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MEETING REPORT

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Todos Santos small RNA symposium

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ABSTRACT

Worm biologists from the United States, Canada, and the United Kingdom gathered at the Colorado State University Todos Santos Center in Baja California Sur, Mexico, April 3–5, 2019 for the Todos Santos Small RNA Symposium. Meeting participants, many of whom were still recovering from the bomb cyclone that struck a large swath of North America just days earlier, were greeted by the warmth and sunshine that is nearly ubiquitous in the sleepy seaside town of Todos Santos. With only 24 speakers, the meeting had the sort of laid-back vibe you might expect amongst the palm trees and ocean breeze of the Pacific coast of Mexico. The meeting started with tracing the laboratory lineages of participants. Not surprisingly, the most common parental lineages represented at the meeting were Dr. Craig Mello, Dr. Gary Ruvkun, and Dr. Victor Ambros, whom, together with Dr. Andy Fire and Dr. David Baulcombe, pioneered the small RNA field. In sad irony, on the closing day of the meeting, participants were met with the news of Dr. Sydney Brenner's passing. By establishing the worm, *Caenorhabditis elegans*, as a model system Dr. Brenner paved the way for much of the research discussed here.

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Introduction

Small non-coding RNAs – microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), and small-interfering RNAs (siRNAs) – are involved in some of the most fascinating biology on earth. While they are well known for their roles in development, transposon silencing, and, in some instances, providing immunity to viruses, small RNAs affect a host of biological processes, from DNA elimination to transgenerational epigenetic inheritance. The tiny roundworm, *Caenorhabditis elegans* has been at the forefront of small RNA research since the discovery of the first miRNA, *lin-4*, in *C. elegans* in the early 1990's [1,2], followed by the discovery of RNAi in the late 1990's [3]. Thus, it is only appropriate that the first Todos Santos Small RNA Symposium (Figure 1) centered on recent progress to understand the incredibly diverse and complex roles of small RNAs in gene regulation and development, using *C. elegans* as a model system. We highlight here the research presented at the meeting, focusing on seven major themes that emerged: 1) miRNA-mediated gene silencing, 2) endogenous and exogenous RNAi pathways, 3) Argonaute proteins, 4) piRNA formation and function, 5) small RNAs in development, 6) germ granules and germline immortality, and 7) small RNAs in parasitic nematodes.

miRNA-mediated gene silencing

The miRNA lin-4 was discovered in 1993 but it wasn't until 7 years later that the second miRNA, let-7, was discovered in worms [4]. However, unlike lin-4 which is worm-specific, let-7 is perfectly conserved across a wide range of animals, including humans [5]. The discovery of let-7 launched the miRNA field, which has since matured and continues to advance our understanding of fundamental processes underlying development and disease [6].

Dr. Amy Pasquinelli (University of California, San Diego) kicked things off with a presentation on her group's efforts to identify miRNA-target recognition rules and particularly the role of compensatory pairing outside of the seed region of the miRNA (positions 2–8) [7]. Although considerable progress has been made over the past two decades, it is still not fully understood what defines a functional miRNA target. It is particularly puzzling why, despite most miRNAs interacting with only partial base-pair complementarity to their targets, some miRNAs, such as let-7 and miR-1, are perfectly conserved across their entire length. Dr. Pasquinelli also discussed her recent work on poly(A) tail length and its impact on mRNA stability and gene expression. Her lab has shown that most mRNAs have relatively short poly(A) tails – 30–90 nucleotides. Interestingly, mRNAs with shorter poly(A) tails tend to be more highly expressed and more efficiently translated than their long-tailed counterparts [8]. She speculated that poly(A) tail length and, hence, poly(A) binding protein (PABP) occupancy may influence the ability of the miRNA complex to regulate a bound target. Dr. Anna Zinovyeva (Kansas State University) presented on her recent efforts to identify factors that modulate miRNA-induced silencing

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Figure 1. Todos Santos small RNA symposium participants gathered at the entrance to the Colorado State University Todos Santos Center. Participants listed from left to right: (back row) Richard Davis, Kailee Reed, Lindsey Winkenbach, Antony Jose, Dustin Updike, Shawn Ahmed, Carolyn Phillips, Martin Simard, Weifeng Gu, Thomas Duchaine, (middle row) John Kim, Amy Pasquinelli, Anna Zinovyeva, Christopher Hammell, Sylvia Fischer, Heng-Chi Lee, Swathi Arur, Dieu An Nguyen, Amy Buck, Wen Chen, Alicia Rogers, Rachel Doser, Julie Claycomb, (front row) Xantha Karp, Elaine Youngman, Scott Kennedy, and Taiowa Montgomery.

complex (RISC) activity. She identified a protein with predicted RNA-binding domains (KH-homology domains) and prion-like domains that interacts with the miRNA Argonaute, ALG-1, and regulates miRNA activity, demonstrating that the molecular mechanisms of the miRNA pathway are still not fully understood more than 25 years after this pathway was first discovered.

