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Delving into the diversity of silencing pathways

Symposium on MicroRNAs and siRNAs: Biological Functions and Mechanisms

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The Keystone Symposium on MicroRNAs and siRNAs: Biological Functions and Mechanisms took place between 28 January and 2 February 2007, in Keystone, Colorado, USA, and was organized by D. Bartel and S. Grewal.

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Introduction

This Keystone Symposium highlighted the diversity of mechanisms by which small interfering RNAs (siRNAs) are generated and regulate gene expression, and the diverse roles that microRNAs (miRNAs) have in development, cell differentiation and the pathogenesis of human diseases. Since the first meeting of this series in 2002, progress in the field has been remarkable. Crucial developments include the elucidation of steps in the biogenesis of repeat-associated small interfering RNAs (rasiRNAs), new ideas about how siRNAs silence gene expression, how miRNAs repress translation, and the importance of siRNAs as tools in biological research and therapeutics.

The discovery of siRNAs, which silence gene expression at the transcriptional and post-transcriptional level in a sequence-dependent manner, has revolutionized the biological sciences. Since their discovery, it has become apparent that various classes of siRNA exist, which differ in their origin and mode of biogenesis. They all associate with proteins of the Argonaute family, which mediate their functions. Multicellular organisms encode several Argonaute paralogues, which fall into two main clades: the Argonaute and the PIWI-like proteins (Carmell *et al*, 2002). The elucidation of steps in the biogenetic pathway of small regulatory RNAs that associate with the PIWI-like proteins was a highlight of this meeting.

'Ping-pong' mechanism for rasiRNA biogenesis

PIWI-like proteins are required for the establishment and maintenance of the germline. In *Drosophila,* these proteins are thought to have a role in silencing retrotransposons and other repetitive genetic elements, thereby preserving the integrity of the genome. Last year, several groups reported that small RNAs associated with these proteins do not have the characteristic 21-nucleotide length of miRNAs or siRNAs, but are 25–30 nucleotides in length (reviewed by Parker & Barford, 2006). Furthermore, the biogenesis of these RNAs is independent of Dicer (Vagin *et al*, 2006), raising the question of how they are generated.

G. Hannon (Cold Spring Harbor, NY, USA) and M. Siomi (Tokushima, Japan) have provided at least a partial answer to this question (Brennecke *et al*, 2007; Gunawardane *et al*, 2007). Both groups have analysed small RNAs associated with the *Drosophila* PIWI-like protein Argonaute 3 (AGO3). Similarly to the two other *Drosophila* PIWI-like proteins—PIWI and Aubergine (AUB)—AGO3 is expressed mainly in the germline, and associates with small 24–27-nucleotide RNAs that are complementary to AUB-associated small RNAs in their first ten nucleotides. The PIWI- and AUB-associated RNAs match the antisense strand of retrotransposons and repetitive

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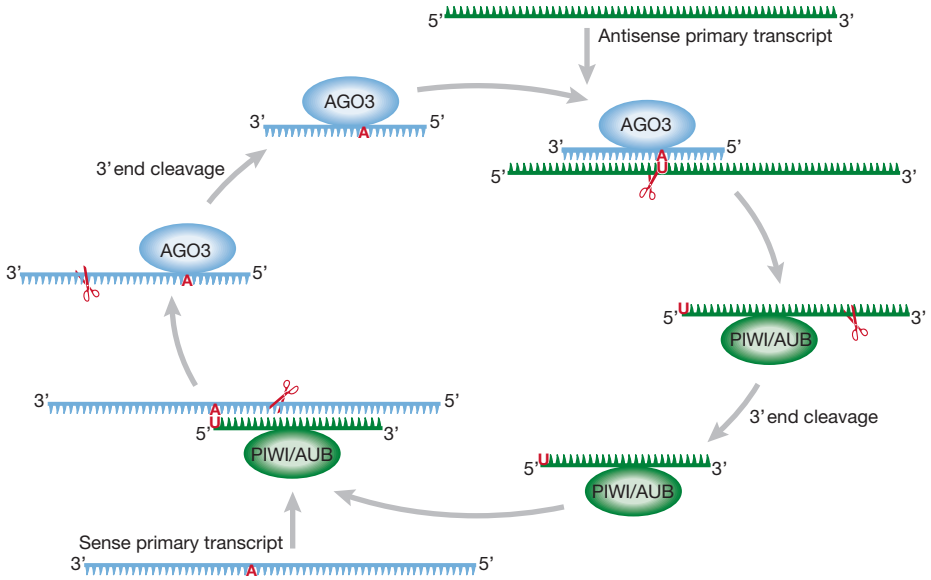

