

# The impact of non-coding RNAs

## Workshop on New Functions of Regulatory RNAs in Pro- & Eukaryotes

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The EMBO workshop on the New Functions of Regulatory RNAs in Pro- & Eukaryotes took place between 13 and 15 January 2009, in Vienna, Austria, and was organized by R. Schroeder, D. Barlow & E. Westhof.

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translation, these important milestones have drastically changed our view of RNA to be an enzymatically active molecule. Since then, several abundant small 'non-mRNAs' have been discovered such as the small-nuclear RNAs of the spliceosome and small-nucleolar RNAs that are involved in rRNA modification. More recently, other small RNAs—such as siRNAs and microRNAs—have been shown to inhibit translation through direct interactions with the mRNA. At the beginning of the new millennium, the impact of RNA on the regulation of gene expression was foreseeable. The hunt for non-coding regulatory RNAs (ncRNAs) is ongoing in prokaryotes and eukaryotes, and large research efforts are being directed towards elucidating the mechanisms whereby they control gene expression. This EMBO workshop highlighted recent findings on the regulatory role of RNA in all kingdoms of life, covering various RNA-based regulatory mechanisms, from chromatin modifications to translational repression and RNA degradation.

### The impact of RNA on DNA

Several talks at the workshop focused on the impact of RNA on heterochromatin formation, DNA methylation, DNA elimination, chromosome silencing and the regulation of telomerase—an enzyme that adds telomeric DNA to the ends of chromosomes.

Heterochromatin has long been thought to be transcriptionally silent; however, it has recently been shown that ncRNAs are transcribed from heterochromatin DNA in various organisms (Buhler & Moazed, 2007). In fission yeast in particular, small RNAs target these ncRNAs, which are transcribed from centromeric DNA (Fig 1). The small RNAs are processed similarly to the small RNAs in the RNA interference (RNAi) pathway, but in a chromatin-dependent manner. Double-stranded RNAs are cleaved by Dicer and loaded into an Argonaute protein complex that targets RNA transcripts. The RITS-siRNA-transcript complex subsequently recruits chromatin-modifying factors that trigger the silent heterochromatic state. An RNA-dependent RNA polymerase complex (RDRC) implements RdRP activity using the transcript from heterochromatin as a template to make the double-stranded RNA necessary for recycling the RNAi process. D. Moazed (Boston, MA, USA) examined the mechanism by which RNAi can lead to heterochromatin formation and showed that when the RITS complex is tethered to a euchromatic transcript, the RNA transcript initiates heterochromatin formation (Buhler & Moazed, 2007). In addition, his group showed that Swi6—a protein

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See Glossary for abbreviations used in this article.

### Introduction

The central role of RNA in translation has been accepted for more than 50 years, although classically the only known roles for RNA were as a template for translation (mRNA) or as part of the translational machinery (rRNA and tRNA). In 1989, S. Altman and T. Cech received the Nobel Prize in Chemistry for their discovery of RNA catalysis; Altman described the catalytic activity of the RNA molecule in the RNase P ribozyme of *Escherichia coli* (Guerrier-Takada *et al*, 1983) and, concurrently, Cech observed the self-splicing reaction of the *Tetrahymena* rRNA precursor (Kruger *et al*, 1982). As RNA has generally been considered to have a passive role in

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**Glossary**

$\sigma^{70}$	RpoD, 'housekeeping' sigma subunit of the bacterial RNA polymerase
$\sigma^S$	RpoS, stress/stationary phase sigma subunit of the bacterial RNA polymerase
BC1	small brain-specific non-mRNA
Dcp2	dipeptidyl-carboxypeptidase
DMS3	uncharacterized protein involved in <i>de novo</i> DNA methylation
DsrA	sRNA regulating translation of RpoS
eIF4A	eukaryotic initiation factor 4A
Hfq	host factor for Q $\beta$ phage
HSF1	heat-shock factor 1
HSR1	heat-shock RNA 1
mRNA	messenger RNA
NTP	nucleotide triphosphate
pRNA	RNA product
RdDM	RNA-dependent DNA methylation
RdRP	RNA-dependent RNA polymerase
RITS	RNA-induced transcriptional silencing
<i>rne</i>	RNase E gene
rpoS	RNA polymerase stress and stationary phase $\sigma$ factor
RppH	RNA pyrophosphohydrolase
rRNA	ribosomal RNA
scRNA	small cytoplasmic RNA
scnRNA	small-scan RNA
SELEX	systematic evolution of ligands by exponential enrichment
SINE	short interspersed nuclear element
siRNA	small interfering RNA
SMC	structural maintenance of chromosome
sRNA	small non-coding RNA
TelRNA/TERRA	telomeric RNA
tRNA	transfer RNA

required for heterochromatic gene silencing—is necessary for the RNAi-mediated gene silencing by promoting RNAi. Swi6 associates with the ncRNA transcripts and tethers them to the chromatin, allowing them to become RNAi substrates (Motamedi *et al*, 2008). Overall, Moazed provided further evidence of the importance of RNAi for silencing centromeric DNA in fission yeast.