Endogenous and exogenous RNAi pathways

In *C. elegans*, environmental or exogenous RNAi (exoRNAi) functions as an anti-viral silencing response. Exogenous double-stranded RNAs (dsRNAs) are processed into primary siRNAs by the ribonuclease Dicer [9]. Primary siRNAs trigger production of what are typically more abundant 22-nt, 5′G-containing secondary siRNAs, called 22G-RNAs, through the activity of a protein complex, the Mutator complex, that contains at its core an RNA-dependent RNA polymerase (RdRP) [10]. Thousands of endogenous mRNAs are also regulated by endogenous small interfering RNA pathways (endo-siRNA pathways) involving 22G-RNAs. Production of 22G-RNAs against endogenous gene targets occurs without the addition of exogenous dsRNA, and instead can be triggered by piRNAs or a 26-nt, 5′G-containing class of primary siRNAs called 26G-RNAs. Endo-siRNA pathways play important roles in germline development and fertility [11].

It is not well understood what marks an mRNA for silencing by endo-siRNA pathways. By examining wild isolates of *C. elegans* for polymorphisms that lead to differences in 22G-RNA levels for target genes, Dr. Elaine Youngman (Villanova University) determined that nonsense mutations can trigger entry of a transcript into the 22G-RNA endo-siRNA pathway, pointing to a link between nonstop mRNA decay and RNAi. Dr. Weifeng Gu (University of California, Riverside) presented on a class of 22G-RNAs produced from 3′UTRs that seem to defy the genetic requirements of known endo-siRNA pathways, suggesting that they may represent a new or hybrid small RNA pathway. Dr. Antony Jose (University of Maryland) described functional mosaicism of exoRNAi. His results indicate that there is considerable variability between cells in the efficiency of exoRNAi. He also discussed work from his lab demonstrating that the RNA-dependent RNA polymerase *rrf-1* is not essential for all somatic RNAi, particularly intestinal RNAi, and that *ego-1*, which is thought to function specifically in the germline, can partially compensate for loss of *rrf-1* activity in the soma.

Dr. Sylvia Fischer (Boston Children's Hospital) discussed the RNA editing genes, ADARs, and their genetic interaction with the MOV10 helicase, *eri-6/7*. The ADAR genes and *eri-6/7* are individually not essential for viability but, surprisingly, disrupting both pathways leads to vulval rupture and reduced fertility. ERI-6/7 functions in the embryonic branch of the 26G-RNA pathway, which seems to compete with other small RNA pathways for components of the RNAi machinery [12]. In the absence of RNA editing, the endo-siRNA machinery appears to mount a hyperactive response to endogenous double-stranded RNA (dsRNA), which is normally edited by the ADARs, leading to imperfect base-pairing within the dsRNA and thus reduced efficiency for processing by Dicer [13,14]. Supporting this model, disrupting the exoRNAi pathway rescues the sterility of *eri-6/7*-ADAR combinatorial mutants. High levels of perfectly base-paired dsRNA in ADAR mutants may siphon factors, such as Dicer, away from the miRNA pathway, which could lead to reduced expression of miRNAs and upregulation of their targets, and possibly causing the developmental defects observed in ADAR, *eri-6/7* mutants.

It is unclear how an mRNA is processed or biochemically modified to serve as a template for the production of siRNAs. Dr. Scott Kennedy (Harvard Medical School) presented new evidence that the nucleotidyltransferase MUT-2, a key component of the Mutator complex, modifies the 3′ ends of mRNA templates with poly(UG) tracks. This so-called pUGylation is required for siRNA production from an exogenous RNAi template and thus likely represents a key step in exoRNAi.

Argonaute proteins

Small RNAs are unified by their association with proteins in the Argonaute/Piwi family. *C. elegans* was initially described as possessing 27 Argonaute genes, spanning three subfamilies: AGOs, PIWIs, and WAGOs [15]. Many of the Argonautes are specialized for one pathway or another and several remain to be characterized. Recently, chemical modifications to Argonautes, such as phosphorylation and methylation, have been shown to affect their activity as well, further adding to the complexity of the system [16,17]. New data presented at the meeting reveals the extent to which Argonaute modification may impact small RNA mediated gene silencing in *C. elegans*.

