NON-CODING RNA

Gene regulation by antisense transcription

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Abstract | Antisense transcription, which was initially considered by many as transcriptional noise, is increasingly being recognized as an important regulator of gene expression. It is widespread among all kingdoms of life and has been shown to influence — either through the act of transcription or through the non-coding RNA that is produced — almost all stages of gene expression, from transcription and translation to RNA degradation. Antisense transcription can function as a fast evolving regulatory switch and a modular scaffold for protein complexes, and it can 'rewire' regulatory networks. The genomic arrangement of antisense RNAs opposite sense genes indicates that they might be part of self-regulatory circuits that allow genes to regulate their own expression.

In recent years, our view of RNA has markedly changed — from regarding these molecules solely as intermediates of genetic information to appreciating their variety of functions that are independent of their protein-coding potential[1–7]. One of the best-characterized non-coding RNAs (ncRNAs) that mediate gene regulation is X-inactive specific transcript (*XIST*), which has a key role in mammalian X chromosome inactivation[2]. More recently, the development of high-throughput approaches has revealed pervasive transcription in all genomes that have been investigated so far. This phenomenon produces numerous types of previously unknown ncRNAs and challenges our traditional definitions of genes and functional regions of the genome[8–11]. Some classes of short ncRNAs (<200 nucleotides in length) are already accepted as fundamental players in gene regulation; these include small interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs) (reviewed in REF. 12). However, we have only begun to understand the functions of the vast majority of long ncRNAs (>200 nucleotides in length)[10,11,13,14].

In this Review, we focus on antisense transcripts, which is a class of long ncRNAs. Antisense transcripts are transcribed from the strand opposite to that of the sense transcript of either protein-coding or non-protein-coding genes. Here, we refer to the originally annotated transcript as the sense transcript and the more recently identified one on the opposite strand as the antisense transcript. The study of gene regulation by antisense transcription is particularly intriguing, as their genomic arrangement immediately indicates that they may act on each other. Antisense transcripts have previously been reviewed for bacteria[15,16], plants[17] and humans[18]. Therefore, we go beyond particular species or taxonomic groups to discuss the diverse biological roles of antisense transcription, as well as its implications on gene regulation, genome architecture and evolution.

We begin by discussing the characteristics of antisense transcripts and how they can be identified, and review the advances and challenges in the genome-wide characterization and functional annotation of these transcripts. We then describe different mechanisms of gene regulation by antisense transcription and the biological effects of such regulation. We discuss how antisense RNAs may have advantages over other gene regulators (such as transcription factors) for integrating multiple kinds of regulatory signals, establishing on–off (that is, bistable) switches and even 'rewiring' gene regulatory networks. We end by discussing the evolutionary implications of antisense transcription and its consequences for genome organization.

Characteristics and expression

Antisense transcripts were initially discovered in bacteria more than 30 years ago[19]; soon after this, examples were found in eukaryotes[20]. Only with the introduction of genomic approaches less than 10 years ago did it become apparent that antisense transcripts are widespread throughout the genomes of a range of species[21–23]. Notably, more than 30% of annotated transcripts in humans have antisense transcription[24]. However, antisense transcripts are generally low in abundance and are, on average, more than 10-fold lower in abundance than sense expression[24].

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In contrast to protein-coding mRNAs, which accumulate in the cytoplasm[25], antisense transcripts preferentially accumulate in the nucleus[26]. However, some antisense transcripts have been found to be associated with chromatin[27,28] and in a range of distinct locations, including the mitochondria and the cytoplasm[25].

