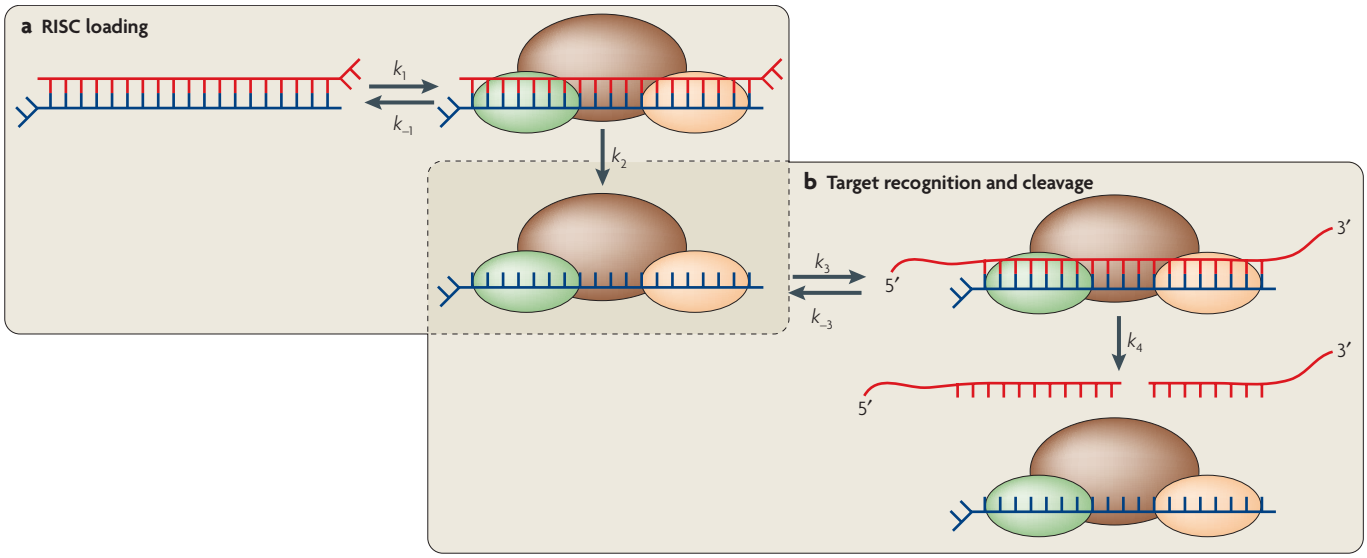

Figure 5 | **Kinetic checkpoints for RISC assembly and function.** RNA-induced silencing complex (RISC) assembly and function can be divided into at least two catalytically controlled steps, and for simplicity only two kinetic checkpoints are considered. **a** | The first kinetic checkpoint involves RISC loading. After Dicer processing of double-stranded (ds)RNA, RISC is assembled on the ~21-nucleotide (nt) RNA helix, the helix is unwound, the passenger strand is destroyed[81–83], and the guide strand is incorporated into RISC. The catalysis of RISC loading is governed by k_2, which can be influenced by the thermodynamics of the RNA duplex[47,79,80,84–89]. **b** | The second kinetic checkpoint involves target recognition and cleavage. RISC finds its mRNA target, cleaves the mRNA, and is recycled for other events. The catalysis of target cleavage and product release is governed by k_4, which can be influenced by several factors such as the target structure[92,103,104], structural reorganization of RISC, product release and other facilitators of RISC recycling. Long dsRNA or hairpin RNA, which are Dicer substrates, are better RNA interference agents than 21-nt small interfering (si)RNAs (REFS 18–20; C. Chu and T.M.R., unpublished observations), but the kinetic steps (k_2 or k_4) that are affected by pre-Dicer triggers are not known. dsRNA binding by RISC is denoted by k_1 and RISC binding to the target mRNA is denoted by k_3.

were the rate-limiting steps in enzymatic catalysis. RISC catalysis can involve at least two conformational transitions. First, the active site of AGO2 cleaves the target RNA and makes the target–guide-strand duplex accessible for unwinding activity that is required for product release. Second, the RISC–guide-strand orients itself into a catalytically active complex after the target strand is removed. It is important to note that these analyses of RISC catalysis do not take into consideration the time and conformational limitations that are involved in locating and binding to the correct target RNA *in vivo*.

