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Demystifying Small RNA Pathways Meeting Review

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The Keystone Symposium entitled "RNAi and Related Pathways," organized by Craig Mello (University of Massachusetts), Phillip Zamore (University of Massachusetts) and James Carrington (Oregon State University), was held in Vancouver, British Columbia. The meeting participants reviewed recent reports and presented new advances in our understanding of the widespread role of small noncoding RNAs in gene regulation.

The first hints of unconventional homology-mediated silencing phenomena surfaced during the early and mid 1990s in plants, fungi, and nematodes (Guo and Kemphues, 1995; Napoli et al., 1990; Romano and Macino, 1992; Smith et al., 1990; van der Krol et al., 1990). However, it was not until the end of the decade that the mechanism and broad utility of RNA interference (RNAi)-related gene regulation started to be fully realized. The microRNA (miRNA) field also quietly existed as a single worm gene throughout most of the 1990's (Lee et al., 1993). By the turn of the century, though, RNAi and miRNAs had caught the attention of many researchers intent on exploring the broad biological and therapeutic potential of gene regulation mediated by ~22 nucleotide noncoding RNAs. As keynote speaker David Baulcombe suggested, the romance period during the early years of the twenty-first century has now developed into a marriage state for investigators of RNAi and related pathways with the mysteries giving way to tangible complexities. Overlap and redundancy in regulatory mechanisms and small RNA guides, the unknown abundance and types of small RNAs, and expanded RNAi/miRNA factor gene families were repeating themes at the 2006 RNAi and Related Pathways Keystone Meeting. The intertwined nature of different types of small RNA pathways and other forms of gene regulation gave way to diverse topics in most plenary sessions. In this meeting review, I highlight some of the outstanding talks in a thematic rather than chronological order (Figure 1).

RNAi Mechanism

The long sought mechanism of RNAi has matured to the atomic resolution. This process uses small interfering RNAs (siRNAs) to guide *R*NA-*i*nduced *s*ilencing *c*omplexes (RISC) for cleavage of complementary target messenger RNAs (mRNAs). The endonucleolytic cleavage, or "slicing," of the target mRNA usually severs the phosphodiester bond that links the tenth and eleventh nucleotides base-paired to the siRNA. RISC is a multiprotein complex that includes Argonaute proteins at its core. Identification of the RISC factor responsible for slicing target mRNAs was greatly aided by structural studies of Argonaute homologs. The PIWI domain common to all Argonaute proteins contains an RNaseH fold (Parker et al., 2004; Song et al., 2004), and predicted catalytic residues within this motif are required for RISC slicer activity (Liu et al., 2004; Meister et al., 2004). David Barford (Institute of Cancer Research, London) and Dinshaw Patel (Memorial Sloan Kettering Cancer Center) each summarized their insights into the function of the PIWI domain based on impressive independent crystal structures of the *Archaeoglobus fulgidus* Piwi protein bound to an siRNA-like duplex (Ma et al., 2005; Parker et al., 2005). The structures show that the PIWI domain places the 5′ half of the guide for solvent exposure, likely to accommodate target recognition, and positions the scissile phosphate bond between target strand nucleotides 10 and 11 into the nuclease active site. Additionally, unpairing of the first nucleotide of the guide strand and anchoring by PIWI domain residues are important for efficient cleavage activity. These models refine our understanding of the molecular mechanism of RISC activity and may contribute to the design of optimized siRNAs and small molecules that could selectively modulate Argonaute function.

The slicer function of some Argonaute proteins can play dual roles in the RNAi pathway. In addition to cleaving the target strand of an mRNA-siRNA duplex, the discarded passenger strand siRNA can also be sliced in half by Argonaute (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). Mikiko Siomi (University of Tokushima, Japan) and Phillip Zamore (University of Massachusetts) each showed that *Drosophila* Ago2 can cleave the passenger strand of an siRNA duplex and that this event facilitates RISC maturation. It remains to be explained how cleavage of the passenger strand favors its displacement from Ago2.

