

RNA Control of Epigenetic Processes

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Epigenetics has become a subject of intense interest, as it deals with the contextual information that is superimposed on the relatively stable underlying genomic sequence by the modification of DNA (and RNA) and the modulation of chromatin structure. While not usually explicitly acknowledged, there is often an ambiguity about whether the term 'epigenetic information' refers mainly to that which is intrinsically acquired and transmitted within the trajectories of differentiation and development (as only a subset of genes are active within any given cell), including phenomena such as parental imprinting, or that which is acquired as a consequence of gene-environment interactions, although the two pathways probably intersect, at least in part. It is likely that most epigenetic changes that underlie developmental processes are driven by internal feed-forward programs embedded within the genome (as a kind of first derivative of DNA information), and that the role of gene-environment communication (including that conveyed by cell-cell signalling) is to supplement and to fine-tune these endogenously programmed epigenetic cascades (1), as well as to respond to external physiological parameters.

The fine control of chromatin structure is one of the major hallmarks of eukaryotes and of gene regulation during multicellular development (2). Chromatin architecture is altered by methylation of the DNA and by various types of modifications to histones (the so-called 'histone code'), including compound patterns of methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, carbonylation, deimination and proline isomerisation at various residues (3). The major focus of the field to date has been on how such 'marks' are distributed in relation to features such as active / inactive promoters / transcriptional states and vary in different developmental and disease (e.g. cancer) states (see references 4,5 for examples), studies which are now being extended genome-wide (the 'epigenome') (see reference 6 for an example). However, there has been comparatively little attention paid to how such modifications are differentially regulated and precisely targeted to tens, if not hundreds, of thousands of different genomic loci and positions in different cell lineages during normal (and abnormal) growth and development.

It is becoming apparent that the missing link may be RNA (7,8), and that non-protein-coding RNAs (ncRNAs), which dominate the developmentally-regulated transcriptional output of mammals and other complex organisms, are likely to play a major role in directing relatively generic chromatin-modifying enzymes and complexes to their sites of action, although the mechanisms involved are not at all understood. It is also clear that ncRNAs regulate many other levels of gene

expression during development. Indeed, the mammalian genome, rather than being viewed as islands of protein-coding genes in a sea of evolutionary junk, may be better thought of as an RNA machine (9), wherein the majority is expressed as ncRNAs in a developmentally-regulated manner (for recent reviews, see references 10,11), and that these RNAs are central to the genetic and epigenetic processes that orchestrate the exquisitely precise patterns of gene expression during the ontogeny of complex organisms (1).

Evidence for RNA-Directed Regulation of Chromosome Structure and Chromatin Architecture

The evidence that RNA-directed processes help to orchestrate chromatin architecture and epigenetic memory is growing rapidly and is already compelling. There are only a limited number of enzymes (DNA methylases, histone methylases, acetylases, deacetylases, etc.) and repressive and permissive (Polycomb-group and Trithorax-group) chromatin-modifying complexes involved, none of which are known to have affinity for particular DNA sequences. However, these modifications must be purposefully directed to different positions in different loci in different cells, which implies that there must be another layer of information to guide this process. Although there is evidence of association of chromatin-modifying proteins with transcription factors at promoters (12), this does not account for the highly complex patterns of modifications that are observed both at discrete loci and over entire domains across the genome as a whole (6). In the absence of an army of sequence-specific DNA binding proteins associated with or recognised by chromatin-modifying complexes, the obvious and most logical candidate is RNA.

In support of this, it is known that RNA is an integral component of chromatin (13) and that many of the proteins involved in chromatin modifications have the capacity to bind RNA or complexes containing RNA (Fig. 1). These include DNA methyltransferases and methyl DNA binding domain proteins (14), heterochromatin protein 1 (HP1) (15), domains commonly found in chromatin remodelling enzymes and effector proteins, such as SET, tudor domains and chromodomains (8,16), and the multi-KH domain protein DPP1, which suppresses heterochromatin-mediated silencing in *Drosophila* (17). Interestingly, some transcription factors also appear to have a high affinity for specific RNA-DNA complexes (18). The vast majority of the genomes of all metazoans, from worms to humans, and probably plants, is transcribed, mainly into ncRNAs with complex patterns of overlapping and interlacing sense and antisense transcripts from both strands (see 10,11). While sometimes dismissed as

transcriptional noise, these transcripts potentially provide a rich source of regulatory molecules to guide the epigenetic trajectories of development (1).