In *Tetrahymena thermophila*, small RNAs known as scRNAs are involved in RNA-mediated DNA elimination during conjugation. These RNAs are transcribed bidirectionally from the micronucleus chromosome; they also recognize and bind to complementary sequences on nascent transcripts from the parental macronucleus DNA, suggesting a mechanism for the recognition of eliminated sequences (Mochizuki *et al*, 2002). K. Mochizuki (Vienna, Austria) described a helicase known as Ema1 that is required for both DNA elimination and heterochromatin formation (Aronica *et al*, 2008). Ema1 enhances the degradation of those scnRNAs that are homologous to the parental, non-eliminated sequences. The scnRNAs that survive are transported to the developing new macronucleus, interact with nascent transcripts and possibly recruit chromatin-modifying factors. Mochizuki proposed that the modifications on the chromatin might be the signal for DNA elimination by some factor yet to be discovered.

In *Arabidopsis*, ncRNAs had been shown previously to trigger promoter methylation. M. Matzke (Vienna, Austria) and colleagues were able to detect small secondary RNAs that are

products of RdRP activity on the ncRNAs. These secondary RNAs are responsible for chromatin silencing through cytosine methylation. The ncRNAs might provide a platform for siRNAs to trigger DNA methylation and the spreading of silencing to regions further downstream of the chromatin (Daxinger *et al*, 2009). In addition, Matzke and colleagues searched for components of RdDM involved in the silencing of developmental genes (Kanno *et al*, 2008), and identified and mapped six genes that are important in this process. One of them is an uncharacterized protein, DMS3, which shows similarity to the hinge domain of SMC proteins. Matzke proposed that this domain could dimerize and is probably responsible for the DNA-binding activity of the RdDM machinery. The evidence that she provided suggests that DMS3 is a general component of the RdDM machinery.

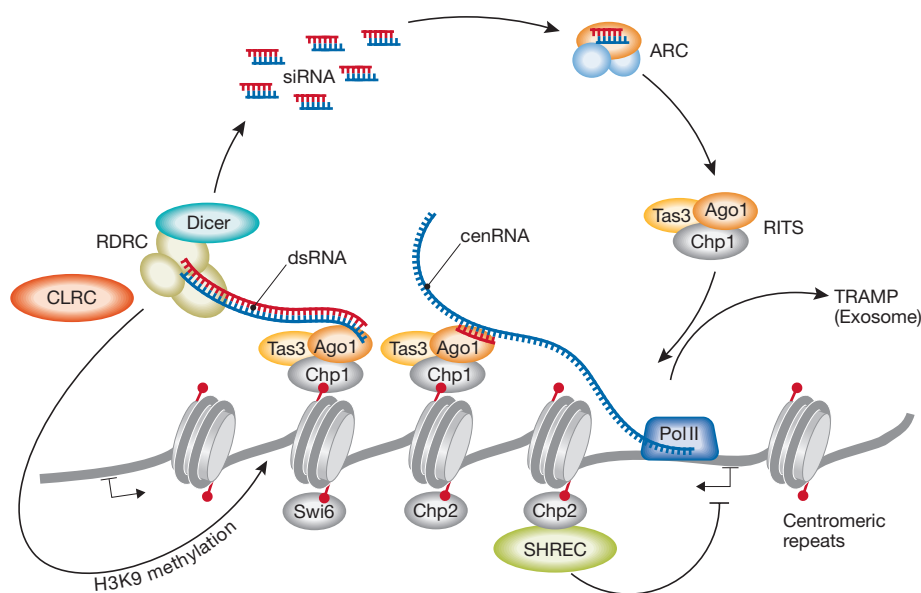
S. Schoeftner (Madrid, Spain) discussed the discovery of TelRNA—also known as TERRA—which is transcribed directly from telomeric DNA (Schoeftner & Blasco, 2008). TelRNAs have been shown to inhibit telomerase activity by binding directly to the RNA component of telomerase. As telomerase has been implicated in tumour growth, the TelRNA has the potential to be used as an anticancer agent. Schoeftner showed that the expression of TelRNA is regulated by telomere length, stress conditions and chromatin structure, and that the TelRNAs are associated with the inactive X chromosome, which could point towards an additional role in X inactivation.