In *C. elegans* and plants, the potency of RNAi is boosted by the amplification of siRNA guides and the systemic spread of the silencing signal. Craig Mello (University of Massachusetts) presented evidence that the Argonaute member RDE-1 (*R*NAi *d*efective) is required to initiate silencing induced by double-stranded RNA (dsRNA), but other family members perform the downstream job of using amplified secondary siRNAs to maintain the silenced state. Worms have over 25 Argonaute genes, and the heroic creation of strains combining deletion mutations in six of them was necessary to assign some of these genes roles in the RNAi pathway. Interestingly, overexpression of single Argonaute family members rescues the RNAi-defective phenotype of the sextuple mutant, even if the gene lacks catalytic residues important for slicer activity. The issue of whether worms produce typical RISC cleavage products is yet to be resolved. Craig Hunter (Harvard University) updated meeting attendees on the genes that emerged from the clever screen preformed by his lab to identify mutants specifically defective in systemic RNAi in *C. elegans*. The SID-1 (systemic RNA*i* *d*efective) multipass

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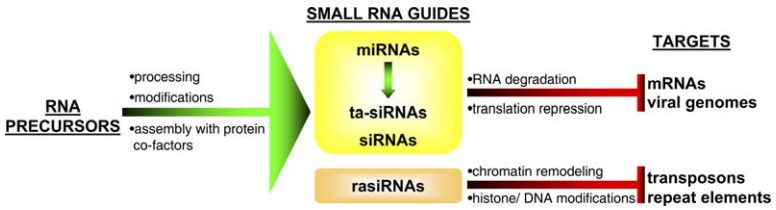

Figure 1. Expression and Function of Small RNAs

Precursors of small RNA guides include miRNA primary transcripts, longer double-stranded RNAs for generating siRNAs, and, in the case of some rasiRNAs, the substrate transcripts have not been characterized. Often multiple processing events and, at least in plants, modifications help generate the small ~22 nt guide RNAs that assemble with protein partners. Some plant miRNAs set the phase for the production of ta-siRNAs. Typically, miRNAs and siRNAs inhibit mRNA and viral gene expression by promoting RNA degradation or translational repression. The rasiRNAs primarily direct silencing of transposons and repeat elements by guiding chromatin remodeling and modifications of histones and DNA at homologous loci.

transmembrane protein appears to mediate both import and export of the systemic signal. Expression of this protein in *Drosophila* S2 cells enables uptake of dsRNAs, preferably 50 base pairs or longer (Feinberg and Hunter, 2003). This observation may be relevant for defining the nature of the endogenous RNAi signal that spreads in *C. elegans*.

Silencing and Defense

The complex interplay between RNAi factors and the viral proteins that evolve to thwart them was a prevalent theme at the meeting. Multiple silencing pathways have been well established in plants as defense mechanisms against viruses. In his keynote address, David Baulcombe (The Sainsbury Laboratory) reported a new mechanism viruses can use to evade silencing. Some luteoviruses encode a silencing suppressor that has E3 ubiquitin ligase activity, which may tag Argonaute proteins for destruction by the proteosome, crippling the plant's RNAi defense mechanism. Olivier Voinnet (Institut de Biologie Moleculaire des Plantes) explained a plant defense mechanism whereby DCL2 (*D*icer *l*ike) can substitute for DCL4 when it is inhibited by the Turnip Crinkle Virus p38 gene product. Additional evidence that variable and often combined Dicer activities are involved in mounting plant silencing defenses supports his proposal that diversification of Dicer proteins in plants may be driven by adaptation to viral suppressors. Herve Vaucheret's (Insititut Jean-Pierre Bourgin) analysis of combinations of Dicer mutations revealed that there are redundancies and, surprisingly, antagonistic effects on small RNA pathways in this expanded gene family in plants. Thus, the dependence on distinct small RNA pathways for gene regulation and viral defense has resulted in expanded numbers and complex interplay for plant silencing factors.

Animal viruses also can foil or utilize RNAi pathways for their own selfish needs. Shou-Wei Ding (University of California, Riverside) demonstrated that the B2 protein of the Flock House Virus (FHV) blocks the RNAi defense systems in *Drosophila* S2 cells and in *C. elegans* (Lu et al., 2005). Additionally, adult flies with mutations in the RNAi factors DCR-2 or R2D2 exhibit enhanced disease susceptibility and shortened lifespans following viral infection, supporting Ding's conclusion that RNAi significantly contributes to innate immunity strategies in invertebrates. Animal viruses can also harness the cellular RNAi machinery to optimize infectivity. Chris Sullivan (University of California, San Francisco) from Don Ganem's lab described the contribution of polyomavirus miRNAs to infection (Sullivan et al., 2005). Murine and primate polyomaviruses express late-phase miRNAs that inhibit early viral transcript accumulation to reduce antigen production and optimize infectivity in animals. Remarkably, the murine polyomavirus encodes miRNA genes that are conserved in function but not in sequence or genomic positions with the primate-like polyomaviruses.

