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RNA silencing in Monterey

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Summary

The tenth annual Keystone Symposium on the Mechanism and Biology of Silencing convened in Monterey, California, in March 2011. Those seeking some West Coast sunshine were, unfortunately, met with incessant precipitation throughout the meeting. Nevertheless, attendees were brightened by enlightening and vigorous scientific discussions. Here, we summarize the results presented at the meeting, which inspire and push this expanding field into new territories.

Key words: microRNA, piRNA, siRNA

Introduction

Central to understanding the process of development is knowledge of how gene activity can be controlled precisely and robustly. Of course, transcriptional regulation is crucial for development, and the functional complexities of DNA-binding proteins, their cofactors, and chromatin structure remain actively pursued. However, the past decade has witnessed a sea change in appreciating the contribution of post-transcriptional gene control by non-coding RNAs. Many advances in this field were first discussed during symposia in the annual Keystone series on RNA silencing, which convened most recently this spring in Monterey. Although the meeting covered various aspects of the non-coding RNA world, emphasis was placed on the microRNA (miRNA), small interfering RNA (siRNA) and Piwi-interacting RNA (piRNA) pathways.

We divide this review into five themes. First, we discuss new mechanistic insights into the biogenesis and post-transcriptional regulation of miRNAs and siRNAs. Second, we report the latest views on the still-controversial topic of how animal miRNAs mediate silencing. Third, we describe new biological functions of miRNAs during animal development and physiology. Fourth, we recount strong progress on elucidating piRNA biogenesis and function. Fifth, we consider the involvement of plant and fungal small RNAs in transcriptional silencing. Together, these talks united researchers in diverse disciplines and model systems, and their collective progress has broad implications for post-transcriptional gene regulation during development and metabolism, its dysfunction during disease and its possible exploitation for therapy.

miRNAs and siRNAs: biogenesis and post-transcriptional regulation

The biogenesis of miRNAs and siRNAs has been well characterized over the past decade (Fig. 1) (Kim et al., 2009). Briefly, primary miRNA transcripts (pri-miRNAs) bearing miRNA hairpins are first cleaved by the nuclear RNase III enzyme Drosha and its cofactor Dgcr8. The resulting pre-miRNA hairpins are exported to the cytoplasm and cleaved by the RNase III enzyme Dicer to generate ~22 nucleotide (nt) duplexes. One strand (the mature miRNA, or 'guide strand') is preferentially loaded into an Argonaute (Ago) protein, which associates with Tnrc6 to yield the RNA-induced silencing complex (RISC). siRNA biogenesis, by contrast, involves cleavage of double-stranded RNAs (dsRNAs) by Dicer and loading of mature siRNAs (guide strands) into Ago2 (Eif2c2), which is specialized for target cleavage ('slicing'). Many talks highlighted outstanding questions regarding miRNA and siRNA biogenesis and regulation (Fig. 1).

Dicer processing

Canonical Dicers have a common architecture that includes a helicase domain, a PAZ domain and two RNase III domains. Studies of *Giardia* Dicer revealed that its PAZ domain binds the RNA 3′ end to position the RNase III domains for substrate cleavage (MacRae et al., 2007; Macrae et al., 2006). However, the role of the N-terminal helicase domain has been less clear. Brenda Bass (University of Utah, UT, USA) reported that the helicase domain of *Caenorhabditis elegans* Dicer (DCR-1 – WormBase) enables it, upon recognition of substrates with blunt termini, to engage Dicer for processive cleavage (Welker et al., 2011). By contrast, RNAs with a 3′ overhang (such as pre-miRNAs) are not cleaved processively and do not require the helicase domain; these data help explain the in vivo importance of the helicase domain in processing nematode siRNAs, but not miRNAs.

Previous work from Narry Kim (Seoul National University, Korea) showed that Dicer-mediated cleavage (referred to as 'dicing') of *pre-let-7* was inhibited by 3′ oligouridylation (Heo et al., 2009). However, her group recently found that human Dicer (DICER1) is not deterred by short 3′ additions. These studies led to the discovery that human Dicer can not only measure cleavage from the 3′ end, but can also recognize the 5′-phosphate to 'count' from the 5′ end (Park et al., 2011). In collaboration with Dinshaw Patel (Memorial Sloan-Kettering Cancer Center, NY, USA) structural studies identified both a 5′-phosphate-binding pocket in the PAZ-containing region of human Dicer, which might serve as an anchor point for measuring duplexes from the 5′ end, and a helical segment implicated in orienting the duplex RNA.

David Bartel (Whitehead Institute, MA, USA) previously reported that some budding yeasts, though notably not *Saccharomyces cerevisiae*, harbor a functional RNAi pathway (Drinnenberg et al., 2009). However, budding yeast Dicers lack most domains found in canonical Dicers, including the PAZ domain. How, then, does this Dicer accurately generate 23-nt siRNAs? Joint efforts by the Bartel and Patel groups showed that multiple Dicer dimers (the functional units of Dicer in budding yeast) bind cooperatively to the interior of the dsRNA substrate, with the distance between active sites of adjacent dimers determining siRNA product length (Weinberg et al., 2011). This 'inside-out' mechanism, starting in the middle of dsRNA and working outwards, differs from the canonical mechanism observed with all other Dicers, which starts at dsRNA ends and works inwards.