**The impact of RNA on transcription**

Over the past few years, ncRNAs have been shown to regulate gene expression at the transcriptional level through a direct interaction with the transcriptional machinery, resulting in either transcriptional activation or transcriptional repression. Many of these ncRNAs are upregulated in response to stress and therefore seem to control a given stress regulon.

E. Nudler (New York, NY, USA) introduced HSR1, which is a thermosensing RNA involved in activating transcription after heat shock. HSF1 is an inactive monomer that must trimerize to activate heat-shock genes and was previously shown to require the HSR1 RNA to form the active trimer complex upon heat shock (Shamovsky *et al*, 2006). Nudler determined that the HSR1 RNA senses fluctuations in temperature, which is a nice example of how the sensitivity of RNA structures to environmental stimuli can be utilized in gene regulation. Nudler isolated a mutant HSR1 that is temperature independent and constitutively active, probably because it can form only the active structure. His group was also able to design a small-hairpin RNA to target HSR1, thereby inhibiting the expression of heat-shock genes. More temperature-sensing RNAs—which have therapeutic potential—will probably be discovered in the near future.

The molecular mechanism by which bacterial 6S RNA inhibits the transcription of  $\sigma^{70}$ -dependent promoters was discussed by K. Wassarman (Madison, WI, USA). The double-stranded structure of the 6S RNA has a large central bulge that resembles the conformation of DNA in the open complex during transcriptional initiation (Wassarman, 2007). Wassarman showed that the 6S RNA forms a stable complex with the RNA polymerase under starvation conditions. However, when nutrients—for example, NTPs—are available, the 6S RNA can act as a template for the synthesis of the 20-nucleotide (nt)-long pRNA, which leads to its release from the RNA polymerase and to its degradation. Wassarman also



**Fig 1** | The nascent transcript model for small interfering RNA-mediated heterochromatin formation in fission yeast. The RITS complex mediates heterochromatin formation by associating with nascent transcripts, through its interaction with an siRNA and its association with methylated nucleosomes. These interactions result in the recruitment of the RDC and Dicer, which produce more siRNAs, thereby enforcing a feedback loop. Chromatin silencing requires HP1 proteins (Swi6 and Chp2), which promote the RITS/nascent transcript interaction and recruit deacetylase complexes. Modified from Moazed (2009) with permission from the author and publisher. Ago1, argonaute 1; ARC, argonaute siRNA chaperone; cenRNA, centromeric RNA; CLRC, Clr4 methyltransferase complex; dsRNA, double-stranded RNA; H3K9, lysine 9 of histone 3; Pol II, polymerase II; RDC, RNA-dependent RNA polymerase complex; RITS, RNA-induced transcriptional silencing; SHREC, a histone deacetylase complex; siRNA, small-interfering RNA; Tas3, adapter protein that links Ago1 and Chp1; TRAMP, a polyadenylation complex that stimulates exosome activity.

showed that region 4.2 of  $\sigma^{70}$ —which binds to the  $-35$  promoter region—is also crucial for 6S RNA binding. However, individual residues contribute differentially to the interaction with DNA and/or RNA, suggesting that RNA recognition and binding differ from recognition of the  $-35$  element in promoter DNA.

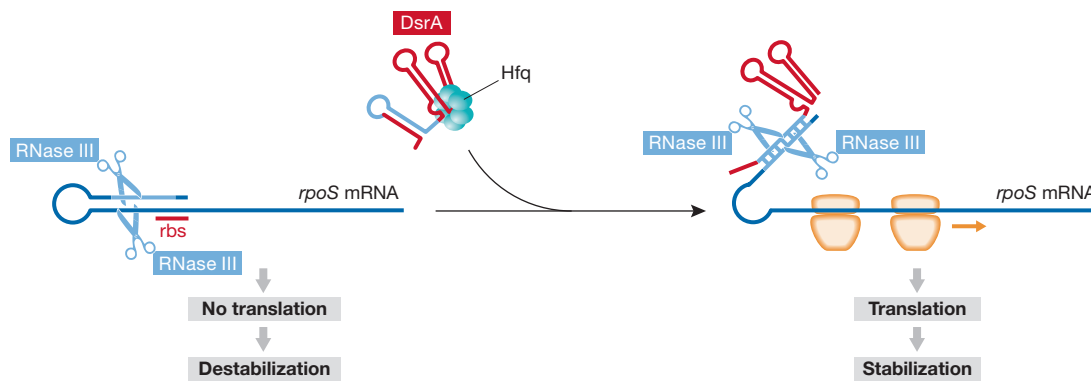
J. Kugel (Boulder, CO, USA) focused on the SINE-derived human Alu RNA and mouse B2 RNA. Both Alu and B2 RNAs are upregulated after heat shock and bind directly to RNA polymerase II (RNAPII) to inhibit transcription. Interestingly, the Alu and B2 RNAs can be treated as modular, and their binding to polymerase and transcriptional repression activities are separable (Mariner *et al* 2008). Kugel showed that the insertion of a transcriptional repression domain from Alu RNA into B1 RNA—which is an ncRNA that can bind to polymerase but not repress transcription—confers transcriptional repressor activity to B1 RNA. Separable distinct domains are not new to the protein world; however, this phenomenon is not as common in the RNA world because RNA has a tendency to misfold when entire domains are deleted.