RNAi for Therapy

Taking a cue from endogenous viral defense systems, several groups are developing RNAi strategies to combat viral infections and treat human diseases. Judy Lieberman (Harvard Medical School) presented her lab's recent demonstration that siRNAs targeted against lethal Herpes Simplex 2 Viral genes offer potent protection from infection in mice (Palliser et al., 2006). Respiratory viruses are a major target for RNAi therapies being developed by Antonin de Fougerolles and his group at Anylam Pharaceuticals, Inc. Hope for restoring vision loss due to ocular neovascularization was offered by Pamela Pavco and her group at Sirna Therapeutics, Inc. Injection of siRNAs that target vascular endothelial growth factor receptor 1 (vegfr1) caused downregulation of vegfr1 mRNA levels and significant reduction in neovascularization in a mouse model system (Shen et al., 2006). Rene Bernards (Netherlands Cancer Institute) presented the large-scale RNAi screens his group is using to study cancer-related genetic pathways. A primary goal is to use their short hairpin RNA (shRNA) vector libraries to identify genes whose loss of function makes cancer cells selectively drug sensitive.

Biological Function of miRNA Pathways

In his keynote address, Victor Ambros highlighted the complexities involved in defining biological roles for specific miRNAs. Many miRNAs belong to families that can share functions in convergent, divergent, or linear regulatory pathways. Three different *C. elegans* miRNAs share sequence homology with *let-7* miRNA but exhibit different temporal expression patterns from *let-7* and show functional redundancy among themselves but not with *let-7* in target specification (Abbott et al., 2005). A new role for the founding miRNA gene, *lin-4*, was presented by Frank Slack (Yale University). He showed that *lin-4* miRNA regulates the maturation of a worm neuron and raised the therapeutic possibility that modulating miRNA activity may be a way to reset neuronal identity.

The surprising dearth of phenotypes that often result from loss of a single miRNA can be at least partially explained by the multilayered and compensatory

mechanisms that regulate development. Richard Carthew (Northwestern University) described the complex interplay among transcriptional, posttranscriptional, and posttranslational systems that respond to epidermal growth factor receptor signaling to promote photoreceptor differentiation in the *Drosophila* eye (Li and Carthew, 2005). Mir-7 functions in this pathway, but loss of the miRNA has little negative effect on eye development because the other layers of regulation compensate. The utilization of redundant gene regulatory mechanisms to assure proper developmental programs was also a theme of Eric Lai's (Sloan Kettering Institute) presentation. Ectopic expression of the *Drosophila* miRNA iab-4 induces homeotic transformation of halteres into wings, due to direct downregulation of Ultrabithorax. However, as this miRNA gene appears to be dispensable for viability, back-up mechanisms likely exist to prevent substantial misexpression of iab-4 targets (Ronshaugen et al., 2005).

William Theurkauf (University of Massachusetts) demonstrated the important lesson that phenotypes resulting from defective RNAi factors do not always directly relate to small RNA pathways. Axis specification in *Drosophila* oocytes is regulated by asymmetric RNA localization. This process is disrupted by mutations in the armitage, spindle-E and aubergine genes, which encode helicases and an Argonaute protein important for efficient RNAi (Cook et al., 2004). Surprisingly, second mutations in the ATR and Chk2 kinase DNA damage response genes rescue the asymmetry but not the RNAi defects in all three mutations. Thus, the critical function of the RNAi pathway during oogenesis is to suppress a DNA damage response, which in turn disrupts axis specification.

Cell lineage specification and axis formation in Zebrafish also appears possible without miRNA function (Giraldez et al., 2005). Instead, miRNAs are required to undergo normal morphogenesis. The first detectable miRNA, mir-430, in developing zebrafish was proposed by Antonio Giraldez from Alexander Schier's lab (Harvard University) to play a role in eliminating maternal mRNAs at the mid-blastula transition. Many of the genes upregulated in the absence of mir-430 are maternally inherited and contain predicted mir-430 target sites. Giraldez presented evidence that miRNAs may accelerate the decay of target mRNAs by promoting their deadenylation. Ronald Plasterk (Hubrecht Laboratory, The Netherlands) reviewed some of his group's impressive in situ analyses of miRNA expression patterns in zebrafish embryos (Wienholds et al., 2005) and discussed their progress in defining functions for specific miRNAs. About half of the known zebrafish miRNAs are expressed in brain tissues. Although morpholino knockdown of single miRNAs often produced no detectable phenotypes, this strategy has implicated some miRNAs in specific biological functions, such as touch perception. These talks vividly demonstrated that zebrafish has emerged as a powerful system to understand the function of vertebrate miRNAs.