The best characterized example of holo-RISC is the multiprotein complex in *D. melanogaster* (reviewed in REF. 91). To understand the kinetics of mRNA-target recognition and cleavage by intact holo-RISC, activated siRISC complexes programmed by specific siRNAs were obtained *in vivo* by transfecting cells with siRNAs and preparing cell extracts for biochemical and kinetic analyses[92]. A comparative analysis of catalytic parameters for RISC-enzyme complexes (TABLE 2) showed interesting features that can be used to understand the structure and function of siRNAs, and perhaps to design more effective siRNAs.

The kinetic parameters clearly indicate that holo-RISC (assembled with siRNAs *in vivo*), minimal RISC (assembled *in vitro*) and recombinant RISC are catalytically different enzyme complexes. Minimal and recombinant RISCs have similar K_m and k_{cat} values, but have lower K_m and higher k_{cat}/K_m values than the holo-RISC. If K_m reflects the binding strength of RISC to its target mRNA[46] and if both minimal and recombinant RISCs have similar K_m and higher k_{cat}/K_m values than the holo-RISC, one might ask what factors contribute to holo-RISC having a lower k_{cat}/K_m value compared with the minimal and recombinant RISCs. There are two possible explanations for the greater catalytic efficiency of minimal and recombinant RISCs. First, these enzyme complexes are more efficient than holo-RISC at the product-release step. And second, minimal and recombinant RISCs have more structural flexibility than holo-RISC to accommodate the conformational changes needed for catalytic cleavage of RNA. It is not clear at this point whether the lower efficiency of target cleavage by holo-RISC is due to the presence of additional protein components that are absent in minimal and recombinant RISCs.

Another important finding of these kinetic analyses is that the presence of target mRNA did not significantly influence either the kinetic properties of RISC or the amount of activated RISC produced in HeLa cells. Whether siRISC was programmed by siRNA sequences that targeted cyclin-dependent kinase-9 (*CDK9*) or green fluorescent protein (*GFP*) mRNA, the siRISC displayed similar catalytic parameters (K_m, V_{max} and k_{cat}). Quantification revealed cellular concentrations of siRISC between 2.5 and 3 nM, which is in agreement with previously estimated concentrations of *let7 D. melanogaster* siRISC[46] and miRISC[93], using the same

Table 2 | **Kinetic analysis of RISC enzymes**

RISC	K_m (nM)	V_{max} (nM s^{-1})	[RISC] (nM)	k_{cat} (s^{-1})	k_{cat}/K_m (nM^{-1} s^{-1})	Reference
Human holo-RISC–*CDK9*	15.42	3.47×10^{-3}	2.5	1.39×10^{-3}	9.0×10^{-5}	92
Human holo-RISC–*GFP*	14.32	3.52×10^{-3}	3.0	1.17×10^{-3}	8.2×10^{-5}	92
Human minimal RISC	2.3	7.1×10^{-3}	0.4	17.9×10^{-3}	7.8×10^{-3}	90
Human recombinant RISC	1.4	1.3×10^{-3}	0.15	8.7×10^{-3}	6.2×10^{-3}	134
Drosophila melanogaster let7	8.4	7.1×10^{-3}	1.0	7.1×10^{-3}	8.4×10^{-4}	46
Human RNase H	38	Not reported	N/A	5×10^{-2}	1.3×10^{-3}	135

CDK9, cyclin-dependent kinase-9; *GFP*, green fluorescent protein; N/A, not applicable; RISC, RNA-induced silencing complex.

quantification methods. *CDK9* siRNA can knock down endogenous *CDK9* mRNA with ~90% efficiency[94], and cytoplasmic extracts that contained 2.5-nM RISC possessed similar kinetic properties as *GFP* siRISC, indicating that once RISC is programmed by a given siRNA sequence it can go through multiple rounds of catalysis *in vivo* and remain active *in vitro* without compromising its catalytic efficiency[92]. These results indicate that siRISC has a 2–4-day half-life as an assembled active enzyme complex.

How does human holo-RISC compare with other enzyme complexes, and what can be learned about the possible mechanism (or mechanisms) of RISC function? A detailed kinetic study using *D. melanogaster* embryo lysates and *let7* siRISC reported a V_{max} of 7.10×10^{-3} (REF. 46), which is remarkably similar to that of human minimal RISC (TABLE 2). Comparing the K_{cat}/K_m ratios for human minimal, recombinant or holo-RISC and that for *let7* siRISC from fruitfly extracts clearly shows that the fruitfly RISC enzyme has a greater catalytic efficiency than human holo-RISCs. Because the differences in K_m do not account for the enzymatic efficiencies of human and fruitfly RISC, it is conceivable that the product release or conformational changes that are required for target cleavage in human holo-RISC-enzyme complexes are slow processes. Interestingly, minimal human RISC binds a 21-nt substrate with a K_m of 1.1–2.3 nM and cleaves its target with a K_{cat} of 1.7×10^{-2} s^{-1} (REF. 90). The lower K_m and higher K_{cat} values of minimally active RISC compared to those of human holo-RISC could be due to the use of a small 21-nt substrate instead of a longer capped and polyadenylated RNA target[92].

