

TIMELINE

The rise of regulatory RNA

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Abstract | Discoveries over the past decade portend a paradigm shift in molecular biology. Evidence suggests that RNA is not only functional as a messenger between DNA and protein but also involved in the regulation of genome organization and gene expression, which is increasingly elaborate in complex organisms. Regulatory RNA seems to operate at many levels; in particular, it plays an important part in the epigenetic processes that control differentiation and development. These discoveries suggest a central role for RNA in human evolution and ontogeny. Here, we review the emergence of the previously unsuspected world of regulatory RNA from a historical perspective.

RNA has long been at the centre of molecular biology and was likely the primordial molecule of life, encompassing both informational and catalytic functions. Its informational functions are thought to have subsequently devolved to the more stable and easily replicable DNA, and its catalytic functions to the more chemically versatile polypeptides¹. The idea that the contemporary role of RNA is to function as the intermediary between the two had its roots in the early 1940s with the entry of chemists into the study of biology, notably Beadle and Tatum², whose work underpinned the one gene–one enzyme hypothesis (FIG. 1 (TIMELINE)). This idea later matured into the more familiar one gene–one protein concept and became widely accepted despite the prescient misgivings of experienced geneticists, notably McClintock³. The concept that genes encode only the functional components of cells (that is, the ‘enzymes’) itself had deeper roots in the mechanical zeitgeist of the era, which was decades before the widespread understanding of the use of digital information for systems control.

Although the one gene–one protein hypothesis has long been abandoned owing to the discovery of alternative splicing in the 1970s, the protein-centric view of molecular biology has persisted. Such persistence was aided by phenotypic and ascertainment bias towards protein-coding mutations in genetic studies and by the assumption that these

mutations affected *cis*-acting regulatory protein-binding sites⁴. However, this view was challenged by the discovery of nuclear introns and RNA interference (RNAi), as well as by the advent of high-throughput sequencing, which led to the identification of large numbers and different types of large and small RNAs, the functions of which are still under investigation.

“emerging evidence suggests ... that the amount and type of gene regulation in complex organisms have been substantially misunderstood”

In this Timeline article, we examine the history of, and report the shift in thinking that is still underway about, the role of RNA in cell and developmental biology, especially in animals. The emerging evidence suggests that there are more genes encoding regulatory RNAs than those encoding proteins in the human genome, and that the amount and type of gene regulation in complex organisms have been substantially misunderstood for most of the past 50 years.

Early ideas for the role of RNA

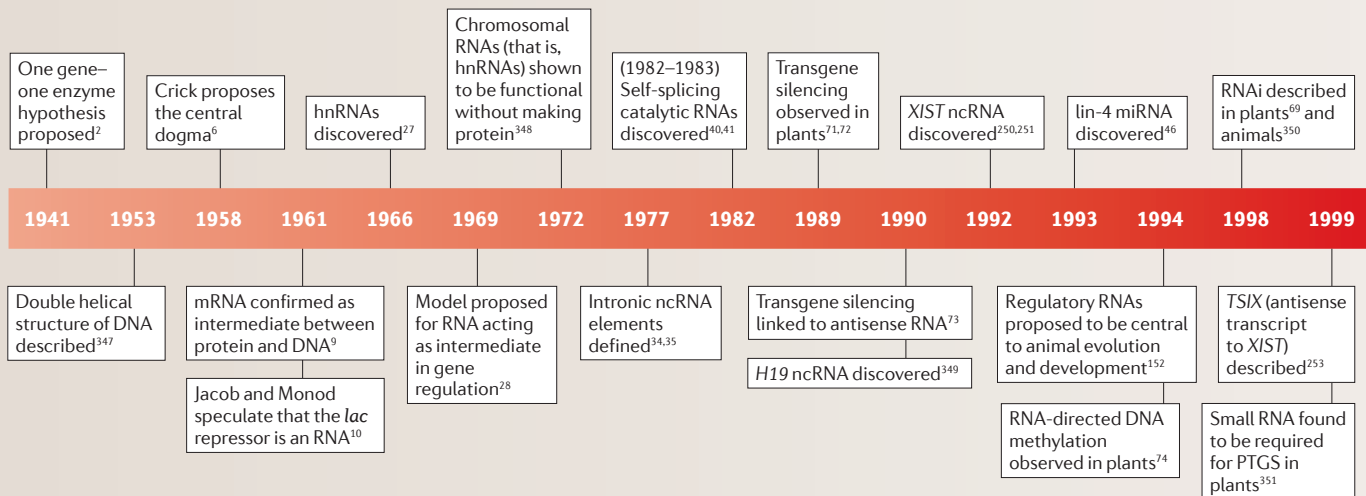
RNA — the central dogma and gene regulation. After the elucidation of the double

helical structure of DNA in 1953 (REF. 5), the following years were preoccupied with deciphering the ‘genetic code’ and establishing the mechanistic pathway between genes and proteins: the identification of a transitory template (mRNA), an adaptor (tRNA) and the ribosome ‘factory’ comprised of ribosomal RNAs and proteins for translating the code into a polypeptide. In 1958, Crick published the celebrated central dogma to describe the flow of genetic information from DNA to RNA to protein, which has proved remarkably accurate and durable, including the prediction of reverse transcription⁶. Nonetheless, in conceptual terms, RNA was tacitly consigned to be the template and an infrastructural platform (with regard to rRNAs and tRNAs) for protein synthesis or has at least been interpreted in this way by most people since that time.

In the mid-1950s, the link was established between rRNA (which is highly expressed in essentially all cells) and the structures termed ribosomes as the platform for protein synthesis⁷. The roles of tRNA and mRNA were experimentally confirmed in 1958 (REF. 8) and 1961 (REF. 9), respectively. The latter occurred in the same year that Jacob and Monod published their classic paper on the *lac* operon of *Escherichia coli*¹⁰, which was the first locus to be characterized at the molecular genetic level. These studies confirmed that at least some, but presumably most, genes encoded proteins and supported the emerging idea that gene expression is controlled by regulating the transcription of the gene, as indicated by the locus encoding the *lac* repressor in the repressor–operator model. At the time, Jacob and Monod did not know the chemical identity of the repressor and speculated in passing that it “may be a polyribonucleotide” (that is, RNA)¹⁰. However, Gilbert later showed that the repressor is a polypeptide that allosterically binds to the lactose substrate, and the brief idea faded¹¹.

These studies reinforced and extended the concept that proteins are not only enzymes but also the primary analogue components and control factors that constitute the cellular machinery. This, in turn, has led to the prevailing transcription factor

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AGO, Argonaute; AIR, also known as *AIRN* (antisense of *IGF2R* non-protein coding RNA); CRISPR, clustered regularly interspaced short palindromic repeat; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; ENCODE, Encyclopedia of DNA Elements; EZH2, enhancer of Zeste 2; H19, H19 imprinted maternally expressed transcript; HDAC1, histone deacetylase 1; hnRNA, heterogeneous nuclear RNA; *HOTAIR*, HOX transcript antisense RNA; lncRNA, long non-coding RNA; miRNA, microRNA; ncRNA, non-coding RNA; piRNA, PIWI-interacting RNA; PRC2, Polycomb repressive complex 2; PTGS, post-transcriptional gene silencing; RNAi, RNA interference; TGS, transcriptional gene silencing; tiRNA, transcription initiation RNA; XIST, X inactive specific transcript.

paradigm of gene regulation, including the derived assumption that combinatorial interactions would provide an enormous range of regulatory possibilities¹² that are more than enough to control human ontogeny. However, this assumption has not been substantiated theoretically or mechanistically, and both the observed scaling of regulatory genes and the extent of the regulatory challenge in programming human developmental architecture seem to be different from these expectations¹³. In this context, it is noteworthy that genome-wide association studies have shown that most haplotype blocks influencing complex diseases are outside the known boundaries of protein-coding genes¹⁴.