Dr. Martin Simard (Université Laval) used mass spectrometry to identify 30 phosphorylation sites on the miRNA Argonaute ALG-1 that correspond to conserved residues in human AGO2. Phosphorylation at the C-terminus of ALG-1 appears to be required for binding to target mRNAs. Hyperphosphorylation at the C-terminus was correlated with premature release of mRNA targets by ALG-1 [18]. Phosphorylation of specific residues in the MID domain, which forms a binding pocket to accommodate the 5′ end of a small RNA, alters miRNA binding. Dr. Carolyn Phillips (University of Southern California) is examining the spatial organization of Argonautes within the cell and is particularly interested in Argonautes associated with germ granules. She demonstrated that localization of multiple germ granule-associated Argonautes is driven by their association with small RNAs. She also identified dimethylarginine sites on several Argonautes and is currently exploring whether dimethylation affects their localization to germ granules. The results thus far suggest otherwise.

Dr. Julie Claycomb (University of Toronto) described her ambitious 'Argonomics' project in which her lab has epitope tagged each of the *C. elegans* Argonautes using CRISPR-Cas9 and characterized their small RNA interactors and molecular functions. This is the first comprehensive analysis of Argonaute-small RNA interactions in the worm. Her initial results reveal a surprising degree of promiscuity within the Argonaute family, pointing to considerable fluidity between the different small RNA pathways. Both Dr. Claycomb and graduate student Dieu An Nguyen (Dr. Carolyn Phillips laboratory, University of Southern California) presented on two distinct isoforms of the gene-licensing Argonaute CSR-1 and their different roles in development. The long isoform is restricted to the spermatogenic germline where it seems to have an effect on spermatogenesis genes. Using mass spectrometry, the group of Dr. Thomas Duchaine (McGill University) identified a new family of Argonaute-interacting proteins, called nuclear AGO-interacting proteins (NIPs). As the name suggests, the NIPs interact with the nuclear Argonautes, particularly NRDE-3. Upon loss of some of the NIP family members, somatic nuclear RNAi through NRDE-3 is selectively enhanced. In the absence of other NIPs, mutant worms are defective in transgenerational RNAi inheritance and transposon silencing.

piRNA formation and function

piRNAs are well-known for their roles in defending the genome against transposons and preserving fertility. *C. elegans* produces thousands of piRNAs, each from its own autonomous transcript and, because of incomplete complementarity in target recognition, they have the ability to target multiple mRNAs. Notably, unlike other animals, seemingly few piRNA targets in the worm are transposons [19]. As such, a major focus of the *C. elegans* small RNA field is to identify the roles of piRNAs in gene regulation and development.

Previous work from Dr. John Kim's group (Johns Hopkins University), identified distinct populations of male and female piRNAs in the hermaphroditic worm and that sex-specific expression was driven by a single nucleotide within an upstream regulatory motif [20]. In a new unpublished study, he identified a transcription factor expressed in the male germline that binds the piRNA regulatory motif to promote piRNA expression. A 5′C within the motif seems to promote binding by the transcription factor, which could explain the sex-specific expression within the pathway that he observed in an earlier study. Dr. Tai Montgomery (Colorado State University) described his efforts to identify the roles of piRNAs in regulating germline gene expression using RNA-seq on dissected gonads from wild type and *prg-1* mutant worms. His results suggest that *C. elegans* piRNAs have widespread effects on the transcriptome, including roles in suppressing somatic genes in germ cells and promoting proper histone gene expression. Postdoctoral Fellow, Dr. Alicia Rogers (Dr. Carolyn Phillips laboratory, University of Southern California) described her work identifying somatic gene misexpression in the germlines of *mut-16* mutants grown at elevated temperatures. piRNAs trigger mRNA entry into the 22G-RNA endo-siRNA pathway, for which *mut-16* is an essential factor. Thus, it is possible that the somatic gene misexpression underlying both *mut-16* and *prg-1* mutants has common roots.

Small RNAs and development

Small RNAs were first discovered for their role in regulating developmental timing. The miRNA *lin-4* suppresses the *lin-14* mRNA to promote transition between early larval stages [1,2]. Since their discovery, small RNAs have been implicated in nearly every developmental process [11,21,22]. Thus, it is not surprising that development emerged as a theme of the meeting.

Dr. Swathi Arur (The University of Texas MD Anderson Cancer Center) talked about her work on the link between extracellular signal-regulated kinases (ERKs) and small RNA biogenesis. In previous work, her lab showed that ERK-dependent phosphorylation inhibits Dicer's activity toward 26G endo-siRNAs in the germline and this is required for proper oogenesis [23]. In recent work, her lab discovered that Drosha, another endonuclease involved in processing miRNAs, but not siRNAs, is also regulated by the RAS/ERK signaling pathway. Phosphorylation of Drosha is seemingly important for the onset of embryogenesis, although its specific role and targets are currently under investigation.