RNA plays a central role in DNA methylation and transcriptional silencing, via the RNA interference (RNAi) pathway, both in plants (19,20) and in animals (21), with associated alterations to chromatin structure involving Polycomb recruitment in *Drosophila* (22) and in human cells (23). Heterochromatin formation is also regulated by small RNAs (24), including the PIWI-interacting RNAs (piRNAs), which epigenetically control transposon activity from flies to vertebrates (25). Interestingly it has recently emerged that PIWI not only interacts with heterochromatin protein 1a (HP1a) (26), but is also required for the formation of euchromatin in some subtelomeric regions in *Drosophila* (27). RNAi-related processes, including RNAi-dependent histone modification and recruitment of Polycomb complexes, have been shown to be involved in heterochromatin assembly and chromosome dynamics in fission yeast (28), heterochromatin formation and programmed DNA elimination in *Tetrahymena* (29,30), and heterochromatin formation and nucleolar organisation in *Drosophila* (17,31). The nuclear organisation of chromatin insulators is also affected by the RNAi machinery (32) and recent deep sequencing studies have shown that double-stranded RNAs formed by sense-antisense transcripts originating from inverted repeats, bidirectional / antisense transcripts from retrotransposons, pseudogenes and mRNAs are processed into small RNAs that have regulatory functions, possibly including epigenetic pathways, in mouse oocytes and *Drosophila* somatic cells (33-35).

RNA-directed DNA methylation (RdDM) is well characterised in plants, whereby RNA Pol IV transcripts are processed by DICER-LIKE3-dependent endonuclease to generate 21-24 nucleotide small RNAs that are incorporated into ARGONAUTE4 (36) to guide DRM1/2 methylation activity to the region of genomic DNA homologous to the siRNA sequence (37). Promoter-directed siRNAs can induce promoter methylation and transcriptional gene silencing, allowing plants to regulate transcription of genes during development (38). siRNAs may also be incorporated into the RdDM pathway to silence transposons and repeat portions of the genome (39,40), analogous to the silencing of retrotransposons by piRNAs in animals (25,41). Deep sequencing has revealed that one-third of all methylated DNA sequences correlate with small RNAs in *Arabidopsis* flowers (42). Indeed, the epigenetic plasticity afforded by RdDM may have contributed to the evolution of flowering plants, with a recent study showing that small RNA-mediated RdDM controls the epigenetic differences underpinning phenotypic differences between two closely related *Arabidopsis* ecotypes (43).

Long ncRNAs are also involved in many epigenetic processes. These include RNA-directed programmed genomic rearrangements in ciliates (44), imprinting in mammals and the global activation or repression of sex chromosomes for dosage compensation in insects and mammals, respectively (45), as well as the regulation of ribosomal DNA copy number (46), T-cell receptor recombination (47), and maintenance of telomere integrity (48).

Many regulatory regions affecting chromatin structure and the expression of adjacent protein-coding genes are transcribed in

spatially- and temporally-regulated ways (49,50). At least some of these non-coding transcripts play important roles in the activation of gene expression by targeting global protein regulators such as HP1, Ash1 and the chromatin insulator protein CP190 to the cognate sequences in *cis*-regulatory response elements, including Polycomb- and Trithorax-response elements (PREs and TREs) (22,32,50,51). Proteins of the Polycomb group (PcG) and Trithorax group (TrxG) are important global regulators of transcriptional silencing and activation and mediators of epigenetic memory in development, best characterised in homeotic loci (52). Many PREs and TREs are themselves transcribed as ncRNAs (53) and *Hox* gene loci exhibit complex patterns of non-coding transcripts on both strands (54). The activation of the *HoxA* genes is also accompanied by intergenic antisense ncRNA transcription (55). It was recently shown that over 200 long ncRNAs associated with human *HOX* gene clusters are co-linearly expressed along developmental axes, and that one of these ncRNAs (termed *HOTAIR*), originating from the *HOXC* locus, recruits Polycomb complexes to repress gene expression of the *HOXD* cluster *in trans* (56), indicating that non-coding transcription is not simply altering local chromatin structure (57). Moreover, a recent study of ncRNAs associated with tumour suppressor genes focused on a long antisense RNA associated with the p15 locus and found that it specifically acts to alter histone methylation to silence the expression of the gene (58), with important implications for tumourigenesis. Very few of these ncRNAs (of which there are tens of thousands (10)) have been studied, and there are likely to be many more that are involved in similar pathways. The potential involvement of RNA (and inherited variations in loci encoding these RNAs) in epigenetically-mediated human disease is also presaged by the observation that a particular type of thalassemia involves silencing of the α -globin gene HBA2 and methylation of its associated CpG island early in development, which is mediated by the transcription of an antisense RNA associated with an abnormally juxtaposed gene (59).