miRISC assembly and function

miRNAs are assembled into miRISCs that contain Dicer, Argonaute proteins, TRBP (HIV-1 transactivation responsive element (TAR) RNA-binding protein) and the dsRNA-binding protein PACT (BOX 2). The assembly of these components into miRISC has been implicated in miRNA functions[4,18,95–98]. As mentioned above, miRNAs can function like siRNAs and induce mRNA cleavage when the miRNA sequence is completely complementary to its target mRNA[11,51,52].

The two crucial steps in miRISC assembly are the selection of the miRNA sense or antisense strand for miRISC, followed by the potential destruction of the other strand. The mechanisms of these two steps are not completely understood. It is also not clear whether the sense strand is destroyed in all species. miRISC that is assembled with the guide strand binds to the target mRNA in its 3′ UTR to block translation. Translation inhibition by miRISC raises two basic questions. First, how much complementarity between the guide strand and target site is required for gene silencing? Second, how many miRISCs are needed to inhibit translation?

Complementarity between guide strand and target. Based on our understanding of target cleavage by siRISC, as discussed above, it is reasonable to propose that at least one helical turn of A-form RNA (which corresponds to 11 bp) is required for high-affinity target recognition and binding of miRISC. The exact position of this helical turn is flexible in the ~22-nt guide strand in miRISC. In addition, there must be a bulge in the region of the tenth nt from the 5′ end of the guide strand to avoid target cleavage. If the complementarity is less than 11 nt, then the affinity of miRISC for its target and the selectivity of gene suppression would be compromised.

However, Doench and Sharp[99] reported that the ability of miRNA to suppress translation largely depends on the binding free energy between the first 8 nt of the 5′ end of miRNA and its target mRNA. In light of this finding, one could imagine that an miRNA would suppress the 3′ UTRs of a large number of genes. Indeed, recent microarray-analysis studies show that miRNAs can downregulate many target mRNAs containing sequences that are complementary to positions 2–7 of the 5′ end of the miRNA, known as seed sequences[100]. This lack of specificity in gene silencing by miRNA could be viewed in two ways: as an intrinsic problem associated with miRISC function that is harmful to cells under certain conditions, or as an important switch to control tissue-specific populations of actively translated mRNAs. In support of the first view, siRNAs have recently been shown to cause widespread off-target gene silencing, which was mediated by seed-sequence complementarity between the guide strand and the target mRNA[67]. Remarkably, incorporation of a 2′-*O*-methyl group at the second position of the guide strand reduced most off-target gene-silencing effects of siRISC[72].

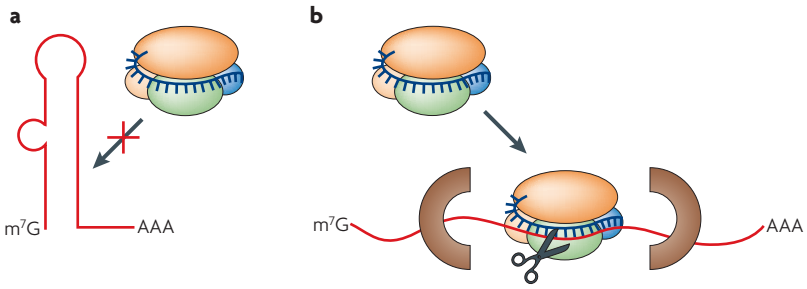

Figure 6 | **Mechanism of RISC–target interactions.** Activated RNA-induced silencing complex (RISC) primed with guide-strand RNA finds and cleaves accessible targets with higher efficiencies than targets that are inaccessible due to their secondary structure or protein binding[92,103,104]. **a** | A stable stem–loop RNA secondary structure prevents access to RISC. HIV-1 transactivation responsive element (*TAR*) mRNA target is not efficiently cleaved by RISC that has been assembled with a *TAR* siRNA. **b** | Disrupting the stem–loop structure enhances RISC access. RISC probably finds its target by a diffusion-control mechanism and cleaves target mRNA efficiently when the target site is accessible. 2′-*O*-methyl oligonucleotide clamps (in brown) make the *TAR* mRNA structure accessible for RISC binding and block RISC from scanning the target-mRNA sequence.