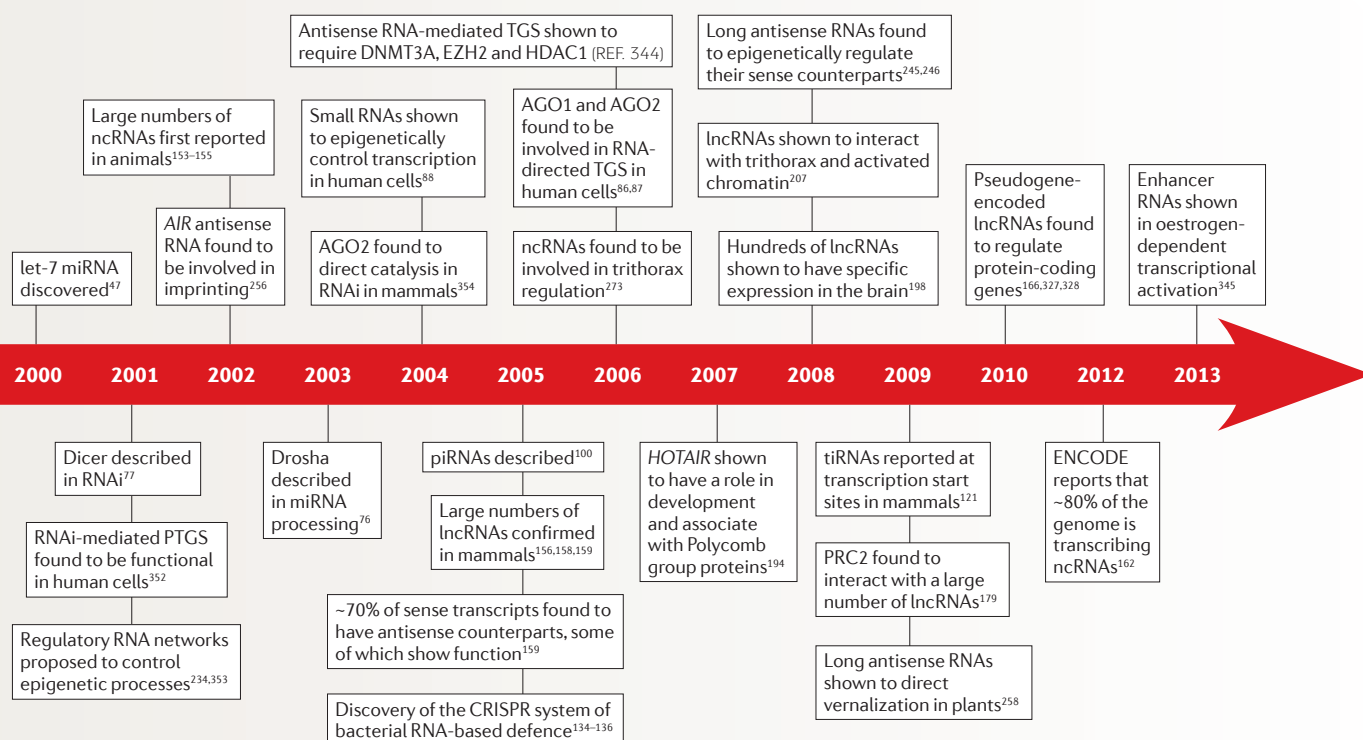
Small nuclear RNAs and small nucleolar RNAs. Following the discovery and functional description of tRNAs and rRNAs, new classes of common small RNAs in the nucleus were identified by biochemical fractionation¹⁵. Many of these small RNAs were found to be part of ribonucleoprotein (RNP) complexes (reviewed in REF. 16). One class — the small nuclear RNAs (snRNAs) (FIG. 2) — was later found to be a central cofactor in RNA splicing¹⁷ (see below) and was therefore given the newer designation

as spliceosomal RNAs. The snRNAs U1, U2, U4, U5 and U6 participate in various RNA–RNA and RNA–protein interactions in the assembly and function of canonical spliceosomes: U1 and U2 recognize the 5' splice site and the branch point, respectively, followed by the recruitment of U4, U5 and U6, which displace U1 and interact with U2 (through U6) as well as the 5' and 3' splice sites (through U5)¹⁸. A set of less abundant snRNAs (U11, U12, U4atac and U6atac) and U5 are found in a variant 'minor' spliceosome termed U12-type¹⁹.

Other small RNAs were found to be localized to the nucleolus and to guide the methylation (the box C/D subclass) and pseudouridylation (the box H/ACA subclass) of rRNAs, tRNAs and snRNAs^{20–22} (FIG. 2). The chemical modifications of rRNAs, tRNAs and snRNAs proved to be essential in ribosomal and cellular function, particularly in tRNA and mRNA maturation, and in pre-mRNA splicing (which requires modification of the U2 snRNA). Notably, the disruption of small nucleolar RNAs (snoRNAs) was found to cause a loss of processing of the 5.8S, 18S and 28S (or 25S in plants) rRNAs²⁰. Early studies found that some snoRNAs are subject to parental imprinting and/or differentially expressed (for example, in the

brain^{23,24}), and that they seem to target a wide range of RNAs (including mRNAs²⁵), which suggests a regulatory role. Related small RNAs have also been identified in subnuclear structures called Cajal bodies (which process telomerase RNA), and these were termed small Cajal body-specific RNAs (scaRNAs)²⁶. However, none of these studies suggested anything other than that the role of RNA was limited to protein synthesis.

The emergence of heterogeneous nuclear RNAs. The first hint that RNA may have additional roles in complex organisms was the discovery of heterogeneous nuclear RNA (hnRNA)²⁷ and the observation that the complexity of this population, as determined by denaturation–renaturation hybridization kinetics, was much greater in the nucleus than in the cytoplasm. The existence of hnRNA and the concomitant discovery of the large amount of repetitive sequences (that is, different classes of retrotransposon sequences with similar composition that occupy large portions of plant and animal genomes) led Britten and Davidson to speculate in 1969 that animal cells contain extensive RNA-based regulatory networks^{28–30}. Although this hypothesis attracted a great deal of interest at the time, it also quickly



lapsed. Its proponents did not revisit the hypothesis even after the subsequent discovery of introns (see below) and instead focused on regulatory networks controlled by transcription factors^{31,32} or on the importance of transposons in protein evolution³³.

The discovery of introns. The discovery of introns in 1977 (REFS 34, 35) was perhaps the biggest surprise in the history of molecular biology³⁶ (FIG. 1 (TIMELINE)), as no one expected that the genes of higher organisms would be mosaics of coding and non-coding sequences, all of which are transcribed. However, the prevailing concept of the flow of genetic information was not overly disturbed, as the removal of the intervening sequences (that is, introns) and the reconstruction of a mature mRNA by splicing preserved the conceptual status quo; that is, genes still made proteins. In parallel, it was assumed that the excised intronic RNAs were simply degraded, although the technology of the time was too primitive to confirm this. In any case, introns were immediately and universally dismissed as genomic debris, and their presence was rationalized as evolutionary remnants involved in the prebiotic modular assembly of protein-coding RNAs that have remained (and been

expanded by transposition) in complex organisms³⁷. This notion was consistent, at least superficially, with the implication of the C-value enigma that eukaryotes contained varying amounts of DNA ‘baggage’. It is also in agreement with the accompanying conclusion that retrotransposon sequences are mainly ‘selfish’, parasitic DNA^{38,39}.

RNA as a catalyst. A few years later, Cech, Altman and colleagues demonstrated that RNA itself was capable of enzymatic catalysis (that is, they are ribozymes)^{40,41}, which provided evidence in support of the RNA early hypothesis. They also showed that RNA catalysis exists and has persisted in particular contexts, notably at the core of RNA splicing⁴² and mRNA translation⁴³. This finding reinforced both the mechanical concept of molecular biology and the role of RNA as the platform for protein synthesis, but did not give any hint of RNA as a widespread regulatory factor, although that possibility is perfectly feasible. Indeed, there is increasing evidence that catalytic RNA exists in animal and plant cells, in introns, untranslated regions (UTRs) and elsewhere, and that these RNAs may have various roles, for example, in the regulation of post-transcriptional cleavage reactions^{44,45}.

