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meeting report

# The impact of small RNAs

## Microsymposium on Small RNAs

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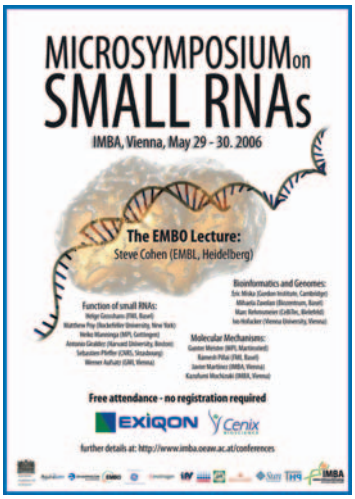


The first Microsymposium on Small RNAs was held at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna, Austria, on 29 and 30 May 2006, and was organized by J. Martinez.

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### Introduction

Our understanding of the RNA-mediated gene-regulatory pathway, known as RNA interference (RNAi), has come a long way since its discovery in the early 1990s. At that time, all knowledge of RNA-mediated silencing was based on one phenomenon: the loss of a non-lethal phenotype after the introduction of exogenous RNA into the affected organism. Since then, various families of non-coding RNAs (ncRNAs) that exhibit analogous repressive effects on gene expression have been identified in most eukaryotic genomes (Cogoni *et al*, 1996; Jorgensen, 2003). Among these are small-interfering RNAs (siRNAs), microRNAs (miRNAs), repeat-associated small-interfering RNAs (rasiRNAs) and scanRNAs (scnRNAs; Mochizuki & Gorovsky, 2004; Tomari & Zamore, 2005). All of these silencing triggers are 21–28 nucleotides in length and assemble into similar RNA-silencing effector complexes; however, they can be distinguished by their mode of inception. siRNAs can be exogenous (derived from viruses) or endogenous (derived from long double-stranded RNA (dsRNA) transcripts) in origin, whereas miRNAs, rasiRNAs and scnRNAs originate exclusively from endogenous sources. The members of the miRNA family of ncRNAs are of special relevance because of their role in development and cell differentiation.

The processing of ncRNAs into mature 21–28 nucleotide RNA duplexes occurs either through the initial processing of the long RNA precursor by the RNase III-like endonuclease Drosha followed by a final maturation step effected by the ribonuclease III enzyme Dicer, or directly by the latter in a one-step splicing event. Once processed, the RNA-silencing trigger is loaded into one of many RNAi effector complexes, all of which share a common component: a member of the RNase H family of proteins called Argonaute (Ago). The final ribonucleoprotein effector complexes function by either repressing gene expression through interacting with a cognate messenger RNA (mRNA) or effecting genome rearrangement (Meister & Tuschl, 2004; Mochizuki & Gorovsky, 2004).

The aim of this microsymposium was to bring together newly established group leaders to discuss their latest findings on many aspects of small RNAs, ranging from their *in silico* prediction to the elucidation of their functional properties *in vivo*.

The two-day symposium began with a historical perspective provided by S. Cohen (Heidelberg, Germany). Cohen described how the initial, fragmented observations of miRNA-expression patterns and their tissue-specific effects on transcript profiles led to the first computational analyses, and the identification of key regions within the approximate 22-nucleotide miRNA sequence that is crucial for target recognition. Cohen went on to emphasize

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the methods by which scientists subsequently expanded these initial approaches to encompass studies detailing not only the anatomical structures of both miRNAs and their cognate target sites, but also the observed reciprocity of their spatial expression. Cohen's belief that spatial and temporal expression profiles of miRNAs and their targets might serve as predictors of miRNA function concluded a speech that touched on many issues covered in greater detail by the ensuing speakers.

The aim of this meeting report is to highlight the ideas and concepts communicated during the microsymposium and to further the discussion on the future of small RNA research.