The search for RNAs that directly bind to RNAPII and possibly regulate transcription was discussed by J. Boots (Vienna, Austria). Boots and colleagues used genomic SELEX to find RNAs transcribed from the human genome that bind with high affinity to RNAPII and mapped more than 11,000 RNA sequences onto the human genome. Many of the RNAs that they identified are expressed and can be co-immunoprecipitated with RNAPII. Boots presented data suggesting that these Pol II-binding RNAs can inhibit transcription.

### The impact of RNA on post-transcriptional regulation

Significant progress has been recently made in the identification of bacterial small ncRNAs. These RNA molecules—which are approximately 50–250 nt in length—are generally encoded in *trans* and interact with their target mRNAs owing to imperfect or short complementarities. Therefore, one ncRNA modulates the stability and/or translation of several different target mRNAs in response to environmental changes (reviewed in Geissmann *et al*, 2009). Their function mostly requires the RNA chaperone Hfq. This hexameric, doughnut-shaped protein has been shown to protect ncRNAs from degradation and to stimulate their interaction with target mRNAs. The regulation of *E. coli rpoS* mRNA—which encodes the stationary phase/stress sigma-factor  $\sigma^S$ —is an example of the intricate post-transcriptional regulation mechanism involving the activity of an ncRNA and Hfq (Fig 2). The translation-initiation region of *rpoS* folds into a complex secondary structure that prevents efficient ribosome binding. The double-stranded region within this structure provides a target site for RNase III cleavage, leading to the degradation of the mRNA. At low temperatures, the level of the ncRNA DsrA increases and it binds to the *rpoS* leader—a process that is mediated by Hfq—thereby disrupting the inhibitory structure and allowing ribosome access and translation of *rpoS* (Repoila *et al*, 2003). In addition, the formed mRNA–ncRNA duplex provides an alternative RNase III cleavage site, leading to the degradation of DsrA (Resch *et al*, 2008).

Several studies have shown that Hfq has a crucial role in virulence and that the underlying regulation is triggered by ncRNAs.



**Fig 2** | Model for post-transcriptional regulation of *rpoS* expression by the non-coding regulatory RNA DsrA and RNase III. See text for details. Adapted from Resch *et al* (2008). Hfq, host factor for Q  $\beta$  phage; mRNA, messenger RNA; rbs, ribosome binding site; *rpoS*, RNA polymerase stress and stationary phase  $\sigma$  factor.

Therefore, ambitious efforts have been made towards the identification of novel ncRNAs in pathogens that regulate the expression of virulence factors. A subset of talks at this workshop addressed different approaches to search for ncRNAs in pathogenic bacteria and reported on the determination of their regulatory networks. P. Cossart (Paris, France) presented a method based on tiling arrays to look for ncRNAs in the Gram-positive food-borne pathogen *Listeria monocytogenes*. By comparing transcripts of strains grown under various conditions, 29 novel ncRNAs were identified, some of which follow the same expression pattern as virulence genes. Surprisingly, the study also revealed the existence of long antisense RNAs that span several genes located on the opposite strand, one of which was shown to downregulate the flagellum locus.

A model organism for Gram-negative pathogens is *Salmonella typhimurium*. By using a co-immunoprecipitation approach in combination with 454 deep sequencing, the group of J. Vogel (Berlin, Germany) detected more than 100 Hfq-binding ncRNAs that are encoded in intergenic regions. To identify potential target mRNAs controlled directly by ncRNAs, pulse-expression studies followed by transcriptome analyses were then performed. These studies indicated that several hundreds (or >20%) of all *Salmonella* genes are regulated by Hfq and ncRNAs (Sittka *et al*, 2008).