Fig 1 | 'Ping-pong' mechanism for rasiRNA biogenesis. A model showing that the 5' end formation of repeat-associated small interfering RNAs (rasiRNAs) associated with Argonaute 3 (AGO3) and Aubergine (AUB; or PIWI) are mediated by AUB (or PIWI) and AGO3, respectively. The mechanism by which the 3' ends of rasiRNAs are generated is still unknown. Scissors represent endonucleolytic cleavage events. This model is based on the studies by Brennecke *et al* (2007) and Gunawardane *et al* (2007).

sequence elements (therefore referred to as rasiRNAs), whereas AGO3-associated RNAs are derived from the sense strand. PIWI- and AUB-associated RNAs show a strong bias for uracil (U) at their 5' ends, whereas AGO3-associated RNAs show a strong preference for adenine (A) at position 10. Together, these observations suggest an amplification loop mechanism whereby the 5' end of AUB- (or PIWI)-associated RNAs are generated by endonucleolytic cleavage of precursor transcripts guided by AGO3-associated RNAs. Conversely, the 5' ends of AGO3-associated RNAs are generated by endonucleolytic cleavage guided by rasiRNAs associated with AUB (or PIWI; Fig 1). In agreement with this model, Siomi and co-workers have shown that both AUB and AGO3 have slicing activity.

Hannon noticed that although 70% of AGO3-, AUB- and PIWI-associated RNAs in *Drosophila* match retrotransposons and repetitive sequence elements, they originate from a relatively small number of loci in the genome. Evidence that two of the genomic loci from which rasiRNAs are transcribed are indeed important for silencing of transposons originates from the observation that they overlap with previously characterized master control loci of known transposons. This includes the *flamenco* locus on the X chromosome that consists of fragments of transposons—including gypsy, ZAM and others—which are all oriented in the same direction and are probably transcribed as a single long transcript. This transcript could be processed by rasiRNAs originating from transcripts in the reverse orientation.

The observations reported by Hannon and Siomi provide important insights into the biogenesis of rasiRNAs; nevertheless, many questions remain. For example, how are the 3' ends of these RNAs generated? How and when does this cycle start? Is it nuclear or cytoplasmic? Both AUB and AGO3 are predominantly cytoplasmic; by contrast, PIWI is predominantly nuclear, and its expression pattern does not overlap fully with that of AGO3, so how are the 5' ends of PIWI-associated RNAs generated in cells that lack AGO3? Finally, how are retrotransposons and mobile genetic elements silenced?

MicroRNA-target prediction: how many targets are 'real'?

MicroRNAs were an important focus of the meeting. There are at least 100 miRNAs in invertebrates and 500 in vertebrates. Computational predictions of miRNA targets indicate that each miRNA might regulate hundreds of different mRNAs. However, several speakers at this meeting pointed out a potential discrepancy between the number of predicted targets and the observation that abolishing miRNA activity does not always result in an overt phenotype. Indeed, R. Plasterk (Utrecht, the Netherlands) showed that inactivating individual miRNAs in zebrafish using morpholino oligonucleotides often resulted in no detectable phenotype.

V. Ambros (Hanover, NH, USA) showed that although *Drosophila let-7* has many predicted targets, the main phenotype associated with the loss of function of this miRNA can be rescued by reducing the expression levels of a single mRNA target. Flies that lack *let-7* have defective courtship behaviour and do not discriminate between males and females. However, this complex neurological phenotype is restored by reducing the expression of Abrupt, a transcription factor regulated by *let-7*, indicating that misregulation of a single, direct target is responsible for the observed phenotype. Similarly, in *Caenorhabditis elegans,* defects associated with the loss of *lin-4* and *let-7* miRNAs are suppressed by mutations that reduce the expression of single targets—that is, *lin-14* and *lin-41*, respectively.