Ago loading and activation

Qinghua Liu (UT Southwestern, TX, USA) recently identified an endoribonuclease that facilitates RISC activation by removing passenger-strand cleavage products (Liu et al., 2009). Composed of two subunits, Translin and Trax, he dubbed this complex 'C3PO'

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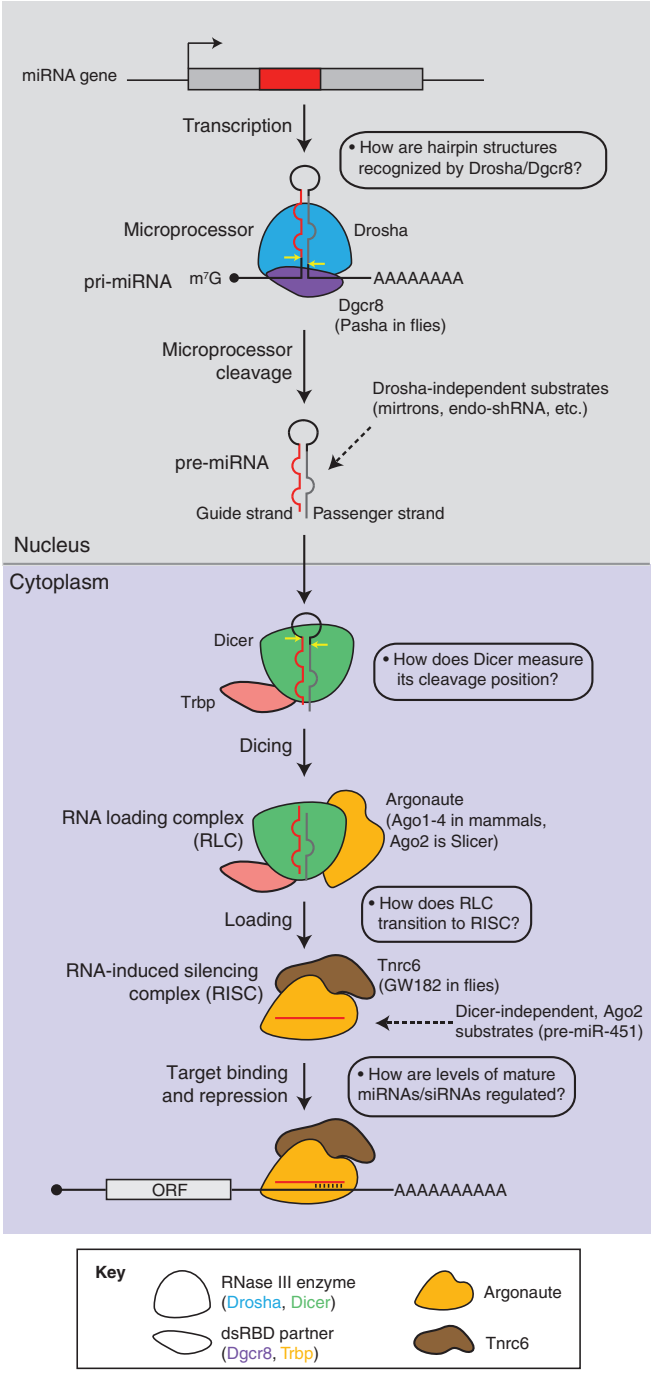

Fig. 1. Animal microRNA biogenesis. Most microRNA (miRNA) genes are transcribed by RNA polymerase II, resulting in the production of a primary miRNA (pri-miRNA). This pri-miRNA is recognized by the 'microprocessor complex' (composed of the RNase III enzyme Drosha and its co-factor Dgcr8) and is cleaved (yellows arrows) by Drosha to give rise to an intermediate called the pre-miRNA. After being exported to the cytoplasm, the pre-miRNA binds to a complex consisting of Dicer (an RNase III enzyme) and Trbp, and is cleaved (yellow arrows) by Dicer to generate a small RNA duplex. The mature miRNA (the 'guide' strand; red) is loaded into Argonaute (Ago) to create a functional miRNA-Argonaute effector complex (RNA-induced silencing complex or RISC). The other strand of the duplex (the 'passenger' or 'star' strand; gray) is degraded. RISC is then guided to its target messenger RNAs (mRNAs) through base-pairing interactions between the guide strand and sequences in the target message, which are found predominantly within the 3′ untranslated region (UTR). The accessory protein Tnrc6 (GW182 in *Drosophila*) mediates interactions with additional proteins to elicit both translational repression and degradation of the target message. Note that some substrates (e.g. mirtrons) generate pre-miRNAs independently of Drosha, whereas some rare miRNAs (e.g. miR-451) are Dicer-independent and mature via Ago2-mediated slicing reactions. Some open questions in the pathway are highlighted in the text bubbles; recent advances that address these questions were presented at the symposium. dsRBD, double-strand RNA binding domain; endo-shRNA, endogenous short hairpin RNA; m^7G, 7-methyl guanosine; ORF, open reading frame; siRNA, small interfering RNA.

(after the chatty Star Wars robot, a nod to their previous designation of the siRNA loading factor R2D2). At this year's meeting, Liu and Patel independently presented C3PO structures. Liu's group crystallized human C3PO and likened it to an elongated Death Star (Ye et al., 2011). Their studies revealed a hollow octomeric complex containing six Translin and two Trax subunits. Patel also observed a central cavity in *Drosophila* C3PO (Tian et al., 2011), half of which he likened to an opened, de-pitted avocado. Intriguingly, the catalytic residues were found on the interior surface, raising questions of how the passenger strand becomes enveloped by C3PO for degradation.

Olivier Voinnet (Swiss Federal Institute of Technology, Switzerland) discussed the transition of Ago from the RNA-loading complex (RLC) into RISC (Fig. 1). Previously, he and Rich Carthew described Ago and its effector partner GW182 (Tnrc6) associating with endosomes and multivesicular bodies (MVBs) (Gibbings et al., 2009; Lee et al., 2009). Voinnet reported that the prion protein (PrP), a known MVB marker, contains extensive GW-repeats resembling the 'Ago hooks' found in other Ago-interacting proteins. Voinnet proposed that PrP directly recruits Ago to the membrane of MVBs. Upon relocalization of Ago and subsequent Tnrc6 binding, a targeting-competent RISC forms. Future studies of the subcellular localization of Ago and its associated proteins should provide more insight into miRNA biology.

miRNA turnover

What determines the fate and longevity of miRNA-Ago complexes? Although most miRNAs seem stable, there is growing interest in unstable miRNAs and the enzymes mediating their decay (Kai and Pasquinelli, 2010; Siomi and Siomi, 2010). Using the cell cycle as a system, Bartel and co-workers identified miR-503 as an unusually unstable miRNA, the levels of which decrease by ~90% during the G0-G1 transition. miR-503 belongs to the extended miR-16 family, several members of which similarly decrease upon this transition. Because this family represses genes that promote the G1-S transition (Cimmino et al., 2005; Linsley et al., 2007), Bartel proposed that high levels of the miR-16 family reinforce G0 arrest, whereas the subsequent downregulation of this family, mediated by the unusual instability of some members, facilitates cell cycle re-entry.