Biogenesis of miRNAs

In animals, Drosha and its partner DGCR8, also called Pasha, recognize and process miRNA hairpin precursors from longer primary transcripts. V. Narry Kim (Seoul National University) defined RNA features important for this maturation step. Optimal processing requires a lower stem region followed by a single-stranded region at the base of the precursor stem, which includes three helical turns and often is interrupted by central unpaired bases; a terminal loop is apparently inessential. These parameters may aid in the identification of additional Drosha substrates and in the design of siRNA producing vectors.

Modification of plant miRNAs appears critical for their existence. Xuemei Chen (University of California, Riverside) showed that *Arabidopsis* HEN1 encodes a methyltransferase that modifies the 2′ hydroxyl of the miRNA terminal 3′ nucleotide (Yang et al., 2006; Yu et al., 2005). This methyl group may protect miRNAs from a 3′ end uridylation activity (Li et al., 2005). Potential HEN1 homologs exist in animals, but methylation of animal miRNAs has not been reported.

Mechanism of miRNA Function

A common mechanism for how miRNAs function was not resolved at this meeting and, in fact, may not exist. As Victor Ambros pointed out in his keynote address, multiple pathways may be available for miRNAs to effectively inhibit target gene expression. Independent studies by Witold Filipowicz (Friedrich Miescher Institute for Biomedical Research) and David Humphreys from Thomas Preiss's group (Victor Chang Cardiac Research Group) led to the conclusion that miRNA regulation of some targets is through inhibition of translation initiation (Humphreys et al., 2005; Pillai et al., 2005). The mechanism by which miRNA complexes interfere with translation initiation is yet to be elucidated. Filipowicz also presented a specific example of an endogenous miRNA target that appears to be regulated at the translational level. Expression of mammalian cat-1 (*c*ationic *a*mino acid *t*ransporter) is subject to multiple levels of regulation (Hatzoglou et al., 2004), which now appears to include a miRNA pathway. Filipowicz reported that miR-122 inhibits CAT-1 protein expression, but not mRNA stability, in liver cells. Interestingly, miR-122-mediated repression of CAT-1 appears to respond to cellular stress.

The eventual fate of many mRNAs targeted for regulation by the miRNA pathway appears to be degradation. Microarray experiments presented by several speakers confirmed that expression of miRNAs is related to downregulation of many mRNAs with predicted target sites. Additionally, regulation of genetically defined targets of the founding miRNA genes in *C. elegans* involves mRNA destabilization (Bagga et al., 2005). To uncover mechanisms by which miRNA targeting can lead to mRNA degradation, Alan Zahler and Guoping Gu (University of California, Santa Cruz) partially purified a *lin-4* miRNA complex from *C. elegans.* When presented with a substrate resembling its natural partially complementary target, the fraction containing the *lin-4* miRNP exhibited cleavage activity but the RNA products were not characteristic of slicer action. Zahler suggested that a yet to be identified nuclease may be tethered to the target by this miRNP.

miRNAs can guide the generation of other small RNA species. James Carrington (Oregon State University) described an unexpected function for several *Arabidopsis*

miRNAs. These miRNAs guide the cleavage of target transcripts to set the phase for *trans*-acting siRNAs (ta-siRNAs) (Allen et al., 2005). Following cleavage, the ta-siRNA substrates are converted to dsRNAs and processed by Dicer to yield 21 nucleotide ta-siRNAs that then target other genes for negative regulation.

miRNA Targets

Significant percentages of animal genes seem to be direct targets of miRNA regulation. David Bartel (MIT) reported that about one-third of human genes are under selective pressure to maintain pairing with miRNA seed sequences—generally nucleotides 2–7 from the 5′ end of the miRNA (Lewis et al., 2005). Comparison of mouse tissue-specific expression patterns of miRNAs and predicted targets led to the conclusions that conserved targets tend to be preferentially downregulated in miRNA-enriched tissues and that genes highly coexpressed with specific miRNAs are depleted of cognate target sites (Farh et al., 2005). Bartel proposed that the extensive depletion of these sites, which are only 7 nt in length, indicates that such sites are often sufficient for miRNA-directed repression and implies widespread nonconserved targeting. Similarly, Nikolaus Rajewsky (New York University) also reported that a high fraction of the human genome is targeted by miRNAs and that there is an inverse correlation between human tissue-specific miRNAs and target mRNAs predicted by his algorithm PicTar (Krek et al., 2005; Sood et al., 2006). He also presented a computational tool that directly correlates 3′ UTR motifs with changes in mRNA levels assayed by genome wide microarray experiments. To validate predictions made by an updated version of the PicTar algorithm, Rajewsky turned to *C. elegans* as a robust experimental system, and presented a platform to test endogenous 3′ UTR-mediated regulation. At least 10% of worm genes have conserved miRNA target sites. Some of the newly predicted targets of the *let-7* miRNA exhibited reporter gene expression patterns that are consistent with direct regulation by this miRNA (Lall et al., 2006).