R. Carthew (Evanston, IL, USA) provided an explanation for the seemingly contradictory observations that although miRNAs have hundreds of targets, loss-of-function mutations in miRNA do not have the expected pleiotropic effects. Studies on the *Drosophila*

miR-7 led Carthew and co-workers to propose that miRNAs are incorporated into robust regulatory networks, and that the loss of one node of the network—for example, the miRNA—has no detectable consequences when flies are grown under standard laboratory conditions. Only when the network itself is impaired would the effects of miRNA loss-of-function be seen (Li & Carthew, 2005).

Another reason why the number of consequential targets for a given miRNA might be smaller than the number of conserved targets is that miRNAs might fine-tune target expression, so their absence results in only minor effects in most circumstances. Finally, miRNA-binding sites might simply be masked by secondary structures or by RNA-binding proteins. R. Schroeder (Vienna, Austria) showed that the accessibility of an siRNA-binding site is crucial for RNA-induced silencing complex (RISC) activity. W. Filipowicz (Basel, Switzerland) showed that binding of a protein known as HuR to the 3' untranslated region (UTR) of cationic amino-acid transporter 1 (CAT1) mRNA during stress conditions relieves *miR-122*-mediated silencing, probably as a result of the displacement of the miRNA (Bhattacharyya *et al*, 2006). Therefore, it seems likely that, in addition to HuR, other RNA-binding proteins are able to counteract miRNA-mediated silencing. If these proteins were to be expressed under specific physiological conditions or in a tissue-specific manner, then immunity or susceptibility to miRNAs could be regulated. This view was further strengthened by data presented by A. Giraldez (New Haven, CT, USA), who showed that silencing of *nanos* mRNA by *miR-430* occurs in somatic cells but not in the germline, and that this effect is mediated by 3' UTR sequences outside the miRNA-binding site. Together, these observations suggest that the presence of a miRNA-binding site is not always predictive of miRNA regulation in the cell type under investigation and that the functionality of miRNA-binding sites should be experimentally validated.

Although, at present, it is not possible to predict the accessibility of miRNA-binding sites *in vivo*, the increasing number of experimentally validated miRNA-binding sites is improving the accuracy of target predictions. D. Bartel (Cambridge, MA, USA) and C. Burge (Cambridge, MA, USA) observed that conserved seed matches are more strongly repressed and are more frequently associated with additional determinants. Bartel found that the position of the miRNA-binding site within the 3' UTR has an important role: sites that are close to the open reading frame or to the 3' end of the transcript are more likely to be regulated than sites more centrally located within 3' UTRs, and sites that are near to each other tend to act cooperatively. Sequence context also influences the likelihood that a site will be regulated *in vivo*: sites that are embedded in A+U-rich sequences are more likely to be regulated. Burge observed that an A or U at position 9 of the target site is over-represented among 'good' miRNA-binding sites regardless of complementarity. The rules that are becoming apparent from these talks are based on the analysis of a large number of human miRNA targets. It would be interesting to know whether they hold true in other organisms such as *Drosophila* in which most 3' UTRs are A+U-rich.

Role of microRNAs in cell differentiation

Several talks addressed the biological role of miRNAs using cellular and developmental genetic approaches in both tissue culture and in living organisms. Some miRNAs have a tissue-specific pattern of expression and this provides a first hint on their potential biological function. P. Sharp (Cambridge, MA, USA) discussed how miRNA expression changes during the maturation of T lymphocytes. These changes can be correlated with stage-specific cell processes, suggesting that miRNAs have important roles in cell differentiation (Neilson *et al*, 2007). In particular, *miR-181* was found to be upregulated in double-positive stage thymocytes. These results are in agreement with the role of *miR-181* in T-cell development as reported by C.Z. Chen (Standford, CA, USA; Chen *et al*, 2004).