The small RNA revolution

The discovery of microRNAs. In 1993, Ambros and colleagues showed the first evidence for small (~22-nucleotide) regulatory RNAs with the discovery of the genetic loci *lin-4* and *let-7*, which regulate the timing of *Caenorhabditis elegans* development^{46,47} (FIG. 1 (TIMELINE)). Although *let-7* is highly conserved from nematodes to humans⁴⁸, very few microRNAs (miRNAs) were discovered genetically^{49,50}, and these RNAs remained interesting idiosyncrasies until the discovery of RNAi (see below). This discovery led to the targeted cloning after size selection of many more miRNAs^{51–53} and the demonstration that these miRNAs act, at least partly, by imperfect base-pairing — typically with the 3′UTRs of target mRNAs — to inhibit their translation and to accelerate their degradation⁵⁴.

Current databases list large numbers of evolutionarily widespread miRNAs⁵⁵, almost all of which had evaded prior detection by genetic screens but many were subsequently validated by reverse genetics. Although many miRNAs can be identified by conservation, it is also evident that many are tissue and lineage specific^{56,57}, and that there may be many more to be discovered.

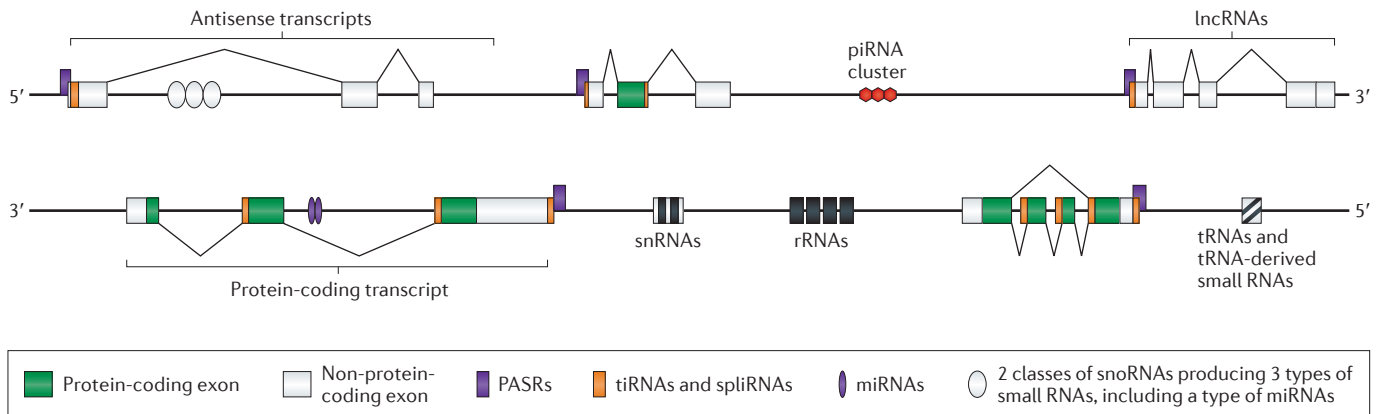


Figure 2 | Complex expression of the genome and examples of non-coding RNA expression. The mammalian transcriptional landscape is represented graphically with genes expressing ribosomal RNAs, tRNAs, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), various protein-coding and non-coding genes (which encode mRNAs and long non-coding

RNAs (lncRNAs), respectively), as well as genes expressing small regulatory RNAs such as microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), promoter-associated short RNAs (PASRs), transcription initiation RNAs (tiRNAs) and splice site RNAs (spliRNAs), snoRNA-derived small RNAs and tRNA-derived small RNAs. The transcriptional units are not depicted to scale.

There is also evidence that many, if not most, protein-coding transcripts are targets for miRNA regulation^{58,59}. In some cases, miRNAs can regulate large numbers of target mRNAs⁶⁰ and, reciprocally, many mRNAs contain target sites for a large number of miRNAs⁶¹, although the implied regulatory logic of this complex multiplex arrangement has not been explained. The targets of miRNAs are usually thought to be mRNAs but may also include other types of RNAs⁶². Biologically, miRNAs have been shown to regulate many physiological, developmental and disease processes, including pluripotency⁶³, epithelial–mesenchymal transition and metastasis⁶⁴, testis differentiation⁶⁵, diabetes⁶⁶, and neural plasticity and memory⁶⁷, among others⁶⁸.

The RNA interference pathway. miRNAs are only one aspect of the phenomenon of RNAi, which silences gene expression after the introduction of sense–antisense RNA pairs. This process was discovered in 1998 in plants⁶⁹ and *C. elegans*⁷⁰ (FIG. 1 (TIMELINE)). These discoveries were presaged by the curious phenomenon of transgene silencing, which is mainly found in plants^{71,72} and linked to both antisense RNA and small RNA-directed DNA methylation, thus indicating transcriptional and post-transcriptional silencing^{73,74}. Mechanistic analyses of these silencing mechanisms showed that exogenous double-stranded RNA (dsRNA) is processed into short fragments (known as small interfering RNAs (siRNAs)) with similar sizes to miRNAs, which implies that miRNAs may represent a similar endogenous system.

This hypothesis was confirmed and led to the elucidation of natural dsRNA precursors in stem–loop structures⁷⁵, as well as the identification of key genes and enzymes involved in their biogenesis and function, notably Drosha⁷⁶, Dicer⁷⁷ and several Argonaute (AGO) proteins⁷⁸. AGO proteins were already known to have central roles in differentiation and development⁷⁹ but are now known to also be involved in defence against RNA viruses in many organisms⁸⁰. Drosha and exportin 5 are involved in the cleavage and export of dsRNA precursors from the nucleus to the cytoplasm⁷⁶, where they are further processed by Dicer to small (21–24-nucleotide) dsRNA moieties. One strand of the dsRNA is loaded into the AGO component of the RNA-induced silencing complex (RISC), which also comprises other proteins⁷⁷. The RISC is guided by the small RNA strand to complementary RNA targets, which are subsequently silenced by translational repression and/or RNA destabilization^{81,82} (FIG. 3).

Although still under discussion, the current view is that siRNAs (and short hairpin RNAs (shRNAs)) — which seem to naturally occur more commonly in plants — act primarily by perfect base-pairing and by AGO-mediated cleavage of complementary target RNAs; hence, they are used widely as experimental tools and potential therapeutic agents⁸³. By contrast, miRNAs have incomplete homology with their target sequences and act primarily at the translational level^{81,82} (FIG. 3).

Both miRNAs and siRNAs are thought to act post-transcriptionally in the cytoplasm, but the existence of AGO in the nucleus^{84–87} and the role of the RNAi pathway in

epigenetic modulation⁸⁸ suggest that the system is more complex and multifaceted than expected. For example, it has been shown that miRNA isoforms are developmentally regulated⁸⁹, that the target ‘seed’ sequence is only one factor in target recognition^{90,91} and that miRNAs can also impose transcriptional gene silencing⁹² (FIG. 3). There is also growing evidence of intersecting pathways, such as RNA editing and modification, in these networks^{93–96}.

PIWI-associated small RNAs. Although most AGO proteins are expressed ubiquitously and associate with both miRNAs and siRNAs, there is a subclass of AGO proteins termed PIWI that are required for germ cell development^{97–100}. PIWI and PIWI-like proteins associate with a distinctive class of small (26–30-nucleotide) RNAs termed PIWI-interacting RNAs (piRNAs), which epigenetically and post-transcriptionally silence transposons in germ cells^{101–110}. PIWI is found predominantly in the nucleus¹¹¹, colocalizes in an RNA-dependent manner with Polycomb group proteins¹¹² and seems to be expressed in other tissues (including the brain¹¹³), which suggests a role beyond genome protection in epigenetic processes^{114,115}.