A large fraction of the mammalian genome is comprised of transposon-derived sequences. Although often pejoratively referred to as 'repeats' and assumed to be non-functional 'selfish' DNA, many transposon-derived sequences are expressed in interesting patterns in development and appear to play a significant role in developmental regulation (39,60). Recent evidence suggests that tissue-specific transcription of at least some of these repeats functions to organise the locus concerned into nuclear compartments as a developmental strategy to establish functionally distinct domains to control gene activation during development (61). These and other observations of the functionality of transposon-derived sequences (which were first described by McClintock as 'controlling elements') calls into question the assumption that ancient repeats may be used as an index of the rate of neutral evolution (unconstrained sequence drift), and therefore also the derived estimate that only 5% of the human genome is under 'purifying' selection. Indeed, it is possible that most of the mammalian genome is functional and under selection as regulatory (RNA) sequences, albeit under different constraints and selection pressures (especially given the central role of regulatory sequences in adaptive radiation) than those encoding proteins, which are limited by relatively strict structure-function relationships (62).

In general, ncRNAs have lower expression levels than mRNAs, which may not be surprising if their function is regulatory. In yeast, many unstable 'cryptic' ncRNAs are barely detectable by conventional expression analysis, but are upregulated upon depletion of exosome components or cofactors (63). Although initially assumed to be transcriptional noise, it was recently found that the expression of these transcripts can be controlled by chromatin remodelling (64), and that some are exported to the cytoplasm (65). Moreover, it was found that specific 'cryptic' RNAs in yeast, which are regulated during chronological aging, direct the histone deacetylase Hda1 to the *PHO84* locus to repress its expression (66). A similar mechanism may be involved in heterochromatic gene silencing (67) and gene activation (68). Hundreds of ncRNAs 'reminiscent of cryptic transcripts in yeast' have been detected in *Arabidopsis* (69) and it seems likely that there are many rare or cell-specific, short half-life, functional ncRNAs operating to regulate gene expression and chromatin architecture in eukaryotes (70).

RNA Editing

Thus far, we have been concerned only with RNA-directed alterations to chromatin structure during programmed development or developmental abnormalities such as cancer. However, RNA is also involved in the transmission of environmental information into the system and into these endogenous epigenetic networks via RNA editing. RNA editing occurs via two classes of enzymes, the ADARs (one of which, ADAR3, is brain-specific) that catalyse adenosine deamination to inosine (71) and the APOBECs (two of which, APOBEC1 and APOBEC3, are specific to mammals, the latter having been greatly expanded and subjected to positive selection in the primate lineage) that catalyse cytidine deamination to uracil (72,73). RNA editing has been a well-recognised phenomenon throughout metazoan evolution and occurs in most, if not all, tissues, but is particularly active in the brain, with a dramatic increase in the incidence of RNA editing during vertebrate, mammalian and primate evolution (71), almost certainly associated with the development of more advanced cognitive abilities. There are well-characterised iconic examples of RNA editing altering the amino acid sequence and splicing patterns of neurotransmitter receptors, presumably to alter the electrophysiological properties of the synapse. RNA editing has also been shown to alter both microRNAs (miRNAs) and their targets (74), indicating that these fundamental circuits can also be dynamically modulated. One cannot imagine that this is a random process and indeed inositol hexaphosphate is complexed within the active site of ADAR2 (75), strongly implying a link to cell signalling pathways. Moreover, the existence of RNA editing in many tissues implies that environmental information is being fed into RNA-mediated pathways in many different contexts, with every reason to expect that at least some of this information will result in both immediate and longer-term epigenetic effects. It has also recently been shown that there is a global reduction of adenosine to inosine editing and complex gene-specific alterations in editing patterns in tumours versus normal tissues, and that overexpression of ADARs results in a

decreased proliferation rate of glioblastoma cells (76).