Dr. Xantha Karp (Central Michigan University) presented new work exploring the roles of miRNAs in regulating cell fate during dauer, a type of developmental stasis that allows worms to survive when food is scarce. Regulation of the *let-7* miRNA family

by DAF-16/FOXO may have a role in preventing the expression of adult cell fate markers during dauer. Dr. Christopher Hammell (Cold Spring Harbor) introduced recent work in which he identified a prion-like (Q/N-rich) protein with RNA binding activity that modulates translation. Interestingly, depletion of the gene can rescue the development defects associated with miRNA mutants, possibly by suppressing translation of miRNA targets, thereby preventing their overexpression.

Germ granules and germline immortality

Within the germline, much of the small RNA machinery resides in germ granules, RNA-protein condensates adjacent to nuclear pores. In *C. elegans* there are three types of germ granules – P granules [24], Mutator foci [10], and Z granules [25,26], – associated with endo-siRNA pathways. While P granules have been studied for several decades, their specific roles in small RNA biogenesis and function are not well understood.

Previous work from Dr. Shawn Ahmed (University of North Carolina) identified a role for piRNAs in preserving germline immortality [27,28]. In the absence of piRNAs, germ cell atrophy occurs, and sterility gradually ensues over multiple generations. The cause of sterility in piRNA mutants is unclear but new work from the Ahmed lab demonstrating P granule disfunction in piRNA mutants points to disruption of germ granules as a possible cause. Dr. Ahmed also presented on a novel but rare class of siRNAs matching to telomeres.

Dr. Dustin Updike (Mount Desert Island Biological Laboratory) and Dr. Heng-Chi Lee (University of Chicago) converged on remarkably similar findings regarding the *C. elegans* Vasa homolog, GLH-1, which is involved in P granule assembly. Both labs made a number of mutations in core motifs in GLH-1 to identify their roles in P granule assembly and disassembly. Some mutations in the DEAD-box motif completely disperse GLH-1, while others cause P granules to detach from the nuclear periphery and form large, stable aggregates. Deletion of the FG motif of GLH-1 increases granule size while reducing contact at the nuclear periphery. Following a similar experimental trajectory, both labs used mass spectrometry to identify GLH-1 interactors, identifying large sets of overlapping proteins. Given that GHL-1 is key P granule component, these factors are likely to represent a large proportion of the constituents of P granules. Importantly, but perhaps not unexpectedly, several small RNA factors were also identified.

Small RNAs in parasitic nematodes

C. elegans was not the only nematode represented at the meeting. One of the more peculiar roles for small RNAs is in DNA elimination, perhaps the most extreme mechanism of gene silencing [29]. This process is best characterized in ciliated protozoans but also occurs in number of multicellular organisms, including some nematodes [30]. Dr. Richard Davis (University of Colorado School of Medicine) is examining a potential link between small RNAs and DNA elimination in the nematode *Ascaris*, which undergoes developmentally-programmed DNA elimination. Notably, *Ascaris suum* has a similar repertoire of small RNA machinery and pathways as *C. elegans*, although they lack piRNAs [31]. Two WAGO-family Argonautes are differentially associated with chromosomes fragments that will be retained or eliminated suggesting Argonautes and small RNAs play a role in DNA elimination in *Ascaris*, but their roles remain elusive. Dr. Amy Buck (University of Edinburgh) uses a host-pathogen model to study the role of small RNAs in cross species communication. Dr. Buck previously discovered that extracellular vesicles filled with miRNAs are transferred between a parasitic nematode, *Heligmosomoides bakeri*, and its mouse host, where they are detected in intestinal cells and may regulate genes important to enable parasite survival in the gut [32]. In new work, she is characterizing the endogenous and secreted small RNAs of *H. bakeri*. Many of the secreted small RNAs detected in mouse hosts are siRNAs, and not miRNAs, derived from repetitive regions of the *H. bakeri* genome and have sequence complementarity to endogenous host genes, including those associated with nutrient absorption, which is important for proper functioning of the immune system [33]. These tantalizing results suggest that rapidly evolving portions of the *H. bakeri* genome produce small RNAs that could manipulate host gene expression in favor of maintaining a cozy home for the parasite within the mouse intestine [34].

Conclusions

As with any stimulating scientific meeting, friendships blossomed, collaborations were formed, and the abundance of new data raised even more questions than were answered. Future meetings will likely grow to include small RNA biologists outside of the United States and Canada and may even be expanded to include other model systems. The organizers, Tai Montgomery, Amy Pasquinelli, Carolyn Phillips and Julie Claycomb welcome suggestions for Todos Santos, 2021.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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