One of the largest bacterial regulatory ncRNAs is the *Staphylococcus aureus* RNAIII, which is 514 nt long. RNAIII is an example of a multiple target regulator that modulates the expression of genes involved in virulence at the post-transcriptional level (Boisset *et al*, 2007). P. Romby (Strasbourg, France) focused on the interaction of RNAIII with several of its target mRNAs that encode virulence factors and one that encodes a transcriptional regulatory protein, Rot. The RNAIII–*rot* mRNA interaction was studied in detail, revealing the existence of several loop–loop interactions that partly overlap the ribosome binding site (rbs), thereby inhibiting ribosome binding. Furthermore, reminiscent of the DsrA/*rpoS* regulation shown in Fig 2, the RNAIII–*rot* interaction creates an RNase III cleavage site within the *rot* mRNA, leading to its degradation. This study illustrates the complexity of RNAIII-dependent regulatory networks in the virulence of *S. aureus*. In addition, Romby described the identification of several novel ncRNAs that might act as regulatory antisense RNAs.

G. Storz (Bethesda, MD, USA) introduced bacterial small RNAs, which repress the synthesis of small hydrophobic proteins that are

toxic at high levels (Fozo *et al*, 2008). Increasing numbers of these mRNA–sRNA pairs, which are classified as type I toxin–antitoxin pairs, are being found. The expression of some of the toxins is induced by stress, implying that the proteins might have a beneficial role in the stressed cells.

In addition to the translational repression mediated by mRNA–sRNA duplexes, RNAs can also inhibit translation through other mechanisms, as indicated by H. Tiedge (New York, NY, USA). BC1 RNA inhibits translation by interfering with the formation of the 48S ribosomal complex in neurons. Specifically, it targets the helicase eIF4A within the 48S complex, and might uncouple the ATP hydrolysis and helicase activity of the eIF4A protein (Lin *et al*, 2008).

### The impact of RNA on degradation

Several talks at the meeting focused on RNA-degradation pathways. B. Seraphin (Gif sur Yvette Cedex, France) presented evidence for a new endonucleolytic activity of the exosome, which is mediated by a different domain than the exonucleolytic activity, and discussed the role of this enzyme in the degradation of cryptic unstable transcripts (CUTS) and other RNAs (Lebreton *et al*, 2008). In the light of this new activity for the exosome, Seraphin suggested that we must revise our view of the mechanism of exosome-dependent RNA processing and degradation to include the cooperation between multiple domains of the exosome. J. Belasco (New York, NY, USA) discussed the mechanism of RNA decay by RNase E. Bacterial mRNA is protected from RNase E-dependent degradation by a 5'-triphosphate, which can be converted into a monophosphate by the action of a pyrophosphohydrolase, RppH. As a 5'-monophosphate stimulates RNase E degradation, this pathway is strongly reminiscent of the process of cap removal in eukaryotes. This parallelism is supported by the structural resemblance of the enzymes RppH and Dcp2, which act on bacterial and eukaryotic 5' ends, respectively (Messing *et al*, 2009). In addition, Belasco gave insights into the intricate regulation of *rne*, which is dependent on elements within the 5'-untranslated region of *rne* mRNA. Similar to a 5'-monophosphate, one of these elements was shown to stimulate degradation through direct binding to RNase E.

Another mechanism of gene expression regulation is based on features of the mRNA, which was introduced by I. Moll (Vienna, Austria). She reported on the formation of protein-depleted ribosomes in the

presence of the antibiotic kasugamycin, which are still active in the translation of mRNAs that lack a 5'-leader (Kaberina *et al.*, 2009). Several stress factors were found to be translated in the presence of the antibiotic. The determination of the 5'-termini of these mRNAs revealed a processing event that renders them leaderless under stress. The data suggest that this mechanism might provide a means to cope with adverse conditions in which the ribosome is not equipped with the whole set of ribosomal proteins required for the translation of canonical mRNAs.

### Summary

Regulatory ncRNAs have been found in all organisms, and vary widely in size and function. The data presented at this EMBO workshop did not focus on a particular system or mechanism, but rather brought together scientists to share ideas, and to compare and contrast how different organisms utilize RNA. This offered a great opportunity to compare differences and similarities in the function of RNA and its regulatory mechanisms. Overall, the meeting revealed a general phenomenon: RNA can take over roles traditionally thought to be reserved for proteins, especially under stress conditions.

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