How many miRISCs are needed to inhibit translation? The second question could be addressed by considering the affinity of miRISC for its target. Evidence supports the notion that several miRNA target sites for high-affinity miRNA–target interactions are required for translation suppression[40]. It is plausible that an miRISC that recognizes an imperfectly matched target with only 7–9-nt complementarity between the guide strand and the mRNA would bind the target with low affinity. So, multiple copies of miRISC could bind the target and compensate for the low-affinity miRISC–mRNA interaction and could initiate an oligomerization event that sequesters the whole RNP and transports it to P-bodies for mRNA degradation (FIG. 2). This sequestration could also explain the cooperative effects of RISC function that enhance translation repression[10]. The minimal binding affinity of miRISC that is needed to silence the expression of a specific gene remains to be determined.

Target accessibility and RISC efficiency

AGO2 has recently been identified as the catalytic engine of RNAi[75,76,101]. The 2.25-Å resolution crystal structure of the Argonaute protein from *Pyrococcus furiosus* shows a crescent-shaped base that is formed by the N-terminal, middle and PIWI domains[77]. The PIWI domain of the Argonaute protein resembles the ribonuclease RNase H[77]. In addition to being structurally related to an Argonaute protein, RNase H produces cleavage products with 3′-OH and 5′ phosphate groups that are similar to the products of mRNA cleavage by RISC[90,102]. Therefore, the catalytic parameters of RISC can be compared with those of human RNase H (TABLE 2), showing clearly that RNase H is 10–20-fold more efficient compared with human RISC. During RNase H catalysis, the enzyme binds and cleaves the target duplex structure, whereas RISC carries a guide-strand RNA that must first recognize and bind the correct target before cleavage.

The influence of target-site accessibility on siRISC catalysis was recently examined in three studies[92,103,104]. In the first study, Schubert *et al.*[103] constructed the target site upstream of *GFP* mRNA and designed the site to be either accessible or embedded in a stem–loop structure. Quantification of reporter-gene knockdown showed that the accessibility of the target site was clearly correlated with RNAi efficiency. When the target site could adopt a stable stem–loop structure, RNAi activities were drastically reduced[103].

In the second report, Overhoff *et al.*[104] designed a large set of siRNAs to target *ICAM1* and *survivin* mRNAs. Target-mRNA regions were classified into accessible and inaccessible structures by iterative computational methods and experimentally confirmed by RNase H mapping[104]. RNAi efficiencies of various siRNAs were analysed in cell culture by measuring the target knockdown and calculating the half-maximal inhibitory concentration (IC_{50}) values[104]. Interestingly, when siRNAs were used against accessible target sites, target suppression was enhanced with much lower siRNA doses[104].

In the third study, Brown *et al.*[92] took a biochemical approach and used the mRNA for HIV-1 TAR as a target for RISC cleavage. *TAR* RNA has a stable, stem–loop structure[105] that is not accessible for cleavage by RISC that has been programmed with a *TAR* siRNA, limiting the efficiency of target cleavage (FIG. 6a). siRNA complementary to the stem region (33–51 nt) of *TAR* was designed based on the hypothesis that this double-stranded stem region would hamper the binding of siRISC to the target site. As expected, cytoplasmic extracts prepared from HeLa cells programmed with *TAR* siRNA exhibited minimal target-cleavage activity (~2%) when monitored *in vitro* using a capped-*TAR* target RNA substrate[92]. To enhance target-site accessibility, the secondary structure of *TAR* was disrupted by annealing 20-nt 2′-*O*-methyl oligonucleotides that were complementary to the 5′ or 3′ regions of the siRNA-binding site of *TAR* (FIG. 6b). These 2′-*O*-methyl oligonucleotides clamped the RNA, interrupting the secondary structure of *TAR* and increasing target-site access. The efficiency of *TAR* cleavage by RISC was measured *in vitro* in the presence of one or of two 2′-*O*-methyl oligonucleotides clamps pre-annealed to the target RNA[92]. Strikingly, the cleavage of clamped *TAR* target RNA increased significantly to ~65% in comparison with free *TAR* target RNA.

Altogether, these three studies highlight the importance of target structure in defining the efficiency of siRISC. As mRNAs are large molecules to which structural and regulatory proteins are bound, it is challenging to develop computational approaches to reliably predict accessible sites for siRISC cleavage. As more data become available from genomic and proteomic analyses of the composition and structure of RNPs, algorithms could be developed in the future to predict accessible sites in mRNAs.

RISC–target interactions

RISC might find its target by two possible mechanisms. RISC could scan the mRNA for target sites from the 5′ end or the 3′ end through a mechanism similar to that used by ribosomes to locate translation-initiation sites[106]. Or, RISC could find its target by a diffusion-controlled mechanism.