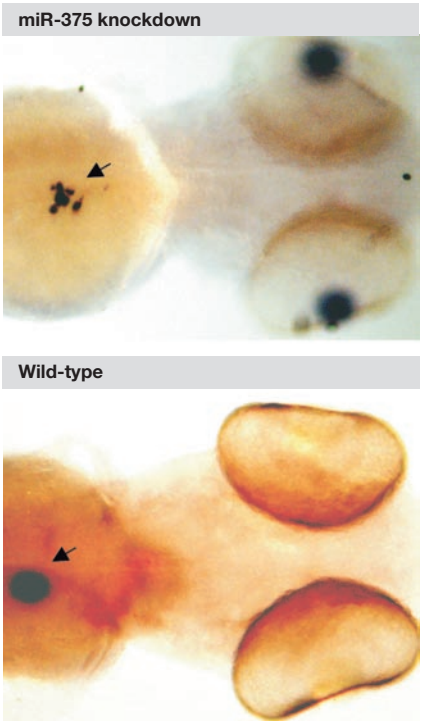

Fig 2 | Role of miR-375 in pancreatic islet cell organization. Knockdown of *miR-375* using a morpholino oligonucleotide complementary to the *miR-375* precursor causes abnormalities (arrows) in pancreatic islet organization in zebrafish. Image courtesy of R. Plasterk and R. Ketting (Utrecht, the Netherlands).

Other ways to elucidate miRNA function is by direct knockout of miRNA genes or by expressing oligonucleotides that interfere with their function. J. Krutzfeldt (of M. Stoffel's group, Zürich, Switzerland) reported a crucial role for *miR-375* in the function of pancreatic islet cells, which secret insulin. For example, newborn mice that lack *miR-375* seem normal, but the adults suffer from hyperglycaemia owing to a decreased pancreatic β-cell mass and an increased number of glucagon-secreting α-cells. The role of *miR-375* in the development of pancreatic islet cells is conserved, as a similar aberrant pancreatic islet morphology was observed by Plasterk in zebrafish embryos injected with morpholino oligonucleotides complementary to the *miR-375* precursor (Fig 2).

Three further studies presented at the meeting illustrate the role of miRNAs in cell differentiation. Sharp showed that knockout of the murine *miR-290–295* cluster leads to embryonic lethality, although some 'escapers' grow to adulthood. Female escapers have no ovaries, indicating an essential role for this cluster in the specification and/or maintenance of the female germline. K. Ivey (San Francisco, CA, USA) found *miR-1* and *miR-133* to have a role in the development

of mesodermal precursors, and A. Dutta (Charlottesville, VA, USA) showed that *miR-206* was required for muscle-cell differentiation in response to serum starvation (Kim *et al*, 2006).

MicroRNAs in human disease

C. Croce (Columbus, OH, USA) emphasized the emerging evidence that miRNAs have important roles in the pathogenesis of several human diseases, including cancers and metabolic disorders. Indeed, many miRNA genes are located in chromosomal regions that are altered in cancer patients. The expression of some miRNAs is reduced in some cancer cells, suggesting that these miRNAs downregulate oncogenes. Croce presented evidence indicating that *miR-15* and *miR-16* act as tumour suppressors in 68% of chronic leukaemias, and that an important target of these miRNAs is B-cell lymphoma 2 (Bcl2; Cimmino *et al*, 2005). A. Krichevsky (Boston, MA, USA) reported that *miR-21* expression is high in solid tumours, and inhibitors of *miR-21* prevent tumour growth (Chan *et al*, 2005). Bartel showed that the loss of *let-7*-directed repression of the high mobility group protein HMG2A oncogene provides a mechanism for transformation (Mayr *et al*, 2007). Therefore, miRNAs regulate various aspects of tumour growth and might represent a new class of therapeutic targets. In addition, changes in miRNA expression profiles correlate with the differentiation state of the tumours, as shown by Croce, suggesting that miRNA profiles could be used for cancer diagnosis.

Mechanisms of microRNA-mediated gene silencing

Despite the remarkable progress we have seen in understanding miRNA function and target prediction, the mechanisms by which miRNAs regulate gene expression remain controversial. Initially, animal miRNAs were reported to repress translation without significantly affecting mRNA levels. More recently, several reports have shown that animal miRNAs can induce significant degradation of target mRNAs (reviewed by Pillai *et al*, 2006). Consistently, transcripts that are upregulated in cells in which the miRNA pathway is inhibited, for example, by depletion of Dicer or Argonaute proteins, are enriched in predicted and validated miRNA targets. Conversely, ectopic expression of specific miRNAs in cells in which they are not normally present leads to a reduction of the levels of transcripts containing binding sites for the miRNA (reviewed by Pillai *et al*, 2006).