## Mechanisms of small RNA-guided gene silencing

Over the past few years, significant progress has been made in understanding the molecular mechanisms of small RNA-guided post-transcriptional gene silencing. In *Drosophila melanogaster* and *Caenorhabditis elegans*, important components such as the RNase III-type enzymes Drosha and Dicer, and the Ago proteins have been identified and characterized. However, their precise functions, as well as many of their interacting protein partners, are still unknown. Four speakers presented new biochemical results on the mechanisms of post-transcriptional gene silencing.

R. Pillai (Basel, Switzerland) presented data on how miRNAs repress the translation of target mRNAs, and how this repression is relieved by cellular stress. He showed that miRNAs inhibit the initiation of translation and subsequently store their cognate mRNAs in cytoplasmic processing bodies (P bodies). For example, the endogenous cationic amino-acid transporter (CAT-1) mRNA is regulated by the liver-specific miR-122a in Huh7 cells and accumulates in P bodies. Interestingly, during cellular stress induced by serum starvation, the CAT-1 mRNA is released from the P bodies and is translated in the cytoplasm. Pillai's group also identified the (A+U)-rich element binding protein HuR as an essential participant in the stress-induced mobilization of the CAT-1 mRNA from the P bodies (Bhattacharyya *et al*, 2006).

G. Meister (Martinsried, Germany) presented new data on the biochemical purification and characterization of Ago-containing protein complexes from human cells. By using size-fractionation experiments, his group has shown that human Ago1 and Ago2, as well as cellular miRNAs, reside in three complexes of different size. The complex with the lowest molecular weight contains Ago2-mediated Slicer and Dicer activity, whereas the complex with the highest molecular weight contains only the latter. Further proteomic analyses identified the two larger complexes as mRNA-containing ribonucleoprotein complexes; these analyses have the potential to reveal new proteins involved in the RNA-silencing pathway and to provide insight into the maturation process of the RNA-induced silencing complex (RISC).

J. Martinez (Vienna, Austria) used a biochemical approach to identify the cellular kinase that phosphorylates the 5′ ends of transfected siRNAs. Those without 5′ phosphate groups are not functional; therefore, this reaction is a crucial step in ectopically induced RNAi. Martinez and colleagues used different biochemical fractionation methods and analysed siRNA phosphorylation activity in the individual fractions. They were able to identify a 45-kDa protein that is essential for siRNA 5′ phosphorylation. Furthermore, cells depleted of this kinase by RNAi showed reduced phosphorylation of siRNAs. The putative role of this kinase in other RNA metabolic pathways awaits investigation.

## Small RNA-induced chromatin modifications

RNAi has been most thoroughly studied with regard to the regulation of mRNA stability; however, more recently it has also been implicated in DNA and chromatin modification, which results in either transcriptional silencing or DNA elimination (Matzke & Birchler, 2005). K. Mochizuki (Vienna, Austria) and W. Aufsatz (Vienna, Austria) shared their most recent findings on the machinery involved in short RNA-induced sequence rearrangement and chromatin modification.

Mochizuki presented data on Dicer-like proteins in *Tetrahymena thermophila*. This species uses siRNA-like scnRNAs to control genome rearrangements that also involve DNA elimination during conjugation. One of the three Dicer-like enzymes found in *Tetrahymena*—dicer-like protein 1 (Dcl1p)—is essential for scnRNA processing from long dsRNAs. Such dsRNAs result from bidirectional transcription at early stages of conjugation. Furthermore, Dcl1p has an important role in the methylation of Lys9 of histone H3, which is a well-established feature of condensed chromatin but, more specifically, is representative of those genomic elements that are eliminated during conjugation.