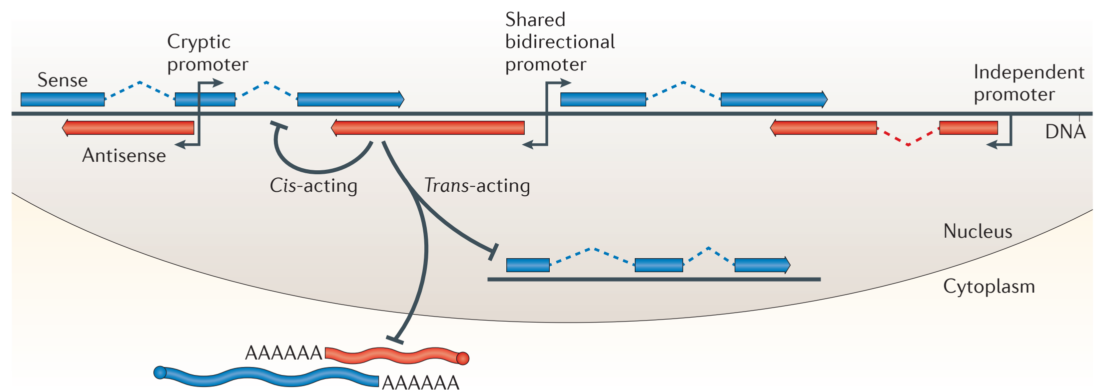
The expression of some antisense transcripts is linked to the activity of neighbouring genes[29,30], whereas many others have distinct expression patterns during different processes, such as cellular differentiation and cancer progression[31], in different environmental conditions or on different genetic backgrounds[29]. Although the apparently low fidelity of transcription initiation suggests that some antisense transcripts arise from transcriptional noise[32], it is clear that many others carry out specific functions[2–7,33]. Before discussing the functional potential of antisense transcripts, it is necessary to understand how they are generated and their intrinsic characteristics.

Expression of antisense transcripts. Antisense transcripts arise from promoters, and their expression is often subject to similar regulation as for other genes. They can arise from independent promoters, bidirectional promoters of divergent transcription units[30,34–37] or cryptic promoters[38–41] (BOX 1). In gene-dense regions, promoter bidirectionality can give rise to a large proportion of antisense transcripts; for example, in yeast, most antisense transcripts seem to originate from bidirectional promoters[34,35]. Promoter bidirectionality, which, until recently, was considered exceptional, has been found to be widespread in species that range from yeast[34,35] to humans[30,36,37], although the degree of bidirectionality is species-dependent; for example, low levels of bidirectionality are observed in *Drosophila melanogaster*[42]. The bidirectional activity of each promoter is influenced by other factors such as the three-dimensional organization of chromatin[43] and the density of polyadenylation signals that surround the promoter[44,45]. Finally, some

Box 1 | **Classification of antisense transcripts**

Antisense transcripts can be classified according to different criteria, such as their origin, genomic orientation, mode of action, length, stability and even the species in which they are expressed. These transcripts have been found to originate from independent promoters, shared bidirectional promoters or cryptic promoters that are situated within genes (see the figure). According to their orientation with respect to sense genes, they can be further classified as head-to-head, tail-to-tail or internal (that is, when they are fully covered by the sense transcripts). Antisense transcripts can exert their function locally, distally, in *cis* or in *trans*, and they can also function in multiple subcellular compartments. *Cis*-acting mechanisms of these transcripts can act either locally (for example, in promoter–gene interactions) or distally (for example, in enhancer–gene interactions). *Trans*-acting mechanisms can also act either locally (for example, antisense transcripts affecting the allele from which they originated and/or any additional allele) or distally (for example, antisense transcripts affecting other genes). Moreover, antisense transcripts can be classified into short (<200 nucleotides) and long (>200 nucleotides) non-coding RNAs (ncRNAs), and stable or unstable RNAs.

Short ncRNAs are accepted as fundamental players in gene regulation. Although they are widespread among eukaryotes, relevant differences exist among species; for example, PIWI-interacting RNAs (piRNAs) are found in animals but not in plants or fungi (reviewed in REF. 12). In this Review, we focus on the much less studied long antisense ncRNAs. Species-specific differences in mechanisms of action might be expected when these mechanisms depend on an accessory machinery, such as the RNA interference machinery, that is not present in all species. As an example, the pairing of sense–antisense transcripts and their consequent degradation by RNase III in Gram-positive bacteria are not seen in Gram-negative bacteria, which suggests a different processing pattern of double-stranded RNAs[108]. Similarly, any effect of an antisense transcript that is mediated by DNA methylation[75,76] is not expected to function in *Saccharomyces cerevisiae*, in which the appropriate DNA methylation machinery is lacking[125]. However, mechanisms of action that are based on the general and highly conserved transcription machinery — for example, transcriptional interference by chromatin modifications — are more likely to be conserved across species. TABLE 1 provides several examples of antisense transcripts with mechanisms that are found in multiple species. So far, few direct comparative studies have been done. It would be interesting to carry out systematic comparative studies that focus on the commonalities and differences of each particular mechanism between species.