Continuing on this theme, Kim reported that miR-141 is rapidly degraded upon loss of cell contact, and that this instability requires a 3-nt sequence in the center of miR-141. By profiling miRNAs following loss of cell adhesion, her group observed destabilization of other miRNAs known to be involved in the epithelial-mesenchymal transition, suggesting dynamic control of miRNA stability during this process.

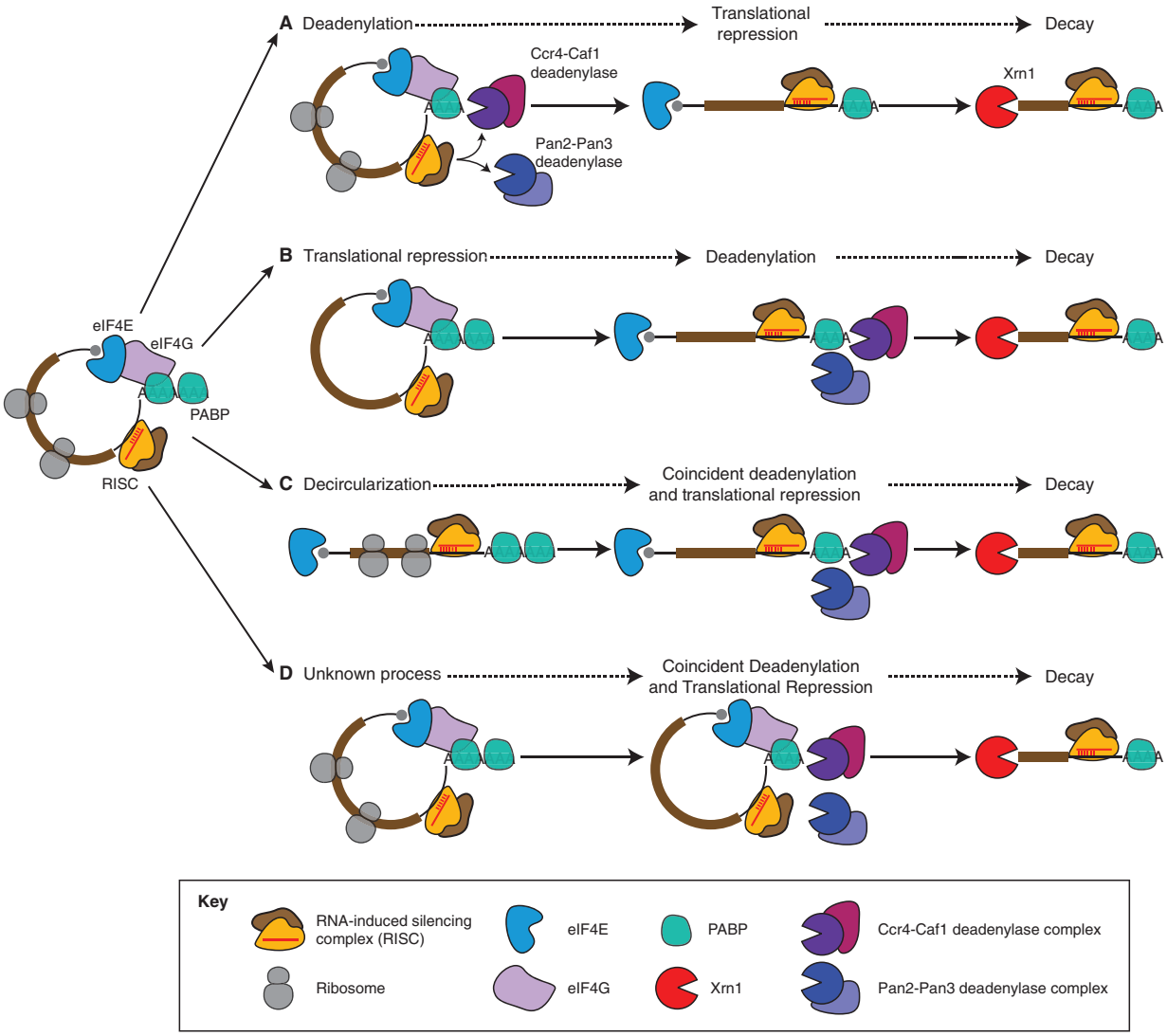

Fig. 2. Proposed mechanisms of microRNA-mediated repression. The majority of microRNA (miRNA)-mediated regulation is due to mRNA degradation. However, the precise mechanism(s) underlying this process remain a topic of lively discussion. Initially, the mature, fully transcribed mRNA is thought be in a circular messenger ribonucleoprotein (mRNP) form composed of, at least, the cap-binding protein (eIF4E), the scaffold protein eIF4G, poly(A) binding protein (PABP), ribosomes and other related proteins not shown here. Once deadenylation, which is mediated by the Pan2-Pan3 and Ccr4-Caf1 deadenylase complexes, has occurred, mRNAs are decapped by the decapping enzyme (not shown) and subsequently degraded by the 5′→3′ exonuclease Xrn1. Current studies aim to elucidate the order and relative contributions of target deadenylation, cap-dependent translational repression and target decay. Evidence for several potential scenarios has been reported, including: (**A**) mRNA deadenylation leading to translational repression and mRNA decay; (**B**) translational repression of target mRNAs leading to deadenylation and decay; (**C**) decircularization of target mRNAs, which then leads to coincident deadenylation and translational repression, and then to decay; and (**D**) an unknown/undiscovered mechanism, which occurs coincidently with deadenylation and translational repression.

Highly stable miRNA-Ago complexes help to protect miRNAs from exonucleases. Ian MacRae (Scripps Research Institute, CA, USA) reported that, surprisingly, in vitro, upon the addition of fully complementary target RNA, guide RNAs were rapidly released from human AGO2 (hAGO2). A few 3′ mismatches inhibited such unloading, thus allowing more Ago-mediated cleavage reactions to be catalyzed in vitro and enhancing silencing in vivo. These results are similar to those from Brian Brown (Mount Sinai School of Medicine, NY, USA), who showed that overexpression of highly complementary targets destabilizes the cognate miRNA (Baccarini et al., 2011). How such unloading interfaces with the trimming-tailing pathway of sRNA degradation (Ameres et al., 2010) remains to be determined.

miRNA-mediated silencing mechanisms in animals

miRNAs act predominantly via target mRNA destabilization, with smaller contributions from translational repression (Guo et al., 2010; Hendrickson et al., 2009) (Fig. 2). Given this, monitoring mRNA changes can identify bulk miRNA targets. Nevertheless, the precise mechanism of repression still eludes the field, and, of all the topics covered at this Keystone symposium, this stimulated the most intense discussions.