Sharp reported that during the maturation of T lymphocytes, upregulation of a given miRNA correlates with the downregulation of transcripts that contain complementary binding sites, again suggesting that mRNA levels are regulated. However, changes in protein and mRNA levels are not always correlated; some targets are regulated mainly at the protein level without detectable changes in the amount of transcript present, whereas others are regulated mainly at the mRNA level. Sharp found that whether miRNAs elicit decay or translational repression depends on the structure of the miRNA–target duplexes (Alemán *et al*, 2007). With a few known exceptions—such as when the miRNA is fully complementary to the target—mRNA decay by miRNAs in animal cells does not occur through endonucleolytic cleavage by the Argonaute proteins, but rather by directing mRNAs to the general mRNA degradation machinery, therefore accelerating their decay (Behm-Ansmant *et al*, 2006; reviewed by Pillai *et al*, 2006).

Although it seems that we are agreed that miRNAs trigger degradation of their targets to differing extents, two questions remain. Is mRNA decay a consequence of translational repression? And, more importantly, how is translation regulated? Recent studies have shown that miRNAs inhibit the initiation of translation, as mRNAs translated through cap-independent mechanisms were shown to escape miRNA-mediated silencing. Other studies have suggested that translation inhibition occurs after initiation, based on the observation that miRNAs and some targets remain associated with polysomes. Furthermore, it has been shown that mRNAs translated through cap-independent mechanisms are subject to miRNA regulation and that regulation occurs by a ribosomal drop-off mechanism (reviewed by Pillai *et al*, 2006).

Two reports at this meeting added a new turn to this already complex picture. M. Kiriakidou (Philadelphia, PA, USA) presented the unexpected observation that the central domain of Argonaute proteins has sequence similarities with the cytoplasmic translation initiation factor eIF4E. This protein binds to the cap structure of mRNAs by stacking the methylated base of the cap between two tryptophans. At the equivalent position of the tryptophans in eIF4E, Argonaute proteins have phenylalanines, which could mediate a similar interaction. Consistently, Kiriakidou showed that human AGO2 binds m^7GTP-sepharose beads and that cap analogues—but not GTP—compete with this binding. Human AGO2 silences the expression of a luciferase reporter when it is artificially tethered to the 3' UTR of the reporter mRNA. When the two phenylalanines are changed to valines, this repression is abolished. Conversely, mutations of phenylalanine to tryptophan, which are expected to increase the affinity of the protein for the cap structure, increased the silencing activity. Kiriakidou's results therefore provide support for a mechanism by which translation is inhibited at the initiation step by displacement of eIF4E from the cap structure.

R. Shiekhattar (Barcelona, Spain) showed that eIF6 and large ribosomal subunits associate with AGO2 in human cells. eIF6 interacts with large ribosomal subunits and prevents their premature association with the small subunit (Ceci *et al*, 2003). Therefore, recruitment of eIF6 by RISC might repress translation at the initiation and/or elongation step by preventing large subunit joining. An explanation for these different models could be that miRNAs regulate gene expression by different mechanisms, although the possibility that some of the discrepancies are due to differences in experimental approaches cannot be excluded.

In contrast to most animal miRNAs, plant miRNAs in general exhibit a more extensive complementarity to their targets and are thought to silence gene expression by promoting endonucleolytic cleavage of their targets in a similar way to siRNAs. O. Voinnet (Strasbourg, France) convinced us that the mode of action of plant miRNAs also includes an important contribution of translational repression to silencing. In a genetic screen designed to isolate genes required for miRNA biogenesis and function in *Arabidopsis*, Voinnet identified mutations that affect miRNA biogenesis, as well as some that affect endonucleolytic cleavage of the mRNA target without affecting miRNA levels. Another class of mutations restored protein expression levels from the reporter, although endonucleolytic mRNA cleavage was still occurring. This indicates that these mutants affect translational silencing, but not mRNA decay by miRNAs. Further analysis of these mutants will certainly provide new insights into how miRNAs silence gene expression.