Other classes of small RNAs in eukaryotes. The molecular genetics, biochemistry and structural biology of the RNAi system are still being unravelled but indicate an ancient, widespread and multilaterally adapted system that controls many cellular processes, the dimensions of which are still being explored. These include potentially lineage-specific

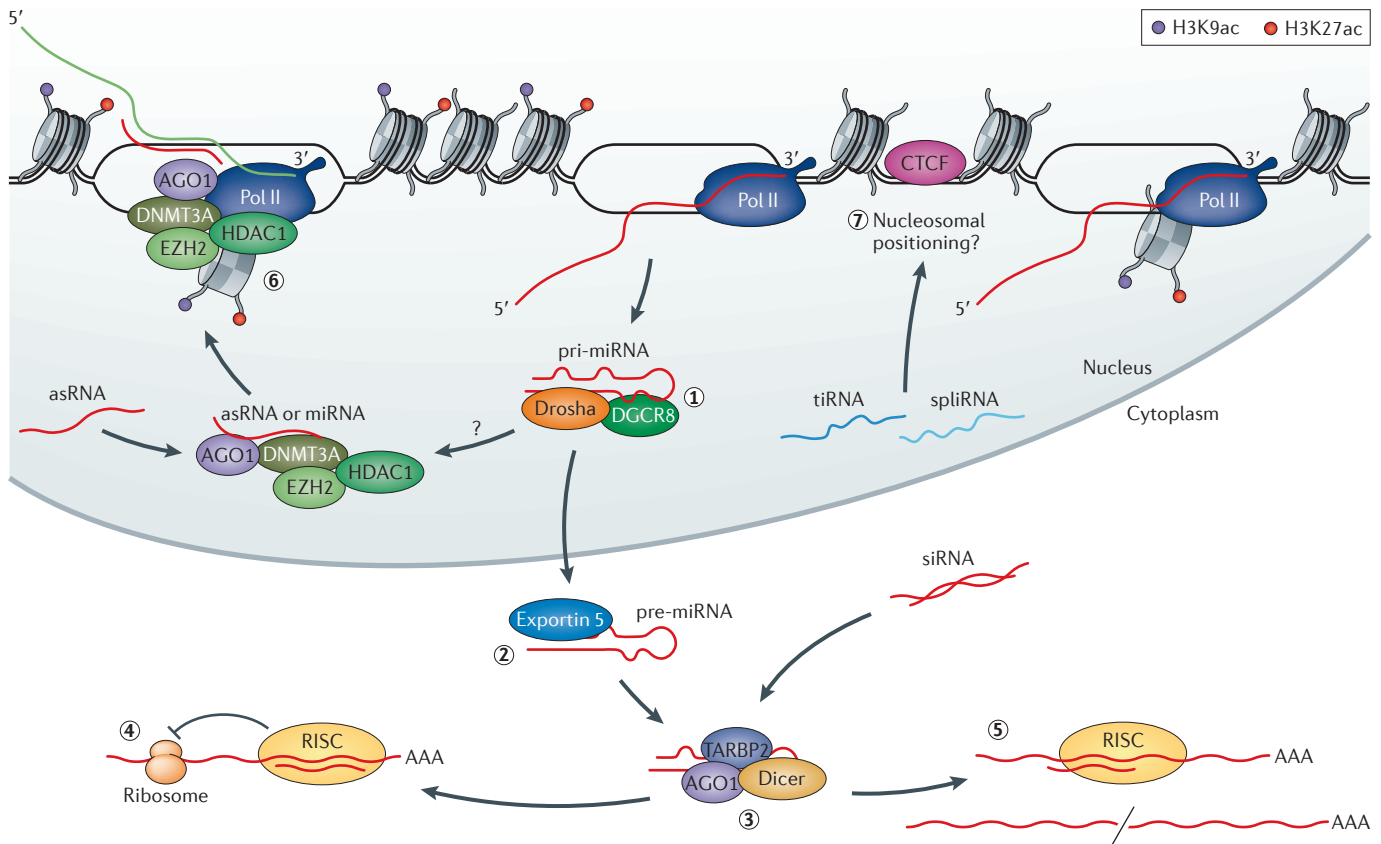


Figure 3 | Functional pathways of small regulatory RNAs. MicroRNA (miRNA) precursors (that is, pri-miRNAs) are expressed as stem-loop structures⁷⁵, which interact with Drosha⁷⁶ and DGCR8 (also known as Pasha) (step 1). They are then processed into pre-miRNAs and exported from the nucleus by exportin 5 (step 2). These transcripts are further processed by Dicer to small (21–23-nucleotide) double-stranded RNAs, one strand of which is loaded into the Argonaute (AGO) component of the RNA-induced silencing complex (RISC) (step 3). Exogenously introduced small interfering RNAs (siRNAs) can also be processed by RISC. The endogenous miRNA or siRNA, or exogenously added siRNA, can then target the repression of translation (step 4) and/or cleavage of homology-containing transcripts^{81,82} (step 5). Some small RNAs are

functional in the nucleus. Exogenously introduced small antisense RNAs (asRNAs) can induce epigenetic silencing of targeted loci^{88,342,343} — a pathway that miRNAs may also use in the nucleus⁹² (step 6). Transcription initiation RNAs (tiRNAs) and splice site RNAs (spliRNAs)^{121,122} are expressed through an unknown pathway that may involve RNA polymerase II (Pol II) backtracking and TFIIS cleavage¹²³ (not shown); tiRNAs and spliRNAs are shown to modulate CTCF-binding factor (CTCF) chromatin localization and to be associated with nucleosome positioning¹²⁴ (step 7). DNMT3A, DNA (cytosine-5)-methyltransferase 3A; EZH2, enhancer of Zeste 2; H3K9ac, histone H3 lysine 9 acetylation; HDAC1, histone deacetylase 1; TARBP2, RISC-loading complex subunit TARBP2 (also known as TRBP).

variations such as the 21U RNAs in *C. elegans*¹¹⁶. Surprisingly, it seems that all snoRNAs from fission yeast to humans produce at least three different subclasses of small RNAs¹¹⁷, one of which has the same size and functions as miRNAs¹¹⁸, and another that is similar in size to piRNAs¹¹⁷. There are also intriguing and recurring reports of tRNA fragments that are produced in tissue-specific patterns¹¹⁹ and that are associated with AGO proteins¹²⁰.

More recently, deep sequencing of small RNA populations has revealed the existence of two other classes of small RNAs in animals but not in plants, which are 17–18 nucleotides in length and associated with transcription initiation¹²¹ and splice sites¹²² (termed transcription initiation RNAs

(tiRNAs) and splice site RNAs (spliRNAs), respectively) (FIG. 3). The origin and function of these RNAs are uncertain, but preliminary evidence suggests that they play a part in nucleosome positioning¹²³ and/or in other levels of chromatin organization¹²⁴. There are also other reports of less distinct classes of promoter-associated RNAs called promoter-associated short RNAs (PASRs)¹²⁵, transcription start site-associated RNAs (TSSa-RNAs)¹²⁶ and promoter upstream transcripts (PROMPTS)¹²⁷, some of which may have a role in RNA-directed transcriptional gene silencing¹²⁸.

Regulatory RNAs in bacteria and archaea. Many small regulatory RNAs have been identified in bacteria, in which

they regulate a wide variety of adaptive responses. Bacterial small regulatory RNAs generally function by simple antisense mechanisms to regulate translation or stability of target mRNAs through altering their secondary structure to expose or sequester *cis*-acting sites^{129,130}. Studies in bacteria have also identified *cis*-acting regulatory RNA sequences known as riboswitches, which act allosterically by binding metabolites to regulate gene expression^{131,132} and almost certainly exist as part of the RNA regulatory landscape in all kingdoms of life.

