

mice deficient in GATA-binding protein 4 (*Gata4*), *Fog2* (which encodes a zinc finger protein) and insulin receptor family genes [15–17]. It will be necessary to examine gene-expression levels on a cell-by-cell basis, and to conduct biochemical and cell transfection studies to investigate further the possible relationship between *Sox9* and *Sox8* expression.

### Sox gene redundancy: a recurring theme

The overlapping functions of *Sox8* and *Sox9* in sex determination are typical of a widespread feature of SOX transcription factor biology. For example, *Sox1–Sox3* have redundant roles in lens induction [18], *Sox5* and *-6* can act interchangeably during chondrogenesis [19] and both *Sox7* and *-17* show functional overlap with *Sox18* during blood-vessel development [20]. In these examples, as with *Sox8* and *-9* in sex determination, it is possible to argue that two or more genes are better than one for an important function. Alternatively, when two or more genes share a role as a result of common ancestry, only one of these will retain that role and the sister gene(s) will ultimately acquire non-overlapping subsets of the functions of the ancestral gene (neofunctionalisation). Either way, the present roles of *Sox* genes are an intriguing example of evolution in progress.

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## RNA-directed transcriptional gene silencing in mammals

Elizabeth H. Bayne and Robin C. Allshire

The Wellcome Trust Centre for Cell Biology, Institute of Cell Biology, University of Edinburgh, Mayfield Road, Edinburgh, UK, EH9 3JR

RNA interference is a highly conserved pathway mediating sequence-specific RNA degradation. In plants, the short RNA intermediates of this pathway can also drive transcriptional silencing of target genes by DNA methylation. Until recently, there was no evidence that a similar pathway operated in mammals; two new studies suggest that small RNAs can direct DNA methylation and chromatin modification in human cells. Although

further investigation is required to determine how widespread RNA-directed DNA methylation is in mammals, the findings raise the possibility that this pathway, far from being merely a curiosity of plant systems, is a conserved mechanism for control of gene expression.

### Introduction

RNA interference (RNAi) is an evolutionarily conserved system mediated by, and targeted against, RNA. The

Corresponding author: Allshire, R.C. (robin.allshire@ed.ac.uk).

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RNAi pathway is triggered by long double-stranded (ds)RNA that is cleaved by an endonuclease named Dicer to produce short interfering (si)RNAs of ~21 nt in length. These siRNAs can be incorporated into an RNAi effector complex – the RNA-induced silencing complex (RISC) – where they direct degradation of RNA with sequence homology to the siRNA.

In plants, siRNAs can also direct methylation of homologous DNA. When this DNA methylation affects a promoter sequence, it can lead to transcriptional silencing of the downstream gene. Recently, results from two groups indicate that siRNA-directed DNA methylation can occur in human cell lines, suggesting a potential additional mechanism for transcriptional regulation in mammals.

### RNA directs transcriptional or post-transcriptional silencing

Silencing based on sequence homology was first recognized in transgenic plants in the late 1980s and, from the outset, it was linked to both transcriptional and post-transcriptional repression. Transgenic plants with multiple copies of the same gene often displayed silencing of all copies of that gene, including the endogenous gene [1,2]. In some cases, this silencing was attributed to transcriptional repression, and in other cases it was due to a post-transcriptional effect [3,4]. Elegant experiments using either plant RNA viruses or hairpin RNAs demonstrated that both of these types of silencing could be triggered by RNA. RNA that is complementary to the coding region of a gene triggered post-transcriptional gene silencing (PTGS) by degradation of homologous RNA [5–7]. By contrast, RNA with complementarity to the promoter sequence of a gene triggered transcriptional gene silencing (TGS) [6,7]. Both types of silencing were associated with heavy methylation of cytosine residues within the homologous DNA, a common mark of transcriptional silencing. In plants, RNA-directed transcriptional silencing is also associated with modification of the associated chromatin by di-methylation of lysine 9 on histone H3 (H3K9Me2) [8].

Although RNA-directed post-transcriptional silencing, or RNAi, is now known to be widely conserved and can be used as a tool for gene knockdown in animal systems [9], evidence for RNA-directed DNA methylation or chromatin modification outside of plants was lacking. However, recent studies in fission yeast and flies have shown that RNA-directed transcriptional silencing is not confined to plants. Fission yeast do not appear to methylate their DNA, but, as in plants, expression of dsRNA induces a silent chromatin state along homologous DNA by H3K9 dimethylation [10]. Moreover, H3K9Me2-mediated transcriptional silencing of some repetitive sequences in both fission yeast and flies is dependent on components of the RNAi machinery [11,12]. Thus, siRNA-directed DNA and/or chromatin modification leading to transcriptional silencing might be broadly conserved.