New regulatory RNAs

The advent of high-throughput and '454' sequencing techniques has boosted the field of small regulatory RNAs. The power of these technologies is particularly impressive when applied to the analysis of

pools of small RNAs present in wild-type backgrounds or in knock-outs of components of silencing pathways. This allows the detection of new regulatory RNAs and also provides important information on their biogenesis. For example, 46 new miRNA hairpins were detected by comparing wild-type and Dicer-knockout embryonic stem cells (Sharp). D. Baulcombe (Norwich, UK) discussed results from a high-throughput sequencing of siRNAs and miRNAs using wild-type and mutant *Arabidopsis* plants. He showed that 90% of plant siRNAs are generated by a plant-specific polymerase—pol IVa+b—and are derived from transposons and retrotransposons. In-depth sequencing analysis also revealed a network of siRNAs in which primary siRNAs lead to the generation of secondary siRNAs. In addition, Baulcombe and co-workers identified miRNAs and miRNA-like RNAs in *Chlamydomonas reinhardii*. Interestingly, they found phased small RNAs in 21-nucelotide increments relative to the start cleavage site, although no recognizable RNA-dependent RNA polymerase has been identified in this organism. J. Carrington (Covallis, OR, USA) reported the identification of 39 new miRNA families in *Arabidopsis* that are not conserved in rice, indicating that these represent new miRNAs (Fahlgren *et al*, 2007).

C. Mello (Worcester, MA, USA) presented results from 454 sequencing of a new class of small RNAs in *C. elegans* that are dependent on the Dicer-related helicase DRH-3. Out of 48,000 RNA molecules, 11,000 were small 22-mers. Of these, 98% started with guanine, approximately 50% hit annotated genes (about 97% antisense and 3% sense) and 50% corresponded to noncoding and repetitive sequences. Therefore, Mello speculated that a significant fraction of the genome is regulated by the silencing machinery.

T. Tuschl (New York, NY, USA) reported sequencing 250 small-RNA libraries from 26 different human organs. 65% of clones in these libraries represent miRNAs. It is estimated that there are 500 miRNA genes in humans, and about 39 of these are not found in any other organism. Carthew reported 101 new miRNAs that are conserved in all 12 *Drosophila* species, and about 212 that are not conserved and are expressed at low levels.

Some general rules can be derived from these genomic studies: ubiquitous miRNAs are generally expressed at high levels, whereas species-specific miRNAs are expressed at low levels, and for some of these, no targets have been identified. The theme that species-specific miRNAs are generally expressed at low levels, belong to single-gene families and have no detectable targets was reiterated several times during the meeting (Carthew and Carrington). N. Rajewsky (Berlin, Germany) proposed that most weakly expressed miRNAs—in particular, lineage-specific miRNAs—have no biological function, and are on an evolutionary path either to generate a new function or to be eliminated. The observation that new miRNAs are expressed at low levels also explains why their acquisition is tolerated, and this in turn favours their evolution (Chen & Rajewsky, 2007).

Diversity of silencing pathways

Silencing pathways have evolved to perform diverse biological functions, some of which seem to be organism- or species-specific. M. Gorovsky (Rochester, NY, USA) reported progress on the elucidation of how the RNA-silencing machinery is involved in germline DNA elimination in *Tetrahymena thermophila*. *T. thermophila* has two nuclei in each cell: a transcriptionally active, polyploid macronucleus, and a transcriptionally silent diploid germline micronucleus. During sexual division, the old macronucleus is destroyed and a new macronucleus is formed from the micronucleus. As part of this process, DNA sequences known as internal eliminated sequences (IESs), which represent 10–15% of the germline DNA, are eliminated. IES elimination requires complementary 28-nucleotide small regulatory RNAs (scan RNAs or scnRNAs), an Argonaute protein (Twi1), a Dicer-like protein DCL1, a DExH-box RNA helicase and additional proteins, some of which have similarities with histone-modification and chromodomain proteins. Gorovsky also identified two other proteins required for this process. These proteins are characterized by the presence of glycine–tryptophan repeats and are therefore reminiscent of GW182 (glycine–tryptophan protein of 182 KDa) and TNRCA–C (trinucleotide-repeat-containing proteins A–C), which interact with the Argonaute proteins in *C. elegans*, *Drosophila* and humans (Behm-Ansmant *et al*, 2006 and references therein).