Aufsatz presented studies in plants to genetically dissect the process by which dsRNA can induce cytosine methylation of sequence-homologous DNA, termed RNA-directed DNA methylation (RdDM). A mutagenesis screen performed in *Arabidopsis* was based on a two-component transgene RdDM model system: a target promoter at one genomic locus becomes methylated and silenced after the introduction of a transcribed promoter hairpin construct that produces promoter-homologous dsRNA and short RNAs. Three mutants were specifically found to affect non-CG methylation of the target promoter: DRD1, a plant-specific SNF2 chromatin remodelling factor, and NRPD1 and NRPD2, which encode the largest and second largest subunits, respectively, of the plant-specific DNA-dependent RNA polymerase Pol IV. Two functionally diverse Pol IV complexes are required for the RdDM of endogenous siRNA-producing loci. The largest subunit of Pol IVa, NRPD1a, is necessary for siRNA generation, whereas the largest subunit of Pol IVb, NRPD1b, is required further downstream for DNA methylation. The RNA-silenced target promoter is associated with hypoacetylated histone H3, but is not enriched in repressive histone H3 Lys9 dimethylation marks. Histone deacetylation is executed by the Rpd3-type histone deacetylase HDA6, which is also required for the maintenance of symmetrical cytosine methylation at the silenced promoter.

## Bioinformatics: finding ncRNAs and their targets

The synergy between computational studies and experimental approaches—most notably using mammalian models—has led to the realization that ncRNAs represent a much greater functional fraction of the genome (Huttenhofer & Vogel, 2006). The turning point in computational 'RNomics' was marked by the discovery of a large number of miRNAs in the year 2001 (Lagos-Quintana *et al*, 2001), which prompted an unprecedented development of computational approaches for the identification of novel ncRNA families. At the meeting, I. Hofacker (Vienna, Austria), M. Zavolan (Basel, Switzerland) and M. Rehmsmeier (Bielefeld, Germany) presented some of the current approaches in computational *de novo* prediction of, as yet, unidentified ncRNAs and miRNA target sites in cognate gene transcripts.

Precursor miRNAs (pre-miRNAs) are characterized by their unique hairpin-like secondary structure. Using this property,

 

Zavolan aims to develop methods for miRNA gene predictions in single genomes. She found that pre-miRNAs generally form stable hairpins irrespective of their flanking regions (Sewer *et al*, 2005). This was evident from the analysis of the genomic sequence of a single species, and is probably due to the constraints imposed by miRNA biogenesis. It might also be related to the observation that pre-miRNAs are more stable than random hairpins (Bonnet *et al*, 2004). Zavolan and her group reduced the sequence and structure of candidate pre-miRNAs into sets of attributes (Pfeffer *et al*, 2005) and trained a support vector machine (SVM) using hairpin structures predicted from ribosomal RNAs, transfer RNAs, random genomic sequences and viral genome sequences as negative sets, and known human pre-miRNAs as a positive set. The SVM was then used to classify candidate pre-miRNAs extracted from various viral genomes. The prediction noted a number of herpesviruses that seem to encode miRNAs, which were validated by cloning studies (Pfeffer *et al*, 2005). The method was also applied to mammalian genomes in the hope of discovering clustered miRNAs (Sewer *et al*, 2005), some of which were later confirmed by cloning results. A web interface to the miRNA prediction tool is available online (www.mirz.unibas.ch). Zavolan also referred to an automated computational tool for the annotation of cloned small RNAs. She described the application of this tool in the analysis of small RNAs expressed at different stages of *D. melanogaster* development (Aravin *et al*, 2003). In total, 48 new miRNA genes, as well as a number of 24–26 nucleotide-long rasiRNAs, were identified.

The identification of miRNA target genes is an essential step towards understanding their regulatory function. Rehmsmeier and colleagues have therefore developed an algorithm called RNAhybrid, which predicts multiple potential binding sites in animal and plant mRNA targets (Rehmsmeier *et al*, 2004). The programme, which is an extension of the classical RNA secondary-structure prediction of two sequences (Zuker & Stiegler, 1981), selects the most energetically favourable binding sites for an miRNA in the 3′ untranslated region (UTR) of a cognate target gene. The statistical significance of a predicted target is assessed by extreme value statistics of length-normalized free energies, a Poisson approximation for multiple binding sites and the number of orthologous targets in comparative studies of multiple organisms. RNAhybrid allows the user to apply uninterrupted base pairing at miRNA positions 2–7 or 2–8 (known as the 'seed'). One recent improvement of RNAhybrid is the disallowance of G:U wobble base pairs in the seed. This is important because they cause a clear reduction in the efficiency of regulation by the miRNA (Brennecke *et al*, 2005). Rehmsmeier also emphasized the flexibility of RNAhybrid in finding non-canonical target sites in the *C. elegans* transcriptome (Kruger & Rehmsmeier, 2006). An interactive version of the RNAhybrid programme is available online (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid).