### RNA-directed DNA methylation in mammals

In mammals, as in plants, transcriptional silencing is frequently correlated with DNA methylation. However, despite non-coding RNAs being implicated in

transcriptional silencing in mammals, for example, Xist RNA-dependent X-chromosome inactivation [13], evidence for RNA-directed DNA methylation has not been forthcoming. Indeed, a previous detailed study found that RNAi in mouse oocytes was not associated with DNA methylation at the target locus [14]. However, two recent papers now present evidence that short RNAs can direct DNA methylation and transcriptional gene silencing in mammalian cells.

Kawasaki *et al.* [15] synthesized a selection of siRNAs specifically targeted against the CpG island (cytosine-rich region) of the E-cadherin gene promoter. They used bisulphite sequencing (Box 1) to show that cytosines within the target sequence were specifically methylated in cells transfected with homologous siRNAs. Furthermore, the siRNAs also induced H3K9 methylation in the target chromatin, although the H3K9Me isoform involved was not indicated. Silencing of the target gene was evident in reduced accumulation of E-cadherin mRNA and protein. The silencing was dependent on the DNA methyltransferases DNMT1 and DNMT3b, suggesting that it occurred at the transcriptional level as a result of DNA methylation.

The Kawasaki study presents a comprehensive analysis of modifications to the E-cadherin promoter. However, it is important to know whether other genes respond in a similar manner to transfected siRNAs. In a complementary study, Morris *et al.* [16] demonstrated silencing of an integrated transgene by transfection of an siRNA with homology to part of its promoter sequence. They also detected methylation of the target DNA, albeit only at one four base-pair site, using a methyl-sensitive restriction enzyme in a PCR-based assay (Box 1). Nuclear run-on assays confirmed that reduced expression of the reporter construct was due to suppression of transcription from the target locus.

Taken together, these two articles suggest that RNA-directed DNA methylation and histone modifications can occur in mammals. However, other reports failed to detect any RNA-directed DNA methylation at homologous genes. In the study by Svoboda *et al.* [14], expression of long dsRNA corresponding to the coding region of a target gene in mouse oocytes triggered PTGS but not DNA methylation. Clearly differences in cell type and/or target site selection (promoter versus coding sequence) could account

#### Box 1. Methods for detecting DNA methylation

##### Bisulphite sequencing

Bisulphite causes deamination of unmethylated cytosine, converting it to uracil, but leaves methylated cytosine unchanged [27]. Sequencing of DNA treated with bisulphite therefore can determine the methylation status of every cytosine in a particular stretch of DNA.

##### Methyl-sensitive restriction enzymes

The activity of some restriction enzymes depends on cytosine residues within their recognition sequence being unmethylated. The methylation status of cytosines that lie within such restriction sites can therefore be assessed by incubating the DNA with a methyl-sensitive enzyme and then assessing the extent of digestion by either Southern blotting or PCR amplification across the restriction site.

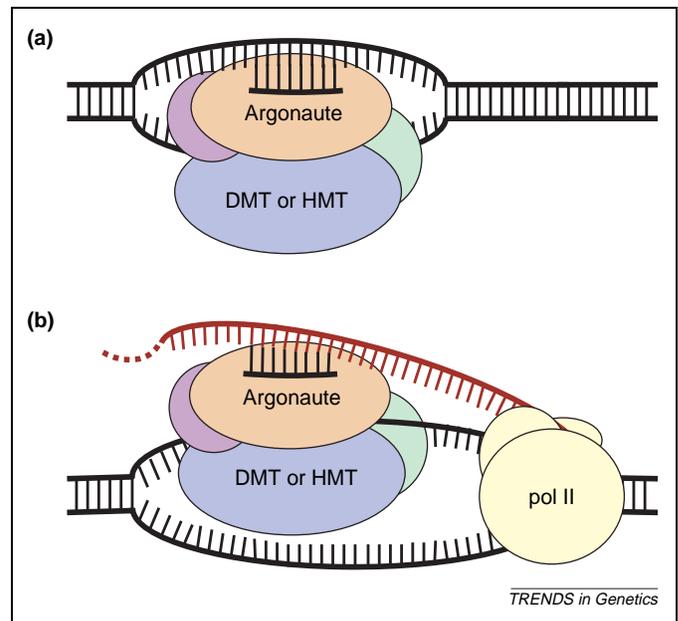
for the different results of these studies; but, at present, it remains unclear how prevalent RNA-directed DNA methylation is in mammalian cells and what parameters affect the efficiency of this process.