In *C. elegans,* the Argonaute protein family has expanded with more than 23 Argonaute paralogues, some of which have roles in primary silencing events and others in transitive RNA interference (RNAi; Yigit *et al*, 2006). Transitive RNAi is the phenomenon by which a small number of RNA molecules can silence a gene over more than 80 animal generations, suggesting an amplification of the primary RNA trigger and the generation of secondary siRNAs. Amplification is mediated by an RNA-dependent RNA polymerase but the precise mechanism by which this enzyme operates has remained elusive. Originally it was thought that the primary siRNA acted as a primer to direct the synthesis of a strand complementary to the target. A. Fire (Stanford, CA, USA) and Plasterk showed that this is not the case (Pak & Fire, 2007; Sijen *et al*, 2007). Both groups have characterized secondary siRNAs and have shown that they carry 5' di- or triphosphates, are of antisense polarity and start a few nucleotides upstream of the primary siRNA. On the basis of these observations, they proposed that each secondary siRNA derives from an independent, unprimed RNA synthesis event by the RNA-dependent RNA polymerase. In the current model, primary siRNAs guide the recruitment of this polymerase to target mRNAs. This leads to the *de novo,* unprimed synthesis of antisense RNAs, which are incorporated into silencing complexes by an unknown mechanism.

Finally, silencing pathways are highly elaborate in plants. The *Arabidopsis* genome encodes six RNA-dependent RNA polymerases, four Dicer-like proteins and 10 Argonautes. Baulcombe proposed that these proteins form functional modules, which combine to produce different silencing pathways that might act at the transcriptional or post-transcriptional levels.

So far, *Drosophila* was thought to be exceptionally simple with only two Argonaute-like proteins: AGO1 having a role in the miRNA pathway, and AGO2 acting in RNAi. P. Zamore (Wocester, MA, USA) reported that silencing pathways in *Drosophila* are not as specific as previously thought and that *miR-277* is loaded into AGO2-containing RISC, although it is processed by Drosha and Dicer-1. However, to be loaded into AGO2-containing RISC, Dicer-2 and R2D2 are required. Further analysis showed that it is the structure of the duplex, rather than the biogenetic pathway, dictating with which AGO protein an RNA duplex associates. For example, a central mismatch in the pre-miRNA disfavours Dicer-2–R2D2 binding and hence AGO2 loading, whereas fully complementary RNA duplexes associating with AGO1 will unwind very slowly. Clearly, the challenge now is to determine which endogenous miRNAs associate with AGO2 and which endogenous targets are regulated by endonucleolytic cleavage.

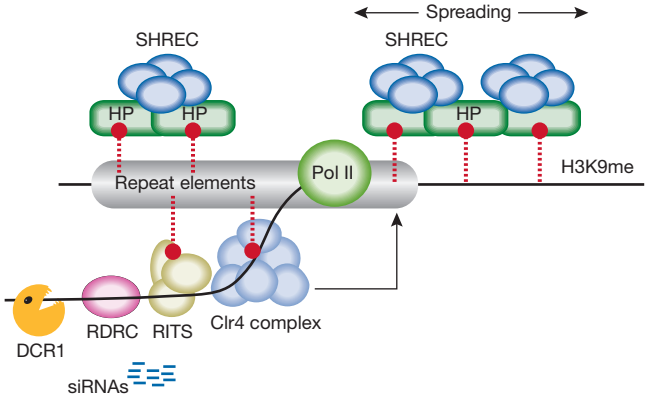

Fig 3 | Role of silencing in heterochromatin formation. The RNA interference machinery, consisting of the RNA-induced initiation of transcriptional gene silencing (RITS) complex, Dicer-1 (DCR1) and the RNA-directed RNA polymerase complex (RDRC), processes transcripts derived from repeat elements into small interfering RNAs (siRNAs). These siRNAs might silence gene expression post-transcriptionally *in cis*. The Clr4 complex methylates histone 3 lysine 9 (H3K9me), which in turn facilitates stable tethering of the silencing machinery to chromatin through RITS and also allows recruitment of heterochromatin-binding proteins (HPs), such as Swi6/HP1. HP binding creates a platform that allows the spread of Snf2/Hdac-containing repressor complex (SHREC) across an entire domain. SHREC can also be recruited to some genomic loci through specific DNA-binding proteins in a process independent of the silencing machinery (not shown). This model is based on the study by Sugiyama *et al*, 2007.