Very recently, the bacterial and archaeal kingdoms have once again surprised us with the sophistication of their molecular machinery. Many bacterial and most

archaeal genomes have loci comprised of regularly spaced repeats that are interspersed by other virus-derived DNA sequences^{133–136} (termed clustered regularly interspaced short palindromic repeats (CRISPRs)). These loci act as an innate immune system by incorporating fragments of viral DNA between the repeats, which are then transcribed and processed to produce small guide RNAs that are linked to their effector complexes through the repeat sequence and that target and destroy viral DNA^{137–140} or RNA¹⁴¹. This system has recently been adapted for RNA programmable sequence-specific genome manipulation in eukaryotes (including mammals^{142–145}) with extraordinary versatility, including targeted gene excision and fusion, as well as engineered CRISPRs that can recruit silencing and activating proteins to target loci^{146–150}. Moreover, the biological ‘arms race’ continues, as bacteriophages encode their own CRISPR system to evade host innate immunity¹⁵¹.

Long non-coding RNAs

The eukaryotic transcriptome. Noting that the density and size of introns (and, as it turned out later, intergenic sequences) increased with developmental complexity, Mattick posited in 1994 that introns had evolved to express an expanding range of *trans*-acting regulatory RNAs (FIG. 1 (TIMELINE)). He postulated that some genes subsequently evolved to express only intronic or exonic regulatory RNAs, and that this RNA-based regulatory system was the essential prerequisite for the emergence of developmentally complex organisms¹⁵². Subsequently, the application of genome tiling array technology and deep sequencing to the characterization of the transcriptome showed that tens of thousands of loci in mammals express long transcripts that do not encode proteins, which are located intergenic, intronic and antisense to protein-coding genes. The initial findings^{153–155} were confirmed in 2005 (REFS 156–159) and extended by the Encyclopedia of DNA Elements (ENCODE) project^{160–162}, all of which showed that the vast majority (at least 80%) of the human and mouse genomes are differentially transcribed in one context or another; other studies also reported similar findings in all organisms examined. Indeed, it seems that most intergenic and, by definition, intronic sequences are differentially transcribed, and that the extent of the transcriptome therefore expands with developmental complexity¹⁶³.

Using more focused deep sequencing methodologies, it has become evident that the full range of the protein-coding and non-protein-coding transcriptome is still vastly under-sampled¹⁶⁴. In addition, many transcripts are not polyadenylated and represent a largely different sequence class^{156,165}, some of which seem to be relevant to development (for example, the *POU5F1* (also known as *OCT4*) transcript^{166,167}). Moreover, 95% of human transcription initiation sites are not associated with mRNA transcription but rather mainly with transcription of non-polyadenylated non-coding RNAs (ncRNAs)¹⁶⁸. These non-polyadenylated transcripts are so far mostly uncharacterized because of the historical use of poly(A) tails to remove the overwhelming rRNA contamination in RNA preparations. This issue is being alleviated by more sophisticated approaches such as cap trapping¹⁶⁹, oligonucleotide subtraction¹⁷⁰ and array capture^{164,171}.

Defining long non-coding RNAs. Long ncRNAs (lncRNAs) are operationally defined as non-protein-coding RNAs that are >200 nucleotides in length, which corresponds to a convenient cutoff in biochemical fractionation and excludes all known classes of small RNAs¹⁷². Transcripts are classified as non-coding if they lack long open reading frames (traditionally >100 codons) and/or do not show codon conservation, although there was considerable uncertainty, as genomic and transcriptomic data were initially limited for comparison. However, recent analyses provide strong evidence that most annotated lncRNAs do not encode proteins; nonetheless, some specify small proteins that had not been identified previously using bioinformatic approaches^{173–175}.

These ncRNAs can be parsed into intronic, antisense or intergenic (that is, large intergenic non-coding RNA (lincRNA)) subsets in experimental studies and databases^{159,176,177}, partly because of mechanistic expectations¹⁷⁸ and because of a desire to reduce ambiguity and overlap with protein-coding loci in functional analyses^{179–181}. However, there is no evidence of any intrinsic difference between RNAs that are intronic, intergenic or antisense, or that overlap with protein-coding transcripts (FIG. 2), for example, in their interaction with chromatin-activating or chromatin-repressive complexes (see below). Nonetheless, ncRNA subclasses will inevitably exist and be defined, some of which may be biased in relation to genomic origin.

Long non-coding RNAs: transcriptional noise or functional? The unexpected discovery of large numbers of non-coding transcripts in eukaryotes, some of which span tens or hundreds of kilobases¹⁸², led to debates about their functionality^{183,184}. In particular, as many lncRNAs were shown to have fairly low evolutionary conservation and low levels of expression, some have posited that they represent transcriptional noise and/or redundant transcripts with no biological importance. This hypothesis remains, at least partly, a possibility. Nevertheless, lncRNAs show a wide range of evolutionary conservation, from ultraconserved ones¹⁸⁵ to primate-specific ones^{186–188}, which can be explained as the result of different structure–function constraints and lineage-specific adaptive radiation¹⁸⁹. Indeed, there is now considerable evidence that lack of primary sequence conservation in lncRNAs does not indicate lack of function^{190,191}, and many lncRNAs show evidence of structural conservation^{192,193}.

Loci that express lncRNAs show all of the hallmarks of bona fide genes⁴, including conservation of promoters¹⁶⁹, indicative chromatin structure¹⁹⁴, and regulation by conventional morphogens and transcription factors¹⁹⁵. Moreover, lncRNAs were found to have a similar range of cellular half-lives as mRNAs¹⁹⁶ and to be differentially expressed in a tissue-specific manner^{158,197}, especially in the brain¹⁹⁸. The study in the brain showed that, although the expression levels of many lncRNAs seem to be lower than those of mRNAs in whole tissues, lncRNAs are highly expressed and easily detectable in particular cell types¹⁹⁸. In addition, lncRNAs were found to have, on average, higher cell specificity than proteins^{165,199}; this is consistent with their proposed role in architectural (as opposed to ‘cell-type’) regulation, in which each cell has a unique positional identity in precisely sculpted organs, bones and muscles²⁰⁰.

Many lncRNAs are alternatively spliced²⁰¹, which is further evidence of the precision of their expression and is hard to reconcile with the suggestion that they are simply transcriptional noise. It should also be noted that some functionally validated lncRNAs can have isoforms that encode proteins²⁰² and that, reciprocally, some (perhaps many) mRNAs have intrinsic functions as *trans*-acting regulatory RNAs^{203–205}. In some contexts, 3'UTRs can be separately expressed and convey genetic functions in *trans*²⁰⁴, and both lncRNAs and mRNAs may be further processed to produce subsidiary species²⁰⁶.

lncRNAs have been shown to be dynamically expressed in a range of differentiating systems, including embryonic stem cells²⁰⁷, muscles²⁰⁸, T cells²⁰⁹, breast tissues^{210,211}, the erythroid system²¹¹ and neurons^{212–214}, as well as in cancer and other diseases^{210,215–222}. Such dynamic expression of lncRNAs is at least partly controlled by conventional transcription factors^{195,213}.

Emerging roles of non-coding RNAs

The validation of ncRNA functions has so far mainly relied on knockdown of candidate ncRNAs. Knockdown of ncRNA expression has proved to be surprisingly easy using chemically engineered antisense oligonucleotides, or using siRNA- or shRNA-mediated approaches, frequently resulting in phenotypic changes in cultured cells, in which most studies have been carried out.