### The 'hows' and 'whys' of RNA-directed DNA methylation

The mechanism of the siRNA-directed DNA methylation observed in mammalian cells remains a mystery. However, models can be proposed on the basis of what is known about siRNA-directed chromatin modification in other systems. In fission yeast, siRNAs are incorporated into a protein complex called the RNA-induced initiation of transcriptional gene silencing (RITS) complex that is required for silent chromatin – 'heterochromatin' – formation [17]. RITS includes the chromodomain protein Chp1, which is essential for H3K9 methylation [18], and the argonaute protein Ago1. Argonaute proteins from other organisms are common components of silencing complexes; therefore, RITS might act as an siRNA-directed effector complex of chromatin modification, analogous to RISC in post-transcriptional silencing. In fission yeast, RITS is presumed to recruit histone methyltransferase activity, and this initiates the silencing of target loci in the absence of DNA methylation. In plants and mammals, in which histone and DNA methylation are tightly interlinked, a RITS-like complex might similarly recruit DNA methyltransferase activity, either directly or as a consequence of histone methyltransferase recruitment.

An outstanding question in all systems is precisely how siRNAs direct effector complexes to target loci. In plants, siRNA-directed DNA methylation is largely confined to the region of RNA–DNA sequence complementarity [19], and it can target promoter sequences that are not thought to be transcribed [6,7]. These observations suggest homologous base-pairing between the siRNA and the target DNA (Figure 1a). However, a recent report showed that plant DNA methylation can be induced by a short RNA with extensive complementarity only to the corresponding spliced transcript, suggesting that RNA–DNA base-pairing is not required [20]. Therefore, the target locus might be located by siRNA binding to the nascent, newly processed transcript (i.e. an RNA–RNA interaction; Figure 1b). This scenario could also explain siRNA-directed histone modification in fission yeast, where all reported target DNA sequences are transcribed [12]. Perhaps target recognition can occur by siRNA binding to either RNA or DNA depending on the circumstances. However, it has not been ruled out that low levels of read-through transcription are required for siRNA-directed promoter methylation and, therefore, that RNA–RNA interactions could account for all RNA-directed chromatin modifications.

Although the studies of Kawasaki *et al.* and Morris *et al.* provide the first indication that mammalian cells have the potential for RNA-directed DNA methylation and associated chromatin modification and transcriptional silencing, the potential significance of this system during normal mammalian development is unclear. However, comparisons with fission yeast suggest that RNA-directed



**Figure 1.** Models for siRNA-directed chromatin modification. DNA (DMT) or histone (HMT) methyltransferase activity is thought to be recruited to target loci by a RITS-like complex that includes an argonaute protein and an siRNA. Two potential mechanisms for target recognition are: (a) siRNA binding to target DNA; or (b) siRNA binding to nascent transcripts produced from target DNA by RNA polymerase II (pol II).

chromatin modification might be important in higher organisms. Endogenous siRNAs in fission yeast correspond to repeat sequences that flank the centromeres (the sites of attachment of chromosomes to the spindle during cell division) [21]. These sequences are normally embedded in heterochromatin that is important for centromere function; mutations in components of the RNAi pathway disrupt this heterochromatin and lead to defects in chromosome segregation [22,23]. Interestingly, mutation of the RNAi component Dicer in chicken cells carrying a human chromosome also causes defects in pericentromeric heterochromatin formation and segregation in that chromosome [24]. Moreover, mouse embryonic-stem cells lacking functional Dicer exhibit reductions in both H3K9 methylation and DNA methylation at centromeres [25]. Together these results suggest that siRNAs might have a conserved role in pericentromeric heterochromatin maintenance. Other potential roles for siRNA-directed chromatin modification in transcriptional gene regulation include regulation of invasive genetic elements such as retrotransposons, which are subject to RNAi-mediated transcriptional silencing in both fission yeast and plants [10,26], and transcriptional control of endogenous gene expression. A role for siRNAs in transcriptional gene regulation is supported by recent work in plants, showing that endogenous short RNAs (microRNAs) direct methylation of a family of genes involved in leaf development [20].

### Concluding remarks

The observation that siRNAs appear to direct transcriptional silencing and DNA methylation in mammals will no doubt add to the growing excitement about the potential therapeutic use of siRNAs to silence the expression of genes that are adverse to human health. It also raises the

possibility that chromatin modifications associated with normal mammalian development are regulated by RNA through a pathway previously believed to be confined to plants. However, it remains to be seen how widespread siRNA-directed silencing and methylation is in mammalian cells, and whether this process is sufficiently efficient and robust to be used as a tool both in biological studies and therapeutics.

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## Dissecting complex traits: the geneticists' 'Around the world in 80 days'

Ariel Darvasi

The Life Sciences Institute, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

The identification of genes mildly affecting quantitative phenotypes constitutes a difficult task that has almost always eluded application, particularly in behavioral phenotypes. Recently, the first study that identified a

gene underlying a QTL affecting anxiety was published. In the course of that study, novel approaches were developed that can significantly reduce the time required to identify such genes. The identification of genes affecting complex traits is expected to provide significant insights into the biochemical mechanisms underlying these poorly understood traits.

Corresponding author: Darvasi, A. (arield@cc.huji.ac.il).  
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