Ago cap-binding and allostery

Although it has been established that the Ago MID domain binds to the 5′ end of miRNAs (Hutvagner and Simard, 2008), the hypothesis that it also binds the 5′ cap of target mRNAs has been both

captivating and contentious (Djuranovic et al., 2010; Eulalio et al., 2008; Kiriakidou et al., 2007). Previous work from Rachel Green's laboratory (Johns Hopkins University, MD, USA) suggested that this domain possesses two distinct, and allosteric, sites: one binding the guide strand, the other binding the 5′ cap (Djuranovic et al., 2010). Crystallization of the MID domain of QDE-2 (the *Neurospora crassa* homolog of Ago) provided structural support for a second ligand-binding site (Boland et al., 2010). However, Filipp Frank (Nahum Sonenberg and Bhushan Nagar laboratories, McGill University, Canada) reported that nucleotides and cap analogs readily interact with the hAGO2 MID domain through the miRNA-binding pocket, but that additional nucleotide interactions outside this region were not observed (Frank et al., 2011).

Elisa Izaurralde (Max Planck Institute for Developmental Biology, Germany) presented new work on the MID-PIWI lobe of QDE-2 (Boland et al., 2011). Siwi also stably bound longer substrates with 1U preference. Excitingly, addition of appropriate lysates to these complexes revealed a processive 3′ to 5′ exonuclease ('trimming') activity that resected the 3′ ends of long substrates yielding 27-nt products. Although structurally similar to *Thermus thermophilus* Ago (Wang et al., 2008), QDE-2 contains a eukaryotic-specific C-terminal insertion within the PIWI domain. She proposed that this insertion can sense the functional state of the protein, i.e. whether or not the guide RNA is bound to target RNA in an extended duplex. In this structure, the binding pocket for the guide RNA 5′ end forms at the MID-PIWI interface, as observed in prokaryotic Ago proteins (Wang et al., 2008). However, the previously proposed cap-binding pocket (Boland et al., 2010) is occluded by C-terminal residues inserting into this interface. Finally, this structure demonstrated that residues previously implicated in allosteric regulation (Djuranovic et al., 2010) are involved in MID-PIWI interdomain interactions and facilitate guide RNA binding by stabilizing this interface.

Rachel Green noted that *Drosophila* AGO1-mediated repression in tethering assays correlates with the binding of miRNAs, m^7G-cap resin and GW182 (Gawky – FlyBase), and she re-iterated a model whereby the second binding site might predominantly regulate Ago function allosterically. She presented new work on Ago proteins bearing PAZ mutations or those lacking a PAZ domain; such proteins were surprisingly functional in tethering assays. During his keynote talk, Patel discussed how formation of an extended duplex within *T. thermophilus* Ago requires the release of the guide strand 3′ end from PAZ (Wang et al., 2009). In agreement with this, Green showed that dAGO1 PAZ mutants, which are impaired for 3′ guide strand binding, show increased and promiscuous cleavage activity. Understanding the importance of miRNA-binding, and the role of allostery, for the ability to mediate repression remains an important goal.

miRNA-mediated repression and deadenylation

Many studies have shown that miRNA binding triggers target deadenylation via Ago and GW182 (Tnrc6) complexes (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Lai et al., 1998; Wu et al., 2006). Deadenylation is an initial step in mRNA decay, which is required for both decapping (and subsequent 5′-3′ decay) and 3′-5′ degradation. Deadenylation also inhibits translation, as synergism between the poly(A) tail and the 5′ cap promotes translation initiation. But what is the mechanistic relationship between target deadenylation and translational repression (Fig. 2)? Is this relationship causal and, if so, does translational repression trigger deadenylation or vice versa? Alternatively, do these processes occur coincidently but independently?

Numerous studies have provided evidence for miRNA-mediated inhibition of canonical translation initiation by various mechanisms (Fabian et al., 2010; Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005). Citing the non-physiological nature of such experiments, Bartel discussed the repression of endogenous long, non-coding RNAs (lncRNAs). If deadenylation is purely a consequence of translational repression, then lncRNAs should be refractory to miRNA-mediated repression. However, Bartel reported that targeted lncRNAs are effectively degraded, suggesting that translational repression is not required for deadenylation. Izaurralde supported the view that miRNAs can mediate deadenylation directly, as her group detected interactions between Tnrc6 and the cytoplasmic deadenylase complexes Pan2-Pan3 and Ccr4-Not. Her studies show that Pan3 bound directly to Tnrc6 and suggest that Tnrc6 and the Pan3-Pan2 complex might form a platform onto which the processive Ccr4/Not deadenylase is recruited.

Thomas Duchaine (McGill University, Canada) described a *C. elegans* extract-based system that recapitulates miRNA-mediated deadenylation and translational repression. The observation that the 3′ untranslated region (UTR) influences both the rate and the extent of deadenylation (Wu et al., 2010) led his group to investigate the 'dialogue' between UTRs and miRNAs. They find that certain alternative poly(A) sites (APAs) in *C. elegans* 3′ UTRs can split cooperative pairs of miRNA binding sites, adding a twist to how APAs can modulate 3′-UTR-mediated repression (Mayr and Bartel, 2009; Sandberg et al., 2008). It will be interesting to integrate these insights with recent genome-wide surveys of *C. elegans* poly(A) sites (Jan et al., 2010; Mangone et al., 2010). Nikolaus Rajewsky (Max Delbruck Center for Molecular Medicine, Germany) also investigated the impact of mRNA-protein (mRNP) complexes on RNA regulation, using PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation). This technique was developed to map RNA binding sites in cultured cells (Hafner et al., 2010) and Rajewsky's group has now extended its usage to intact *C. elegans* animals. They also combined data from published CLIP studies and from the UCSC genome browser to generate a convenient portal for integrated database searches (http://dorina.mdc-berlin.de).