Development and differentiation. Many small ncRNAs^{63–65} and most functionally analysed lncRNAs²²³ seem to have a role in the regulation of differentiation and development. On the basis of studies in cell culture, these include the regulation of apoptosis and metastatic processes^{211,218,220,221,224}, retinal and erythroid development^{211,225}, breast development^{210,226} and epidermal differentiation²²⁷, among many others. Antisense knockdown of some lncRNAs in zebrafish and deletion of sequences that specify lncRNAs in mice have resulted in visible developmental defects^{181,191,228,229}. However, knockouts of the widely expressed nuclear paraspeckle assembly transcript 1 (*Neat1*)²³⁰ or of some of the most highly conserved sequences in the mammalian genome²³¹ have not shown any detrimental effect on development. These results suggest that more sophisticated phenotypic screens are required to delineate functions, especially cognitive ones, because most mammalian lncRNAs are expressed in the brain¹⁹⁸ and many are specific to mammals or primates^{188,232}. A good example is brain cytoplasmic RNA 1 (*BC1*) — a retrotransposon-derived lncRNA that is widely expressed in the brain — the knockout of which causes no visible anatomical abnormality but leads to behavioural changes that would be lethal in the wild²³³.

Epigenetic roles. Consistent with their roles in differentiation and development, a range of genetic and biochemical evidence suggests that a major function of many small RNAs and lncRNAs is the regulation of epigenetic processes^{234,235}, probably by guiding chromatin-modifying enzymes to their sites

of action and/or by acting as scaffolds for chromosomal organization^{179,235–238} (FIG. 4).

RNAs were shown to induce transcriptional gene silencing first in plants^{74,239}, then in fungi²⁴⁰ and human cells⁸⁸, and both small RNAs and the RNAi machinery were implicated in the underlying epigenetic processes^{240–242}. These studies were consistent with the observations that small RNAs interact with Polycomb group proteins²⁴³ and that AGO proteins are found in the nucleus^{86,87} (FIG. 3). In parallel, dating back to 1990, antisense RNAs were shown to affect gene expression, again initially in plants⁷³ and later in animals^{159,166,244–246}. Similar to small ncRNAs²⁴⁷, some lncRNAs have been shown to control alternative splicing^{248,249}. Other naturally occurring lncRNAs were shown to control epigenetic processes *in vivo*, notably in X chromosome dosage compensation^{250–254} and parental imprinting in mammals^{255–257}, and vernalization in plants²⁵⁸. Subsequent studies showed that intergenic and antisense RNAs bind to Polycomb repressive complexes (PRCs)^{194,259–261}, to trithorax chromatin-activating complexes and activated forms of histones²⁰⁷, and to DNA

methyltransferases^{201,262,263}. These observations were writ large in 2009 when it was shown that ~20% of ~3,300 lncRNAs examined were bound by PRC2 and that others were bound by different chromatin-modifying complexes. siRNA-mediated knockdown of PRC2-associated lncRNAs was found to result in gene expression changes, and the upregulated genes were enriched for those normally silenced by PRC2 (REF. 179). Polycomb group proteins were also discovered to bind to RNA with high affinity but low specificity²⁶⁴, which is consistent with the idea that many RNAs interact with these proteins.

One of the notable lncRNAs to emerge — HOX transcript antisense RNA (*HOTAIR*) — is derived from the *HOXC* locus and regulates *HOXD* in *trans*¹⁹⁴. It is involved in cancer metastasis²²⁰ and, when inactivated, results in homeotic transformation *in vivo*²²⁹. lncRNAs have also been shown to act as scaffolds for the assembly of histone modification complexes²⁶⁵, and the widespread alternative splicing of these RNAs suggests that the cargo and/or target specificity can be varied in a context-dependent and differentiation-specific manner.

Glossary

Antisense RNA

A single-stranded RNA that is complementary to an mRNA or a gene.

Encyclopedia of DNA Elements

(ENCODE). An international consortium involved in building a comprehensive list of functional elements in the human genome.

Heterogeneous nuclear RNA

(hnRNA). A type of RNA that is similar to mRNA or pre-mRNA but that is retained predominantly in the nucleus.

Introns

A term first coined by Gilbert to describe the RNA regions that are removed, by being spliced out, to produce mRNAs.

PIWI-interacting RNAs

(piRNAs). Small RNAs that are associated with the PIWI protein complex and that emanated from transposon-like elements

RNA CaptureSeq

A method that combines the ability to capture RNA (that is, to isolate and enrich for certain types of RNA) with deep sequencing technology to mine the human transcriptome.

RNA-directed DNA methylation

An epigenetic process whereby processed double-stranded small (21–24-nucleotide) RNAs guide the methylation of homologous DNA loci.

Small interfering RNAs

(siRNAs). Small interfering, double-stranded RNAs that can be used to suppress homology-containing transcripts in a transcriptional and post-transcriptional manner.

Splice site RNAs

(spliRNAs). Small RNAs that are derived from the 3' ends of exons adjacent to splice sites and that are similar to transcription initiation RNAs (tiRNAs).

Transcriptional gene silencing

The regulation of a gene at the transcriptional level, in contrast to post-transcriptional gene silencing, in which silencing of gene expression occurs at the mRNA or translational level, after transcription has occurred.

Transcription initiation RNAs

(tiRNAs). Small RNAs associated with promoters with peak density at ~15–35 nucleotides downstream of transcription start sites.

Transinduction

A genetic phenomenon whereby mRNA transcription induces transcription of nearby enhancers and intergenic non-coding RNAs.

Transposons

Mobile genetic elements with evolutionary links to retroviruses.

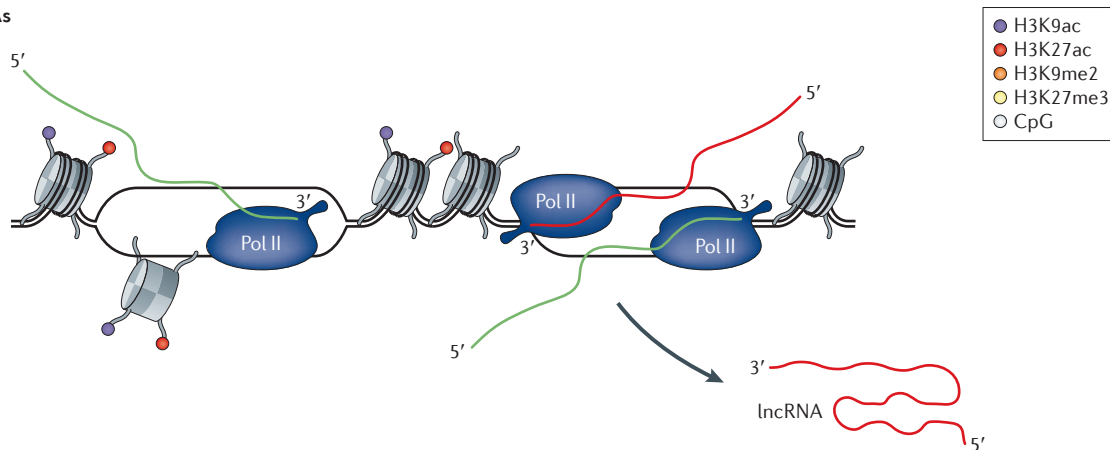
Transvection

A genetic phenomenon whereby non-coding regions can induce transcription of coding regions on other chromosomes.