Intriguingly, two orders of magnitude more RNA editing is observed in human transcripts than in mouse, the vast majority of which occurs in Alu sequences, which are primate-specific and whose genomic distribution suggests positive selection (see 77). While it is sometimes thought that such editing is a means of silencing retrotransposons (74), most Alu sequences are not active as such, and indeed the vast majority of the ~1 million copies in the human genome are in fact unique sequences (78). An alternative interpretation of these observations is that Alu elements provided an important platform for the expansion of RNA editing in primates, driven by and underpinning the development of higher order cognition (77). Since most of these edited elements occur in non-coding sequences, one presumes that they are largely regulatory, affecting brain development and function. Alu RNAs have been shown to act as transacting transcriptional repressors by binding RNA polymerase II (79) and to be involved in the regulation of alternative splicing, translation and mRNA stability (80). Moreover, non-coding RNA expression appears to be particularly active in the brain (81) and it is known that both RNA transport (82) and epigenetic changes, which are generally RNA-directed, are important in memory formation and in informational transformation / reconfiguration processes in general (83).

Intercellular and Intergenerational Epigenetic Signalling by RNA

Most RNA regulatory circuitry is cell autonomous, but recent evidence suggests that RNA may also convey epigenetic information between cells and across generations. It has been known for some time that the epigenetic phenomenon of co-suppression in plants, which is mediated by the RNAi pathway, can be transmitted systemically following grafting of a transgenic onto a wildtype plant (84). Plants use similar pathways to coordinate normal developmental processes (37). Transport of these RNA signals occurs locally via plasmodesmata or systemically via phloem (85), although recent data suggest that the RNA signals involved may be different from conventional miRNAs or siRNAs (86).

Intercellular RNA signalling may also occur in animals. Most animals have orthologs of the *Caenorhabditis elegans* protein Sid1, a transmembrane protein that is required for the systemic spread of RNA interference and which allows the import of double-stranded RNA (dsRNA) into the cell (for recent reviews, see 87,88). There are two paralogs of Sid1 (SidT1 and SidT2) in mammals. Human SidT1 has been confirmed to similarly import dsRNA across the cell membrane. SidT1 and SidT2 are expressed in most tissues and cell types in humans and mice, and exhibit specific expression patterns in the brain, suggesting that they have specialised RNA transport functions, possibly for different types of RNA substrates (88). There is also evidence for RNA transport between neurons and glial cells, although the mechanism is unknown (89). Parenthetically, given the impermeability of the blood-brain barrier to RNA, it may be that this barrier (and the similar barrier in the testis) functions in part to privilege and segregate from systemic circulation the intercellular RNA signalling networks in

these organs, both of which are known to be rich in RNA expression. There is also increasing evidence that RNA transport may be mediated by circulating microvesicles and that such microvesicles can convey developmentally relevant information (see 88). In addition, RNAs can be transmitted between cells in close contact, including from nurse cells to oocytes in *Drosophila* via 'ring canals', between germ cells in mouse spermatogenesis via 'cytoplasmic bridges', and between human embryonic stem cells via gap junctions (see 88). Finally, genetic analysis suggests that specific genes are required for the transport of dsRNA into the germline and for RNAi-mediated gene silencing of germline-expressed genes in *C. elegans*, including PPW-1, which encodes a PAZ/PIWI domain protein of the Argonaute family, as well as three genes whose function is unknown, but one of which (*rsd-3*) has both mouse and human orthologs and contains an ENTH domain commonly found in proteins involved in vesicle trafficking (90,91).

There are many studies in both plants and animals indicating that epigenetic memory may be heritable in both plants and animals, and suggesting that this process is RNA-directed (92,93), as is epigenetic memory generally. As noted already, parental imprinting is

intimately linked to ncRNAs (45) and it is known that RNAi-mediated gene silencing can be inherited for several generations in *C. elegans* (87). Perhaps the most exciting aspect of this area, and one with the capacity to change our view of inheritance and evolution (93), is the recently described phenomenon of 'paramutation', which involves the allele-specific transfer of epigenetic information to cause the heritable silencing of one allele by another and which appears to involve signalling via RNA in maize and mice (see 92,93). Epigenetic inheritance can also be induced by environmental parameters (94,95) and may even affect population dynamics (96). Recent results show that the phenomenon can also be induced by miRNAs and that it is abrogated in mice lacking the gene *Dnmt2* (F. Cuzin, reported at the New RNA Frontiers Conference, Colmar, France, 2007), which is an RNA methyltransferase with important roles in development (97), indicating that epigenetic modification of RNA is part of the mix. Intriguingly, there is increasing evidence that RNA-coupled DNA 'repair' can also occur in eukaryotes (77,98), suggesting that RNA can direct both epigenetic and genetic modifications and that there is a much more dynamic interplay between genomes and the environment than previously envisioned.