In contrast to the apparent broad specificity of animal miRNAs, plant miRNAs are relatively selective of their direct targets. Detlef Weigel (Max Planck Institute) reminded meeting participants that plant targets do include genes with up to five mismatches. Parameters that restrict miRNA-directed cleavage of plant targets include greater than 70% free energy of perfect match, no mismatch at the cleavage site and only one within the seed pairing region, and no more than two consecutive mismatches with the 3′ end of the miRNA (Schwab et al., 2005). The Weigel lab is employing these rules to create artificial miRNAs for targeting specific members of related gene families to study the global effects on gene regulatory networks. He concluded that plant miRNAs have specific effects on a small number of direct targets to orchestrate coordinated responses that are highly connected.

New Small RNAs

Discovery of new small RNAs has skyrocketed in part due to the introduction of "deep sequencing" technologies this past year. In addition to probing the small RNA depths of *Arabidopsis*, David Baulcombe (The Sainsbury Laboratory) also explored Chlamydomonas. His group found evidence of possible miRNAs in this green algae species—perhaps the first demonstration of this small RNA class in a unicellular organism. In *C. elegans*, over 370,000 sequencing reads generated by David Bartel's group (MIT) have revealed new miRNAs and a potentially novel class of small RNAs.

Novel small RNA pathways regulate transposon silencing in *Drosophila*. Phillip Zamore (University of Massachusetts) reported that retrotransposon silencing in *Drosophila* relies on sets of factors and small RNAs that distinguish the mechanism from that utilized by the siRNA and miRNA pathways. Using tiling microarrays, the Zamore group demonstrated that the Y-linked Suppressor of Stellate (Su(Ste)) locus, produces small RNAs of 25–27 nucleotides to silence the Stellate gene on the male X chromosome. Curiously, these repeat-associated RNAs (rasiRNAs) are only generated from one strand in an unphased pattern, which prompts the yet to be answered question of how they are generated.

Transcriptional Silencing

The silencing role of small RNAs is not limited to post-transcriptional regulation of other RNAs. Compelling evidence that small RNAs and the RNAi machinery control DNA and chromatin activities was presented in the final session of the Keystone meeting. Sarah Elgin (Washington University) defined features that initiate heterochromatin formation in *Drosophila melanogaster*. A clever screen for position effect transgene silencing helped uncover the 1360 transposon element on chromosome four that directs heterochromatin formation across distances up to 10 kb away. Both proximity to a copy of transposon 1360 and the RNAi machinery were demonstrated as necessary for efficient heterochromatin formation at a silenced reporter. siRNAs corresponding to the 1360 transposon may provide a local signal for heterochomatin formation.

A role for the RNAi machinery in heterochromatin formation extends to unicellular organisms. Studies in the fission yeast *S. pombe* have revealed that RNAi factors and small RNAs are linked to the recruitment of heterochromatin proteins and histone modifying enzymes at specific genomic loci. Shiv Grewal (National Institutes of Health) showed that RNAi mechanisms and DNA binding proteins are important for establishing heterochromatin at the mating locus (Yamada et al., 2005). He raised the question of how the cell generates transcripts for siRNA production if the homologous locus is thick in heterochromatin. There appears to exist a specialized mechanism that promotes transcription of heterochromatic repeats, which is essential for generation of siRNAs. The siRNAs may provide the inheritable element needed to specify the epigenetic state.

Concluding Remarks

Despite the initial slow momentum in recognizing the existence of small RNAs and their diverse functions, this new field has amassed a faithful and rapidly expanding following of researchers intent on demystifying RNAi and its related pathways. This goal is challenged by the overlapping, redundant, and compensatory features of the biological pathways regulated by small RNAs. Deep sequencing and tiling array technologies indicate

that small RNAs are rich in numbers and types, fulfilling the promise that genomes hold secrets far beyond what we can currently project. Computational methods have proven invaluable for predicting small RNAs and their regulatory targets. Sophisticated bioinformatic approaches, founded on experimental evidence, in combination with rigorous genetic studies will be requisite for guiding our future attempts to assign specific small RNAs and their protein cohorts into complex gene regulatory networks.

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