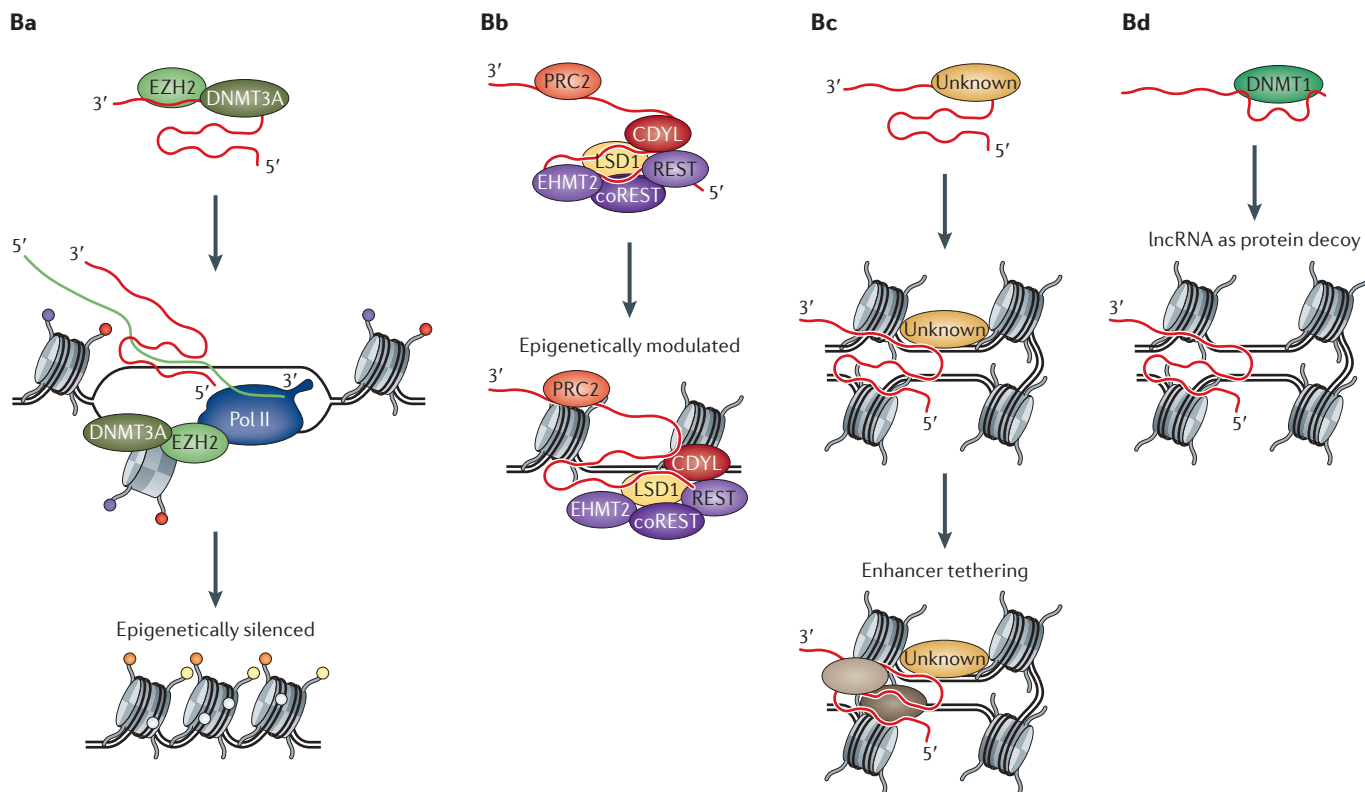
Untranslated regions

(UTRs). Sequences either side of a coding sequence on a strand of mRNA; these can be 5' leader sequences or 3' trailer sequences.

A Transcription of lncRNAs

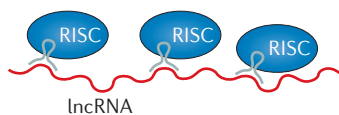


B Nuclear functional lncRNAs

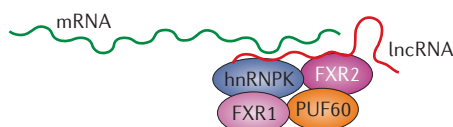


C Nuclear and cytoplasmic functional lncRNAs

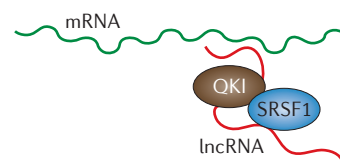
Ca lncRNA as miRNA decoy



Cb lncRNA as translational regulator



Cc lncRNA as splicing regulator



◀ **Figure 4 | Various roles for long non-coding RNAs in cellular regulation.** **A |** Long non-coding RNAs (lncRNAs) are expressed from many loci in the genome — sense and antisense, intronic, overlapping and intergenic with respect to nearby protein-coding loci — and function in both *cis* and *trans*. **B |** Nuclear functional lncRNAs can modulate gene expression both transcriptionally and epigenetically. Some lncRNAs interact with proteins to control the access of chromatin to cellular components and/or guide epigenetic regulatory complexes to target loci, which results in both transcriptional suppression²⁰¹ (part **Ba**) and activation or suppression (that is, bimodal control)¹⁹⁴ (part **Bb**). Proteins involved in chromatin modification — such as DNA (cytosine-5)-methyltransferase 3A (DNMT3A), enhancer of Zeste 2 (EZH2), euchromatic histone-lysine N-methyltransferase 2 (EHMT2; also known as G9a), chromodomain Y-like protein (CDYL), repressor element 1-silencing transcription factor (REST), co-repressor of REST (coREST), trithorax-activating complex MLL1 (REF. 207) (not shown) and Polycomb repressive complex 2 (PRC2) — have been associated with lncRNA-mediated epigenetic silencing^{194,201,265}; the histone demethylase LSD1 (also known as KDM1A) has been associated with activation of silent loci. Enhancer functional lncRNAs tether distal enhancer elements with their promoters^{344,345}, presumably in concert with a protein component that has yet to be determined (shown as 'unknown') (part **Bc**). Decoy functional lncRNAs affect transcription by binding to proteins such as DNMT1 to sequester them from their sites of action, which leads to a loss of maintenance of DNA methylation and gene activation²⁶³ (part **Bd**). **C |** Some lncRNAs can function in both nuclear and cytoplasmic compartments of the cell to affect gene expression and translation of mRNAs. Decoy functional lncRNA complexes affect microRNA (miRNA) targeting of mRNAs (part **Ca**). Some lncRNAs can interact with each other or with mRNAs to sequester small regulatory RNAs, such as miRNAs and therefore RNA-induced silencing complex (RISC), from protein-coding mRNAs^{201,337,338}. Translational regulatory lncRNAs have been observed to recruit protein complexes that consist of heterogeneous nuclear ribonucleoprotein K (hnRNPK), fragile X mental retardation syndrome-related protein 1 (FXR1), FXR2 and Poly(U)-binding splicing factor (PUF60) to homology-containing protein-coding mRNAs, where they bind to and sequester the mRNAs from the translational machinery³⁴⁶ and regulate translation (part **Cb**). lncRNAs can also bind to homology-containing mRNAs and recruit proteins such as QKI and serine/arginine-rich splicing factor 1 (SRSF1), both of which modulate the splicing of the targeted mRNA³⁴¹ (part **Cc**). H3K9ac, histone H3 lysine 9 acetylation; me, methylation; Pol II, RNA polymerase II.

lncRNAs may also be involved in orchestrating the highly dynamic spatial structure of chromatin during differentiation and development^{164,266}, which would explain their often highly cell-specific expression patterns²⁰⁰. Developmental enhancers, as well as Polycomb- and trithorax-response elements, are transcribed in the cells in which they are active^{203,267–272}. These elements may not only be scaffolds for the recruitment of epigenetic regulators²⁷³ but also be the physical mediators of the complex phenomena of transvection and transinduction²³⁴.

Moreover, many lncRNAs show the properties of enhancers¹⁸⁰. These RNAs might guide the physical looping that occurs between enhancers, target promoters and exons with precise positioning of nucleosomes^{274–278} to control transcription and alternative splicing^{237,279,280}. Indeed, the emerging picture is of a chromatin and transcriptional landscape that is exquisitely and precisely controlled in four dimensions by a range of regulatory RNAs that assemble fairly generic (albeit often cell- or differentiation state-specific) enzyme complexes and isoforms to their sites of action in a context-dependent manner²³⁸.

A substantial proportion of lncRNAs reside within, or are dynamically shuttled

to, the cytoplasm, which indicates roles in other cellular processes (BOX 1), including the regulation of protein localization²⁸¹, mRNA translation²⁸² and mRNA stability²⁸³.