The zebrafish embryo is another powerful system for investigating mechanisms of miRNA-mediated repression. Here, maternal mRNAs are deadenylated and cleared upon zygotic expression of miR-430 (Giraldez et al., 2006). Antonio Giraldez (Yale University, CT, USA) profiled ribosome occupancy and total mRNA levels at various time points post fertilization in wild-type embryos and in *dicer* mutants (which lack miR-430). Many of the mRNAs that were repressed in wild-type cells contained miR-430 sites: strikingly, these were first translationally repressed and subsequently degraded. Such results are consistent with Green's kinetic data from human cells, in which translational repression was detected prior to either full deadenylation or reduction in target messages. Although some deadenylation of selected zebrafish transcripts was observed, Giraldez proposed that this was insufficient to explain the observed translational repression. He thus favors a model in which deadenylation and translational repression are coincident processes, perhaps initiated by Tnrc6-mediated mRNA decircularization (Fig. 2).

Biological functions of animal miRNAs

Although studies of miRNAs are aided by genome-wide target predictions and convenient methods for transcriptome profiling, understanding miRNA biology in vivo remains challenging. In contrast to mutants of the founding miRNAs lin-4 and let-7, many

other individual miRNA mutants lack obvious phenotypes (Miska et al., 2007). Instead, mutants of core miRNA pathway components provide a means to assess miRNA requirements broadly. Eric Miska (University of Cambridge, UK) exploited a temperature-sensitive allele of *C. elegans Pasha* (*PASH-1* – WormBase), which encodes an obligate cofactor for Drosha (DRSH-1 – WormBase) (Fig. 1), to inactivate miRNA biogenesis with temporal control. This study revealed that miRNA production is continuously required in adult *C. elegans* for diverse behaviors and lifespan. This mutant also provides a unique background in which differential miRNA stability can be examined in the intact animal.

Victor Ambros (University of Massachusetts Medical School, MA, USA) noted that many miRNA mutants do exhibit phenotypes in genetically sensitized or stressed conditions (Brenner et al., 2010). He found previously that *C. elegans* miRNAs of the let-7 'sister' family function redundantly to control the transition between the L2 and L3 larval stages by repressing hunchback-like 1 (*hbl-1*) (Abbott et al., 2005). Surprisingly, they could bypass the requirement for let-7 sister miRNAs by forcing animals through dauer (a stage of arrested development adopted under harsh environmental conditions), a phenomenon termed 'post-dauer suppression' (PDS). A genetic screen for loci required for PDS revealed NHL-2, which augments let-7 family activity (Hammell et al., 2009), and lin-4, which acts in parallel to let-7 sisters in repressing *hbl-1*. This latter finding was unexpected given that lin-4 is not required per se for the L2 to L3 transition during continuous development. Ambros speculated that other miRNA functions might be revealed under the appropriate sensitization.

The group of Eric Olson (UTSW, TX, USA) deleted ~20 miRNAs expressed in heart and/or muscle. Although these knockouts are all homozygous viable, fertile and of normal morphology, they have been a treasure trove for elucidating essential roles of miRNAs under stressed conditions (Small and Olson, 2011). At the meeting, Olson reported how the dysregulation of miR-378 in skeletal muscle affected glucose uptake: its loss normalized glucose sensitivity in an obesity model and was cardioprotective under a high fat diet, raising the possibility of therapeutic manipulation of this miRNA. Olson also exploited *Mir451* knockouts, which he and other groups found to be relatively normal, except that they were defective in regenerating red blood cells (RBCs) following oxidative stress (Patrick et al., 2010; Rasmussen et al., 2010; Yu et al., 2010). In new work, Olson reported that inhibition of miR-451 reduced hematocrit levels in a mouse model of polycythemia vera, a disease involving excess RBCs. These miRNA inhibitors were well tolerated in wild-type mice supporting their potential therapeutic usage.

Eric Lai (Sloan-Kettering Institute, NY, USA) discussed miRNAs embedded within the *Drosophila* Bithorax-complex (BX-C), which encodes conserved homeodomain ('Hox') proteins that execute segmental identities along the anterior-posterior axis. In both mammals (Mansfield et al., 2004; Yekta et al., 2004) and *Drosophila* (Stark et al., 2008; Tyler et al., 2008), Hox miRNAs strongly target Hox mRNAs, yet changes in segmental identity were not observed in a BX-C miRNA deletion mutant (Bender, 2008). In new work, Lai reported that multiple BX-C miRNA targets were strongly de-repressed in the posterior domains of larval brains that lack BX-C miRNAs. Importantly, heterozygosity of these miRNA targets could rescue the sterility of the BX-C miRNA mutant, establishing them as key target genes in the Hox miRNA network.

Elucidating the mysterious piRNA pathway

The Piwi-interacting RNA (piRNA) pathway generates ~24- to 32-nt RNAs that load Piwi-class proteins in gonads (Siomi et al., 2011). Despite a rich collection of piRNA mutants, our knowledge of piRNA biogenesis and regulatory mechanisms remains rudimentary. Several talks highlighted cell biological, genetic and in vitro biochemical strategies to elucidate the piRNA pathway.

Lysate systems for primary and secondary piRNA biogenesis

Biochemistry was instrumental in the mechanistic dissection of the siRNA and miRNA pathways but, until now, the major insight gained from in vitro studies of piRNAs has been that Piwi proteins exhibit slicer activity, i.e. they can mediate target cleavage. This activity drives a feed-forward loop, termed 'piRNA ping pong', that amplifies piRNAs, yielding pairs that overlap by 10 nt (Brennecke et al., 2007; Gunawardane et al., 2007) (Fig. 3).