Hofacker presented the development of a global ncRNA gene-finder that combines structural prediction with comparative sequence analysis. The method is based on the prediction of consensus structures by using aligned sequences implemented in the RNAalifold programme. (Hofacker *et al*, 2002; Washietl & Hofacker, 2004). Hofacker applied the algorithm to ncRNAs from *C. elegans* and several *Saccharomyces* species, and showed that taking into account the co-evolution of interacting molecules improved the prediction of secondary-structure. Hofacker used an SVM model (Washietl *et al*, 2005a) to apply the algorithm to larger genomes. By screening the Comparative Regulatory Sequence Database, Hofacker was able to recover—with high sensitivity and specificity—known ncRNAs across the human, mouse, rat, *Fugu* and zebrafish genomes, and to provide strong evidence for many putative ncRNAs. The method was implemented in the publicly available programme RNAz (www.tbi.univie.ac.at/~wash). In light of the accuracy of RNAz in recovering known ncRNAs, Hofacker annotated the complete human genome for conserved ncRNAs. More than 30,000 structured RNAs were identified, of which approximately 1,000 are conserved across all vertebrate species (Washietl *et al*, 2005b).

## The functional role of small RNAs

Despite the computational prediction and experimental validation of several miRNAs, only a few have so far been assigned a physiological function. Of particular interest is the role, if any, of miRNAs in development and pathogenesis. To address this issue, five speakers presented their most recent findings on a range of pathways believed to be regulated by miRNAs.

H. Grosshans (Basel, Switzerland) presented work on miRNA target identification and validation. He described several novel *in vivo* targets of the *C. elegans lethal-7* (*let-7*) miRNA, and showed that the interaction between this conserved miRNA and at least one of its targets, the proto-oncogene *ras*, is maintained in humans (Grosshans *et al*, 2005; Johnson *et al*, 2005). The finding that reduced expression of *let-7* in lung tumours correlated with increased accumulation of Ras protein indicates that *let-7* might function as a tumour suppressor in the lung. Grosshans also presented results from a functional genomics screen in *C. elegans* that identified genetic interaction partners of *let-7*. Some of these factors seem to mediate *let-7* function, which should provide information on the mechanism of action of this miRNA *in vivo*.

H. Manninga (Goettingen, Germany) reported new findings on the siRNA-induced RNAi pathway in zebrafish embryos and cell lines. Manninga described targeting various endogenously expressed and exogenously transfected genes with siRNAs for his analysis. In the experiments with zebrafish embryos, no effect was detected on either the expression level of a cognate target or the Ago-mediated processing of transfected targets. The embryos did, however, show continually intensifying morphological defects with increasing siRNA concentrations. In contrast to the embryo experiments, classical RNAi experiments using siRNA duplexes in zebrafish cell lines, derived from both adult and embryonic tissues, revealed detectable siRNA-mediated mRNA cleavage. Interestingly, the treatment with siRNAs had no effect on the growth or morphology of the cells. Manninga concluded that siRNA-induced RNAi functions only in cell lines and not in embryos. These contradictory results could be explained by the overloading of RISC after RNA injection into embryos, thereby blocking the functionality of RISC and the ensuing incorporation of mature miRNAs into the complex. Functional miRNAs are essential for the development of the embryo, but only have a minor role in cell lines.