Transcriptional noise
Random fluctuations that are intrinsic to the gene expression process and that cause differences in the levels of specific RNAs among cells in a clonal population.

Cryptic promoters
Weak promoters, the use of which is associated with disruption of chromatin structure. Transcripts produced from such promoters often have unknown functions.

Head-to-head
Pertaining to two transcripts that are divergently oriented and have overlapping 5′ regions.

Tail-to-tail
Pertaining to two transcripts that are convergently oriented and have overlapping 3′ regions.

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antisense transcripts originate from cryptic promoters that are situated within the transcribed region of their sense gene[38–40] or even from the termination region of the sense gene[46].

The expression of antisense transcripts is also subject to regulation at the level of RNA stability; for example, many antisense transcripts in budding yeast are cryptic unstable transcripts, which are targeted for early degradation by the nuclear exosome[34,35]. This control is used in meiosis in budding yeast during which the nuclear exosome is downregulated[47]. Other degradation pathways also affect antisense expression; for example, one class of antisense ncRNAs called XRN1-sensitive unstable transcripts (XUTs) are specific targets of the XRN1 cytoplasmic 5′→3′ RNA exonuclease[48], whereas other long ncRNAs are controlled by 5′ decapping activity[49].

In addition to the generation of antisense transcripts by RNA polymerases using DNA as a template, they can also originate from RNA-dependent RNA polymerase (RdRP) activity[50,51]. Specifically, it has been proposed that, in humans, some antisense transcripts that contain non-genomically encoded polyuracil stretches are generated using mRNAs as templates[52]. However, further research is needed to determine the extent and relevance of RdRP activity *in vivo* and to understand the biological implications of such RNAs.

Structure of antisense transcripts. Aside from their antisense orientation, antisense transcripts do not have unique biochemical features. In general, they lack protein-coding potential, as their sequence is constrained by the overlapping sense transcript. However, there are many examples of pairs of sense and antisense transcripts that only partially overlap and that are both functional mRNAs with protein-coding activity[53–55]. In general, the untranslated regions (UTRs) of these mRNAs, which are devoid of coding potential, overlap with neighbouring genes to mediate functions that are similar to those of other ncRNAs[11,16,53]. Such antisense transcripts are especially common in organisms that have compact genomes[16,54,55]; for example, in budding yeast, up to 40% of genes have antisense transcripts that terminate within their 3′ UTRs[54].

Independent of protein-coding activity, antisense transcripts are similar to other long ncRNAs in that they can contain specific domains that interact with DNA, RNA or proteins[10,11]. The intrinsic flexibility of RNA molecules to evolutionarily rearrange their sequence has led to the suggestion that antisense transcripts, and long ncRNAs in general, can form flexible modular scaffolds in which different domains that interact with DNA, RNA or proteins are combined to form specific functional complexes[10,11,56]. For example, antisense transcripts can recognize their reverse-complementary sequence in RNA or DNA and can also carry protein-binding domains to modulate gene expression[2,3,57].

Identification and functional analyses

High-throughput approaches have become instrumental for both the identification and the functional characterization of antisense transcripts. Nonetheless, these approaches are limited by the overlap of antisense transcripts with the sense gene, the low expression levels of these transcripts[24,25,29,35] and their limited evolutionary conservation[58–63].