Yuki Tomari (University of Tokyo, Japan) described an in vitro system for primary piRNA biogenesis based on *Bombyx mori* (silkworm) Bm-N4 cells. In lysates, Siwi preferentially loaded 27-nt, 5′ monophosphorylated RNAs with 5′ uridine (1U), mirroring the known characteristics of primary piRNAs. Although this doesn't address how long pre-piRNA substrates are initially fragmented, it indicates that piRNA biogenesis need not involve a 5′ U-specific nuclease: Siwi can simply select 1U RNAs. Siwi also stably bound longer substrates with 1U preference. Excitingly, addition of appropriate lysates to these complexes revealed a processive 3′ to 5′ exonuclease ('trimming') activity that resected the 3′ ends of long substrates yielding 27-nt products. These products (but not longer Siwi-bound species) were methylated at their 3′ ends, indicative of genuine piRNA maturation.

Kuniaki Saito, from the laboratory of Mikiko and Haru Siomi (Keio University, Japan), described a complementary Bm-N4 cell-based assay for secondary piRNA production. Saito reported that immunopurified Siwi cleaved a complementary target, yielding an expected 47-nt product that reflected the generation of a secondary piRNA precursor. Strikingly, when performed in lysates, this cleavage reaction produced a 28-nt product, which was interpreted as a mature secondary piRNA generated by piRISC cleavage and subsequent trimming by unknown factors. With these revelations of in vitro piRNA biogenesis, the hunt is undoubtedly on to establish analogous systems from more genetically tractable species, as well as to purify the nucleases and other factors that mediate piRNA biogenesis.

Tudor domain proteins and piRNAs

Several talks highlighted the spatial compartmentalization of piRNA components (Fig. 3), many of which reside in 'nuage'. As Toshie Kai (Temasek Life Sciences Laboratory, Singapore) reminded us, this French term for 'cloud' describes electron-dense perinuclear structures that are a key cellular location for piRNA function. Another relevant cellular structure is the 'Yb body', which was recently inferred to be a location in which Piwi proteins are loaded (Siomi et al., 2011; Szakmary et al., 2009). Many proteins containing Tudor domains localize to nuage or Yb bodies and, importantly, are required for piRNA pathway function (Siomi et al., 2011). Tudor domains contain a conserved structural motif that can bind to symmetrically dimethylated arginines (SDMAs), which are found on most Piwi proteins. Although the functions of most Tudor-containing proteins (TDRDs) are not known, a 'Tudor code' has been postulated to explain how these domains link Piwi proteins to other functional domains on Tudor proteins or associated complexes.

Julius Brennecke (IMBA Vienna, Austria) screened 18 *Drosophila* Tudor genes in the somatic and germline portions of the ovary and focused on functional analysis of Avocado, an MYND-Tudor protein