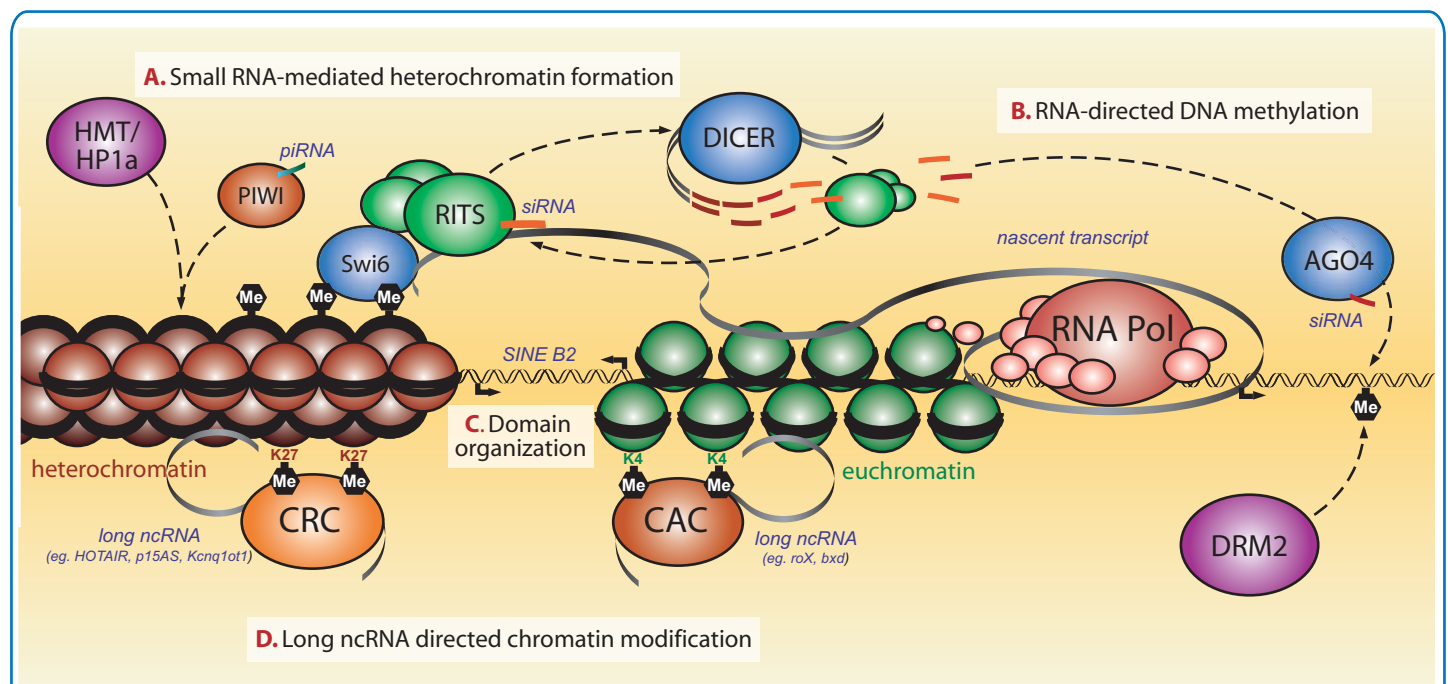


Fig. 1. Simplified representation of RNA-mediated processes that direct chromatin modifications in various eukaryotic organisms.

(A) Small RNAs may direct chromatin modifications. PIWI proteins and piRNAs (PIWI-associated RNAs) interact with HMT/HP1 α (Histone Methyltransferases/Heterochromatin Protein 1 alpha) to induce heterochromatin formation in *Drosophila*. RNA duplexes may be processed in a DICER-dependent manner into siRNAs (short interfering RNAs) that may subsequently direct chromatin modifications, possibly by targeting nascent transcripts or DNA directly. siRNAs may direct histone methylation (Me) via RITS (RNA-induced transcriptional silencing complex) in centromere heterochromatin in fission yeast.

(B) In plants, siRNAs originating from RNA Polymerase 4 transcripts can direct DNA methylation by a DRM2 (Domains Rearranged Methyltransferase 2) dependent mechanism.

(C) The transcription of SINE B2 elements can establish boundaries between euchromatin and heterochromatin domains in mice.

(D) Long ncRNAs (non-coding RNAs) can also recruit chromatin repressor complexes (CRC) or chromatin activating complexes (CAC) to target loci in *cis* or *trans*, thereby regulating the chromatin context of local genes.

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