PIWI domain
A conserved domain that is found in Argonaute-family proteins and that has structural similarities to RNase H. At least in some cases, it cleaves the RNA strand of an RNA–DNA helix.

The scanning model was tested by measuring RISC catalysis of target RNA that contained physical blocks of the 2′-*O*-methyl oligonucleotides that flanked the 5′ and 3′ ends of the cleavage site. If RISC adhered to a directional scanning mechanism, at least one or both of the physical blocks would have decreased cleavage efficiency. In fact, these 2′-*O*-methyl oligonucleotides remained bound throughout the catalytic cycle of RISC and enhanced the cleavage efficiency of RISC by making the target accessible[92]. This result argues against a mechanism of target-site recognition in which RISC scans the length of the mRNA, and it favours a diffusion-controlled mechanism in which RISC binds many sites nonspecifically until the correct target site is found.

RISC catalysis that involves a diffusion-controlled encounter between RISC and its target RNA requires finding the correct target and undergoing the conformational changes that are required for the RNA-cleavage function of RISC, both of which would contribute to decreasing its catalytic efficiency. RISC contains an ssRNA that, upon binding to its target mRNA, becomes a duplex A-form helix, thereby changing the conformation of activated RISC. Only the correct structural change in RISC cleaves the target. Given its K_m of 15 nM (TABLE 2), it is likely that RISC binds sequences nonspecifically and dissociates until it finds the correct target sequence. During these collisions, RISC might bind sequences that are not perfectly matched, but that are partially complementary in the seed and central sequences of siRISC.

As only one helical turn of the A-form helix is required for target cleavage[44–46], this model could also explain the mechanism for off-target effects[67,68]. One way to avoid off-target effects would be to interfere with the cleavage reaction of RISC that occurs when only the seed sequence (nt 2–7 of the guide strand) is base paired with the target mRNA. Indeed, Jackson *et al.*[72] showed that 2′-*O*-methyl modification of the siRNA guide strand at position 2 reduced most off-target effects of RISC. Furthermore, the crystal structure of dsRNA in complex with *A. fulgidus* Piwi[70] indicates that the 5′ region of the guide strand provides a nucleation site for AGO2 recognition and that the interactions between the guide strand and AGO2 set the ruler for cleavage of the target RNA (FIG. 4). It is likely that 2′-*O*-methyl modification of the siRNA guide strand at position 2 interferes with the structural rearrangements of AGO2 that are required for RNA cleavage. To better understand the molecular mechanism for reducing off-target effects, we must determine the crystal structure of AGO2 in complex with the siRNA guide strand that is 2′-*O*-methyl modified at position 2.

The little gene silencer that could

The rationale for RNAi-based therapeutic agents is that new siRNAs could be designed to treat diseases by lowering the concentrations of disease-causing gene products. However, the development of such siRNA-based therapies faces two challenges: first, the identification of chemically stable and effective siRNA sequences, and second, the efficient delivery of these sequences to tissue-specific targets *in vivo* with siRNA amounts that can be translated to clinically feasible doses for humans. Recent advances in understanding the rules for chemically modifying siRNA sequences without compromising gene-silencing efficiencies (TABLE 1) have allowed the design and synthesis of therapeutically effective siRNA molecules that can silence target genes *in vivo*[70,71]. Furthermore, *in vivo* delivery of siRNAs and inhibition of various gene functions have recently been achieved by conjugating cholesterol to siRNA[71] or to oligonucleotide inhibitors of miRNA[107], by forming stable nucleic-acid–lipid particles of siRNA[70], and by assembling lipid–siRNA complexes[108,109]. Based on this rapid progress in understanding the structure and function of siRNAs and their applications in disease models, it is likely that RNAi-based therapeutics will become a reality in the near future.

Over the past few years, the field of RNAi has matured directly from its infancy into adulthood. Several fundamental questions have been addressed about the RNAi mechanism and about the structural and functional features of siRNAs. As a result, RNAi has become a standard experimental tool in virtually every molecular cell biology laboratory, and its therapeutic potential is being widely explored. However, some basic questions are still waiting to be addressed. What are the components of human holo-RISC? What are the cellular functions of RISC? Why does RISC bind to and use an RNA helix that is energetically harder to melt than that of a DNA helix? Why does RISC incorporate ~21-nt RNAs into functional complexes if it needs only 8 nt to suppress translation or 11 nt to cleave its target? How do various RNAs in RISC modulate their specificity? This complexity of the RNAi machinery and its specific role in various pathways highlight a fascinating example of how nature deals with the diversity of functions in life.

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