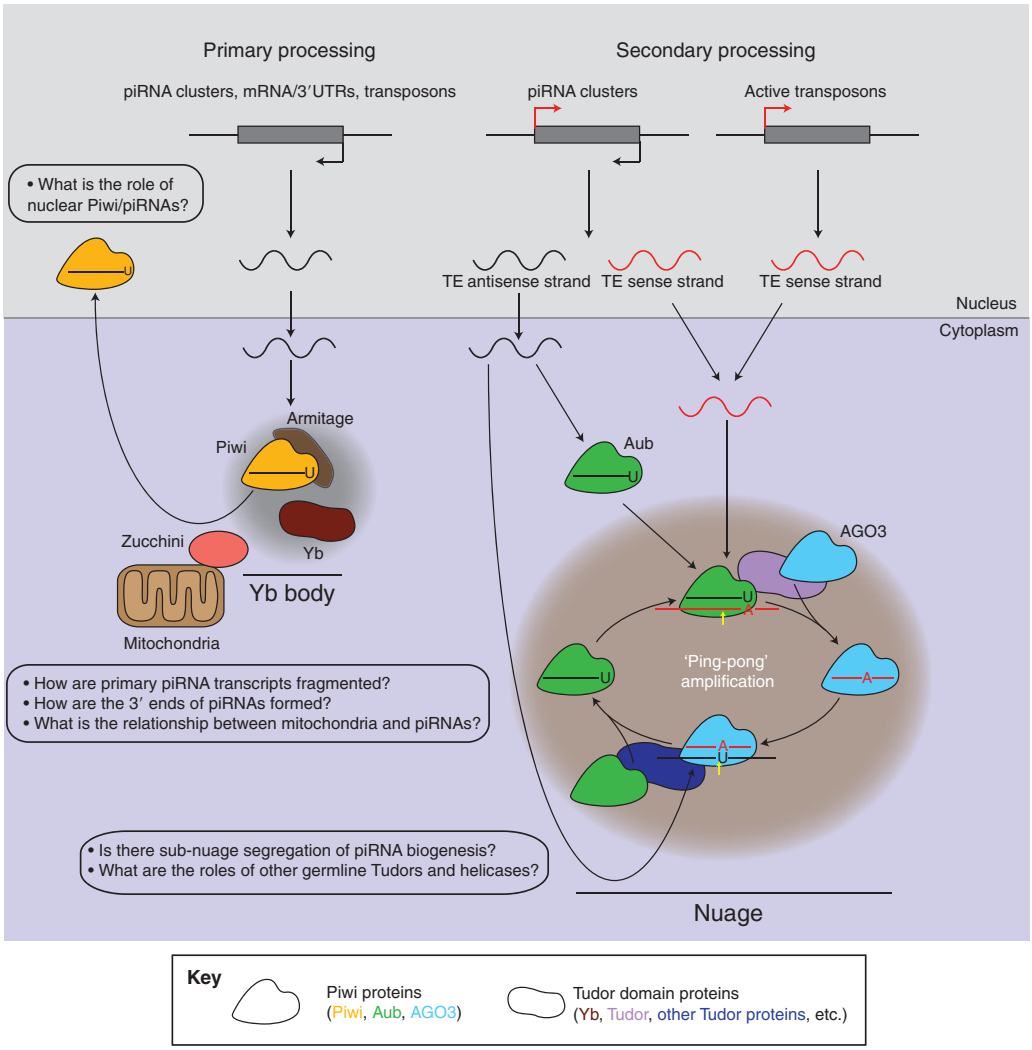

Fig. 3. Biogenesis and function of Piwi-interacting RNAs. The process of Piwi-interacting (piRNA) biogenesis is summarized, and the key regulatory factors and mechanisms involved are shown; for simplicity, only the *Drosophila* orthologs are named. In the primary processing pathway, diverse transcripts, including active transposable elements (TEs), piRNA clusters encoding dead TEs, and 3′ UTRs (untranslated regions) of many mRNAs, are converted into 5′U-biased piRNAs and loaded into Piwi (which associates with the RNA helicase Armitage) in Yb bodies, which exist in proximity to mitochondria. Some Piwi proteins, such as fly Piwi and mouse Piwil4, also accumulate in the nucleus. Primary piRNAs can engage in secondary processing, known as the 'ping-pong' amplification pathway, if antisense counterparts (shown in red) exist, as is the case with piRNA clusters and active transposons. In this pathway, Aub, loaded with primary piRNAs, slices complementary transcripts (as indicated by the yellow arrow) to generate secondary piRNAs. These have an A10 bias, are loaded in AGO3 and can mediate the regeneration of primary 5′U piRNAs. Many 'ping-pong' factors localize to nuage, an electron-dense perinuclear structure that appears to be a key cellular location for piRNA function; additional components include a host of Tudor domain and helicase proteins, not shown. Some open questions in the pathway are highlighted in the text bubbles and progress on these topics was presented at the symposium.

most similar to vertebrate TDRD1. They generated an *Avocado* mutant, which was sterile and displayed upregulation of transposable elements (TEs) in the germline and soma, owing to a defect in primary piRNA biogenesis. Avocado was required for piRNA biogenesis and loading into Piwi, similar to the RNA helicases Armitage and Yb [fs(1)Yb – FlyBase]. In fact, Avocado binds Armitage directly, and both are localized to Yb bodies of follicle cells. Epistasis tests showed that Avocado requires *armi* and *yb* to localize to Yb bodies, whereas loss of the nuclease *zucchini* causes Armitage and Avocado to accumulate in super-sized Yb bodies.

Kai reported mutants of several Tudor proteins, focusing on Tejas (which is most similar to vertebrate TDRD5) (Patil and Kai, 2010), Tapas (which is similar to vertebrate TDRD7) and Kumo (which is similar to vertebrate ring finger protein 17; RNF17). Whereas *Tejas* mutants are sterile and exhibit accumulation of TEs, *Tapas* mutants were fertile with lower levels of TEs. Strikingly, the double mutants exhibited synergistic phenotypes, with nearly complete loss of the ovary within 5-7 days, germarium arrested cysts and increased TE levels. Finally, Kumo might represent one of the most upstream components of the piRNA pathway; Kumo localization to nuage was independent of all known piRNA factors and, reciprocally, the localization of all other piRNA factors was defective in *Kumo* mutants.

Rene Ketting's group (Hubrecht Institute, The Netherlands) found many Tudor proteins in immunoprecipitates of the zebrafish Piwi proteins Ziwi (Piwil1 – Zebrafish Information Network) and Zili

(Piwil2 – Zebrafish Information Network), and used binding assays with SDMA (symmetrically dimethylated arginine) peptides from Piwi proteins to define aspects of the Tudor recognition code. Analysis of Tdrd1 immunoprecipitates revealed short and long RNA populations; the former comprised a mix of mature piRNAs carried in Ziwi and Zili whereas the latter derived from the same genomic regions as mature piRNAs and exhibited signatures of being piRNA targets. This led to a model in which Tdrd1 scaffolds a spatial domain in which Ziwi, Zili and their targets interact. Ketting also reported that Tdrd6 binds specifically to Ziwi and to mainly primary piRNAs, and thus might define a spatial domain relevant to the primary pathway.

Genetic screens for new piRNA factors

A strength of model organism genetics is the capacity to perform unbiased forward screening. Brennecke's group has embarked on a functional genome-wide screen for primary piRNA pathway components using the Vienna *Drosophila* RNAi Center Collection of knockdown transgenes (http://stockcenter.vdrc.at), an ambitious effort poised to yield new factors.

Vasily Vagin (Greg Hannon's laboratory, Cold Spring Harbor Laboratory, NY, USA) screened a collection of female sterile *Drosophila* lines for signs of TE deregulation, and found that loss of a mitochondrial glycerol 3-phosphate O-acyltransferase was detrimental to piRNA-mediated silencing. Encouraged by Hannon to 'make it sexy', Vagin noticed that this mitochondrial enzyme is involved in generating phosphatidic acid (PA). This bears relation to recent reports that MITOPLD, a mammalian homolog of the *Drosophila* piRNA factor Zucchini, localizes to mitochondria, is required for piRNA-mediated silencing and male fertility, and also generates PA (Huang et al., 2011; Watanabe et al., 2011). Vagin speculated on a potential cooperation between metabolic processes generating PA and piRNA biogenesis, and the intriguing possibility that PA might serve as a second messenger that regulates nuage formation and/or piRNA biogenesis.

Possible non-TE functions of the piRNA pathway

Although the major known functional piRNA targets are repetitive elements and TEs, the range of piRNA targets might be broader. For example, the most abundant mammalian piRNAs, expressed in pachytene testis from discrete genomic clusters, do not have obvious targets (Siomi et al., 2011). Similarly, an abundant class of piRNAs derives from the 3′ UTRs of many protein-coding genes in *Drosophila* and vertebrate gonads. These might function as trans-regulatory RNAs, or might exert a cis-regulatory effect on host mRNAs (Robine et al., 2009; Saito et al., 2009).

Anastasios Vourekas (laboratory of Zissimos Mourelatos, University of Pennsylvania, PA, USA) performed high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) on piRNAs and longer RNA fragments associated with the mouse Piwi homologs Mili (Piwil2 – Mouse Genome Informatics) and Miwi (Piwil1 – Mouse Genome Informatics) and the mouse Vasa homolog MVH (Ddx4 – Mouse Genome Informatics). The larger tags comprised many reads mapping to pachytene piRNA clusters, and analysis of Mili large CLIP tags suggested that the larger fragments form by endonucleolytic cleavage and 3′ trimming, in agreement with the lysate-based studies of Tomari and Siomi. Many Miwi large CLIP tags lacking corresponding piRNAs mapped to spermiogenic genes, reminiscent of the hypothesis that Miwi stabilizes these transcripts (Deng and Lin, 2002). However, as most target interactions of Ago proteins are driven by small RNAs, the apparent recruitment of Miwi to spermiogenic transcripts awaits further study.