A. Giraldez (Boston, MA, USA) showed that the maternally derived gene *nanos1*, which is required during germline development and is post-transcriptionally restricted to primordial germ cells, is targeted by miR-430 during zebrafish embryogenesis. The interaction of miR-430 with a cognate target site in the 3′ UTR of the nanos1 transcript is responsible for its deadenylation,

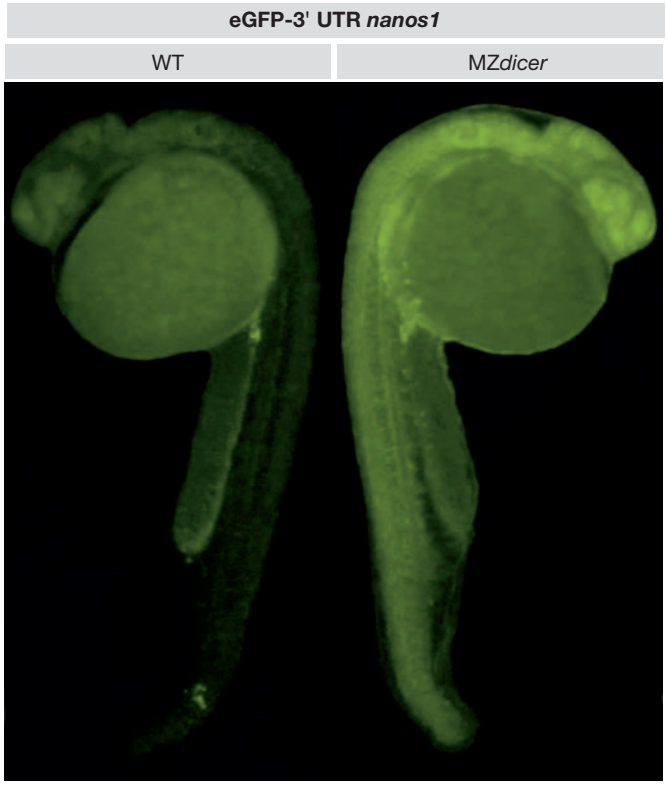


**Fig 1** | Validation of 3′ untranslated region-dependent regulation of *nanos1* by miR-430. An enhanced green fluorescent protein (eGFP) reporter containing the 3′ UTR of *nanos1* is injected into zebrafish embryos. The eGFP-3′ untranslated region (UTR) *nanos1* reporter is repressed in wild-type (WT) embryos compared with maternal-zygotic *dicer* (MZ*dicer*) mutants. Image courtesy of A. Giraldez.

clearance and eventual translational repression in somatic cells. This conclusion is based on the observation that deadenylation and repression, conferred by the interaction of miR-430 and the *nanos1* 3′ UTR, is disrupted in maternal-zygotic *dicer* mutant (MZ*dicer*) embryos (Fig 1). The wild-type phenotype was easily restored by injection of the processed miR-430 duplex into the MZ*dicer* mutant embryos. MiR-430-mediated repression in primordial germ cells is relieved in the context of the *nanos1* 3′ UTR, but not in other 3′ UTRs that contain artificial target sites for miR-430. These results suggest that the context of the *nanos1* 3′ UTR mediates miR-430-induced repression in somatic cells, but antagonizes miRNA-mediated repression in germ cells. Therefore, the equilibrium between miRNA-mediated repression in somatic cells and 3′ UTR-mediated antagonism of miRNAs helps to shape gene expression in somatic compared with primordial germ cells.

The last talk of the symposium was given by S. Pfeffer (Strasbourg, France) on the role of miRNAs derived from DNA viruses. Pfeffer provided an overview of his work on the identification of viral miRNAs (Pfeffer *et al*, 2004, 2005) and showed that there are good indications for viral miRNAs acting in *cis* on their own genome. The identification of cellular targets is more problematic given the lack of conservation of viral miRNAs. Pfeffer is currently investigating the role of Epstein–Barr virus and Kaposi's sarcoma-associated herpes virus miRNAs by combining bioinformatics with cloning and classical biochemical methods. He also presented some preliminary observations that plant viruses might use small RNAs to regulate host genes.

## Concluding remarks

We believe that this meeting achieved its goal by highlighting the interdisciplinary approach of the RNAi field and giving those at the forefront of this exciting new area of research an opportunity to share their latest insights. In light of the tremendous attendance, the organizers are encouraged to make this an annual event, and would appreciate any feedback from the participants and readers of this report.

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