Transcriptional gene silencing

Most of the information we have on how silencing pathways regulate gene expression at the transcriptional level derives from *Schizosaccharomyces pombe* and plants. S. Grewal (Bethesda, MD, USA) previously reported the characterization of the RNA-induced initiation of transcriptional gene silencing (RITS) complex in *S. pombe*, which consists of the Argonaute protein AGO1, Tas3 and Chip1. Grewal showed that RITS associates with heterochromatic regions, and spreads *in cis* in a process requiring heterochromatin-binding proteins (HPs), such as Swi6/HP1 (Fig 3). The *S. pombe* genome also encodes an RNA-directed RNA polymerase RDRC (containing Rdrp1) that, together with components of the RITS complex, localizes in heterochromatic regions of the genome, which are also marked by methylation of histone H3 at lysine 9 (H3K9me) catalysed by the Clr4 complex (Fig 3). Grewal and co-workers have proposed that the RNA-silencing machinery processes transcripts derived from these loci into siRNAs, which might neutralize future invasion by similar sequences. Grewal also described the multi-enzyme effector complex SHREC (Snf2/Hdac-containing repressor complex). This heterotetrameric complex—consisting of Clr1, Clr2, Clr3 and Mit1—mediates heterochromatic transcriptional gene silencing in *S. pombe* by assisting the assembly of higher-order chromatin structures that are crucial for heterochromatin function (Sugiyama *et al*, 2007). SHREC is recruited to various genomic locations through mechanisms involving the RNA silencing machinery and/or sequence-specific DNA-binding proteins (Fig 3).

S. Elgin (St Louis, MO, USA) reported that *Drosophila* HP1a, a protein that localizes to heterochromatin, interacts with the amino-terminal domain of PIWI but not with AGO1–3 or AUB, as shown by a yeast two-hybrid study performed in collaboration with H. Lin (New Haven, CN, USA). HP1 dimerization is required for this interaction. Immunofluorescent staining of polytene chromosomes revealed the presence of PIWI in both euchromatic arms and heterochromatic regions. The heterochromatic staining is sensitive to RNaseH treatment, suggesting that PIWI-associated RNAs might have a role in targeting PIWI to heterochromatic regions through the formation of RNA–DNA hybrids.

In contrast to animals, transcriptional gene silencing in plants involves DNA methylation. *De novo* DNA methylation in *Arabidopsis thaliana* requires components of the silencing machinery including AGO4, Dicer-like-3 (DCL3), an RNA-dependent RNA polymerase 2 (RDR2), and the plant-specific polymerase (pol IVb). M. Matzke (Vienna, Austria) reported the characterization of components of the plant-specific RNA polymerase polIVb. Endogenous targets in *Arabidopsis* were identified and these are mainly in intergenic regions and plant genes located primarily in euchromatin. Matzke proposed a basal inactivation mechanism through this pathway, which can either be reversed to euchromatin or reinforced in heterochromatin.

Conclusions

Small regulatory RNAs are at the forefront of biomedical research. However, many questions remain about their biogenesis, biological function and mechanisms of action. There is no doubt that answers to many of these remaining questions in this fast-moving and dynamic field will be provided in the next meeting of this series, an event to which we can all look forward.

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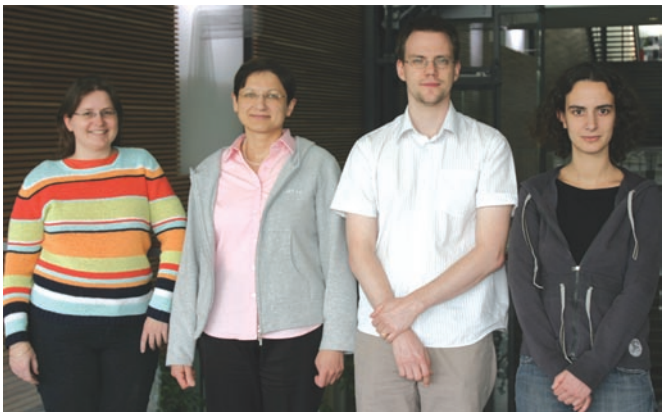

From left to right: Silke Dorner, Elisa Izaurralde, Eric Huntzinger & Ana Eulalio