Martine Simonelig (Institute of Human Genetics, Montpellier, France) reported that piRNA factors are required for timely deadenylation and degradation of maternal transcripts, including the posterior determinant *nanos* (Rouget et al., 2010). Now, she described that, in flies with newly acquired piRNA-mediated immunity to the P element transposon, insertion of a P-piRNA site into the *nanos* 3′ UTR could mediate functional repression of *nanos*. Additional work is needed to determine breadth of piRNA-mediated regulation of mRNAs.

Transcriptional gene silencing in fungi and plants

Although most talks focused on animal systems, RNAi operates much more broadly, and often in ways that are mechanistically distinct between organisms. For example, Kathleen Collins highlighted the fact that various *Tetrahymena* Piwi proteins carry an unexpectedly diverse set of small RNA cargoes (Couvillion et al., 2009), including 3′ fragments of tRNAs (Couvillion et al., 2010). Furthermore, a unique aspect of plant and fungal systems has been the usage of small RNAs to direct transcriptional gene silencing, and we highlight several new findings here.

Plant 24-nucleotide siRNAs

David Baulcombe (University of Cambridge, UK) discussed plant 24-nt siRNAs, which target nascent transcripts and elicit epigenetic modifications of genomic targets. These were implicated in non-cell-autonomous silencing, although definitive proof from *Arabidopsis* grafting experiments has only recently been obtained (Dunoyer et al., 2010; Molnar et al., 2010). Baulcombe now described that in tomato hybrids, 24-nt siRNAs were expressed at much higher levels in hybrids than in either tomato parent, a phenomenon he termed 'transgressive expression'. This expression correlates with target gene silencing and DNA methylation. Moreover, in subsequent hybrid generations, transgressive phenotypes were observed, indicating previously unappreciated functional roles for 24-nt siRNAs in plants.

disiRNAs in *Neurospora crassa*

Yi Liu (UT Southwestern Medical School, TX, USA) recently reported that the *Neurospora* Ago QDE-2 loads small RNAs that are derived from diverse, non-canonical biogenesis pathways (Lee et al., 2010). One such class originated from gene-rich regions that generate siRNAs from both strands, and was unexpectedly generated independently of Dicer. These were thus termed dicer-independent siRNAs (disiRNAs). Their function is not clear but all disiRNA loci checked were cytosine methylated, similar to transposon relics and repeats in *Neurospora*. Surprisingly, methylation at disiRNA loci was reduced but persisted in mutants of all known DNA or histone methyltransferases. Therefore, *Neurospora* disiRNA loci require an unknown nuclease for their biogenesis, and might engage an unknown methyltransferase for their function.

RNAi in *Schizosaccharomyces pombe*

RNAi-mediated transcriptional gene silencing (TGS) has been studied extensively in *Schizosaccharomyces pombe* (Grewal, 2010). Briefly, Dcr1 processes long dsRNA into siRNAs that load Ago1, a component of the RNA-induced transcriptional silencing (RITS) effector complex. RITS is directed to nascent transcripts,

a response that is amplified by the RNA-dependent RNA polymerase Rdp1. Ultimately, the histone methyltransferase Clr4 is recruited and heterochromatic factors such as Swi6 are assembled at silenced regions, including centromeres, telomeres and mating-type loci.

Nick Proudfoot (University of Oxford, UK) earlier reported that loci of convergent transcripts are transiently marked as heterochromatin by RNAi, resulting in cell cycle-modulated gene expression (Gullerova and Proudfoot, 2008). As many RNAi genes are arranged in convergent pairs, does this cell-cycle loop modulate activity of the RNAi pathway? Indeed, 'tandemization' of *ago1* abrogated its cell cycle-dependent heterochromatic marking and regulation, and this induced aberrant mitotic spindles (Gullerova et al., 2011). Proudfoot concluded his talk with unpublished data on TGS in trans, in which convergently transcribed genes expressed from plasmids could silence themselves and endogenous gene copies. Even more tantalizingly, Proudfoot indicated that such a system might also work in human cells.

Marc Bühler (Friedrich Miescher Institute, Switzerland) mapped genomic binding of Dcr1, Rdp1 and Swi6 in *S. pombe* using DNA adenine methyltransferase identification (DamID) (Woolcock et al., 2011). As expected, Swi6, Dcr1 and Rdp1 bound known heterochromatin loci similarly. Surprisingly, Dcr1 (and to a lesser extent, Swi6 and Rdp1) also associated with euchromatic regions. Moreover, although Clr4 is required for accumulation of centromeric siRNAs, the DamID profile of Dcr1 was unaffected in *clr4delta* cells. Bühler hypothesized that although Dcr1 is recruited to chromatin independently of heterochromatin marks, downstream processes, such as Ago-loading, might require histone methylation.

How are the euchromatic loci described by Proudfoot and Bühler, which exhibit transient heterochromatin and subtle RNAi effects, distinguished from canonical heterochromatic loci? Shiv Grewal (NCI, National Institutes of Health, MD, USA) addressed this question through studies of Mlo3, an mRNA export-related factor partially required for centromeric silencing (Zhang et al., 2011). Mlo3 interacts with both the RITS complex (via Clr4) and the nuclear RNA surveillance complex. Grewal proposed that Mlo3 is able to sense aberrant transcripts, which are then either directed to the RNAi pathway or degraded by the exosome, a step presumably influenced by the presence of siRNAs. Curiously, deletion of *mlo3* in RNAi mutants restored silencing and heterochromatin formation, suggesting the existence of a fundamental heterochromatin pathway in which non-coding RNAs can target loci independently of RNAi.

Conclusions

This symposium convened in California, a state whose motto ('Eureka!') is customarily applied to the discovery of gold, but is equally appropriate for the scientific process. As we highlighted above, this annual meeting of the silencing field reliably provides new insights and prompts further questions. The powerful pairing of genome-wide techniques with biochemical, molecular, genetic and structural approaches will undoubtedly maintain progress. The ongoing discovery of processes and relationships, so many of which were unimaginable even last year, highlights the vibrancy of this field and underscores how much remains to be uncovered. Meeting attendees left California, dreaming of the vistas in silencing that are to come.

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Competing interests statement

The authors declare no competing financial interests.

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