RNA modification, evolution and inheritance. Regulatory RNAs may also be influenced by environmental signals and transmitted between cells and generations, which has important implications for understanding gene–environment interactions and evolution. There is evidence that plasticity has been superimposed on RNA-directed epigenetic networks by the expansion of RNA editing, especially during cognitive evolution^{284,285}, and by the use and mobility of retrotransposons^{114,286–289}, which is consistent with the insights of McClintock and of Britten and Davidson. The 'raw material' for evolution is gene duplication and transposition; the latter has the advantage of being able to mobilize functional cassettes in regulatory networks²⁹⁰, which seems to be the main 'driver' of adaptive radiation^{234,291}. Indeed, many lncRNAs may have originated from retrotransposons, and the evolution of mRNAs and lncRNAs may have been accelerated by retrotransposition of functional modules^{292–296}.

Moreover, apart from snoRNA-directed modifications, there are more than 100 other documented modifications of RNA^{297,298}, including cytosine and adenosine methylation that have known physiological and cognitive effects^{299–302}. This indicates an additional layer of RNA informational code and epitranscriptomics — an exciting field that is just beginning to emerge^{303,304}.

There is evidence for systemic transmission of RNA^{305,306} and RNA-mediated epigenetic inheritance in plants and animals^{307–311}. There is also the intriguing possibility of RNA-directed DNA recoding, which may place RNA at the centre not only of gene regulation in the developmental ontogeny of higher organisms but also of both 'hard-wired' and 'soft-wired' somatic and germline evolution^{312–314}.

Conclusions and outlook

Our understanding of the previously hidden and unanticipated world of ncRNAs has greatly expanded in the past two decades. Indeed, in retrospect, it seems that we may have fundamentally misunderstood the nature of the genetic programming in complex organisms because of the assumption that most genetic information is transacted by proteins. This may be true to a large extent in simpler organisms but is turning out not to be the case in more complex organisms, the genomes of which seem to be progressively dominated by regulatory RNAs that orchestrate the epigenetic trajectories of differentiation and development.

The emerging picture is one of an extraordinarily complex transcriptional landscape in mammals and other multicellular organisms. Such a landscape is comprised of overlapping, intergenic and intronic, sense and antisense, small and large RNAs with interlaced exons^{315,316}, which have varying promoters, splicing patterns, polyadenylation sites and localization in different cells and developmental contexts (see below). As there seem to be few distinct boundaries to genes in humans, it might be better to change the focus of analysis to the transcript and to redefine genetic loci as 'fuzzy' transcription clusters^{165,316,317} that are nonetheless semantically anchored or related to an enclosed or nearby protein-coding locus. However, this can only be stretched to a certain extent, and non-protein-coding loci raise problems for existing schema of human genome nomenclature.

Indeed, even the notion of a simple protein-coding sequence needs to be reassessed. It is becoming evident not only that mRNAs can have multiple functions²⁰⁵ but also that

protein-coding sequences themselves can have other embedded functions, as suggested by constraints on synonymous codon usage^{318,319}, including regulatory functions as epigenetic modulators²⁰³, tissue-specific enhancers^{319,320} and transcription factor binding sites³²¹. The possibility, if not likelihood, is that there is a very complex functional and evolutionary interplay between the protein-coding and regulatory functions of RNAs²⁰⁰, and that some lncRNAs may have evolved, at least partly, from protein-coding genes — as in the case of X inactive specific transcript (*XIST*) — by duplication or pseudogenization and the subsequent emergence of paralogous regulatory and/or coding functions^{201,322}. Conversely, new protein-coding capacity may also appear in lncRNAs¹⁷⁴.

The sheer number and diversity of RNAs juxtaposed with their extraordinarily complex molecular functions (FIG. 4) — for example, in regulating epigenetic processes, subcellular organelles, protein-coding and non-coding gene transcription, translation, RNA turnover, chromosomal organization and integrity, and genome defence — suggests that we have a long way to go to understand the structure and functions of what is surely a highly interconnected system. Tens of thousands (if not more) of individual non-coding RNAs exist, and their roles in cell and developmental biology, as well as in brain function, remain to be determined. Moreover, many (if not most) regulatory RNAs have yet to be identified, especially in complex organisms. These include new classes such as the circular RNAs and others that may function as miRNA ‘sponges’

(REFS 62,323–328), the identification of which will require targeted deep sequencing of small and large RNAs that are derived from different genomic locations in various cell types, using targeted techniques such as RNA CaptureSeq^{164,171}.

RNA is not a linear molecule but can fold into complex and allosterically responsive three-dimensional structures that can both recruit generic effector proteins and guide the resulting complexes in a sequence-specific manner to other RNAs and DNA through duplex or triplex formation. Important issues that remain include the identification of functional domains in RNA and their interacting partners, so that we can predict and explain RNA functional interactions in the same way that has already been done by recognition of well-characterized motifs and domains in proteins. One way to do this, which is already underway in many laboratories, is to combine immunoprecipitation of different types of RNA-binding proteins (for example, chromatin-modifying proteins, transcription factors and RNA transport proteins) with deep sequencing of the associated RNAs, followed by analysis of primary and predicted secondary structures, and ultimately by biochemical validation and characterization.

Determination of the structure of RNA species, RNA–RNA, RNA–DNA and RNP complexes will be a rapidly growing field that requires the development of new technologies, such as RNA footprinting using high-throughput sequencing³²⁹ and *in vivo* studies using RNA-based genetic techniques, for example, CRISPR-mediated mutation¹⁴³.

Other objectives include determination of whether small RNA pathways are used in viral defence in humans⁸⁰; the functions of tiRNAs, spliRNAs and snoRNA-derived small RNAs; the roles of piRNAs in retrotransposon dynamics and genome remodeling by retrotransposons in the brain¹¹⁴; the mechanisms and extent of RNA-mediated transgenerational epigenetic inheritance³³⁰; the locations of RNA-binding sites (that is, RNA–DNA duplexes and RNA–DNA:DNA triplexes) in the genome; the crosstalk between different types of regulatory RNAs; the logic and hierarchy of RNA- and protein-mediated regulation of gene expression; and finally, the extent, mechanisms and information content of RNA-mediated communication between cells both within³⁰⁶ and between organisms (that is, ‘social RNA’)³³¹.

Indeed, it seems that RNA is the computational engine of cell biology, developmental biology, brain function and perhaps even evolution itself³¹³. The complexity and interconnectedness of these systems should not be cause for concern but rather the motivation for exploring the vast unknown universe of RNA regulation, without which we will not understand biology.

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Box 1 | Examples of specific long non-coding RNAs and their functions

Long non-coding RNAs have a role in a wide range of biological processes in the cell, for example:

- Template RNAs guide chromosomal rearrangements in ciliates³³²
- Telomeric repeat-containing RNA (TERRA) is involved in telomere biology³³³
- 7S RNA is an essential component of the signal recognition particle, which is involved in protein export³³⁴
- 7SK is a highly expressed structured RNA that acts as a scaffold to assemble a multimeric protein complex containing SR splicing proteins and positive transcription elongation factor b (P-TEFb, which is a cyclin-dependent kinase required for transcriptional elongation by RNA polymerase II and other factors)³³⁵
- Nuclear paraspeckle assembly transcript 1 (*NEAT1*) is an essential component of paraspeckles, which are enigmatic subnuclear organelles that appear in mammalian differentiated cells but not stem cells^{336,337}
- Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is localized to the nucleus and regulates alternative splicing³³⁸ and cell cycle progression³³⁹
- Myocardial infarction-associated transcript (*MIAT*; also known as *gomaflu*) is expressed in an unknown subnuclear structure, possibly a specialized spliceosome, in a subset of neurons³⁴⁰ and has recently been implicated in schizophrenia³⁴¹

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Competing interests statement

The authors declare no competing interests.