Identification of antisense transcripts. The initial discovery of pervasive antisense transcription[21–23] was met with justifiable scepticism[64]. The identification of such transcripts is technically challenging, as it requires strand-specific approaches[65,66] (BOX 2). Therefore, many studies initially mistakenly identified antisense transcripts as sense transcripts. Antisense transcripts and their structure can be directly studied either using strand-specific quantification of RNA abundance[29,35,48,54,55,65,66] or by capturing the process of active transcription using techniques such as global run-on sequencing (GRO-seq)[37] and native elongating transcript sequencing (NET-seq)[67] (BOX 2). Antisense expression can also be indirectly detected through its consequences on chromatin modification states. These indirect measures are independent of the stability or the abundance of antisense transcripts, as they measure the effects of the process of antisense transcription rather than the transcripts themselves. The analyses of chromatin signatures have been powerful for detecting long intergenic ncRNAs[68], but for antisense transcripts, the effectiveness of this approach is limited by the lack of strand specificity of chromatin modification and the generally higher expression levels of the sense transcript.

Functional analyses of antisense transcripts. The function of an antisense transcript can be mediated by either the transcript itself or the act of its transcription. Additionally, it is possible to distinguish between functional effects that are exerted by antisense expression in *cis* (that is, those that affect alleles on the DNA strand from which they are produced, usually locally) and those that are exerted in *trans* (that is, those that affect alleles on different DNA strands) (BOX 1). In cases of antisense effects in *trans*, the interpretation is usually that antisense transcription exerts its effects through the RNA molecules that are transcribed. By contrast, antisense effects in *cis* are often assumed to be due to the act of antisense transcription. However, neither assumption is strictly correct. Regions of antisense transcription can interact with other loci through the three-dimensional organization of chromatin, which can mediate *trans* effects; similarly, antisense transcripts can remain at the locations of their synthesis (such as through stalled polymerases, R-loops or triple helices), which allows the RNA to exert its function in *cis*. Although many examples of *trans*-acting antisense transcripts have been described[3,4,69,70], the fact that both antisense and sense transcripts are transcribed from the same region suggests that antisense transcripts function more frequently in *cis* than other ncRNAs that commonly function in *trans*[10].

A classic approach to determine the function of a gene is to perturb its expression, followed by phenotypic analysis. However, the genomic arrangement of antisense transcripts makes it difficult to perturb antisense expression without also affecting sense expression,

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Box 2 | Genomic techniques for studying antisense expression

Since the discovery of the widespread transcription of non-coding RNAs (ncRNAs), there has been much debate about how much of this transcription is real and how much is simply a result of experimental artefacts[64]. Initial studies had difficulty in distinguishing between bona fide antisense transcripts and artefacts that are derived from their overlapping sense transcripts, which are usually expressed at higher levels. In recent years, several approaches have been developed to measure strand-specific transcription and to minimize experimental artefacts. Furthermore, new genome-wide techniques are delivering promising insights into RNA localization, single-cell expression and chromatin binding.

Transcript abundance

Most techniques that are used for measuring RNA abundance (for example, quantitative PCR or RNA sequencing) require the production of cDNA molecules by reverse transcription as their first step. However, the inherent ability of reverse transcriptase to use either RNA or DNA as a template can result in the production of artefactual double-stranded cDNA, leading to false-positive identification of antisense transcripts. This can be solved by adding actinomycin D[65], a drug that specifically inhibits the DNA-dependent DNA polymerase activity of reverse transcriptase. Many strand-specific protocols that minimize the false-positive identification of antisense transcripts have been developed[65,66]. In the future, the direct sequencing of RNA molecules[24] promises to solve problems that are derived from sample preparation by eliminating the need to produce cDNA.

Strand-specific measurements of RNA polymerases

Chromatin immunoprecipitation is a tool that is commonly used to study the transcription machinery, but as it enriches for double-stranded DNA fragments that are associated with proteins, it lacks strand-specificity. Alternative methodologies can be used to map both the position and the orientation of RNA polymerase. Global run-on sequencing (GRO-seq)[37] measures the presence and orientation of active polymerases that are capable of run-on elongation, whereas native elongating transcript sequencing (NET-seq)[67] allows the measurement of nascent transcripts from engaged RNA polymerases and is independent of their ability to elongate. In both approaches, the RNAs produced by these polymerases are sequenced, thereby achieving strand-specificity.

Subcellular localization

The application of strand-specific techniques to RNAs that are derived from different subcellular compartments[25] (for example, the cytoplasm and the nucleus) helps to define different subpopulations of antisense transcripts, and thereby provides clues about their potential mechanisms of action (for example, those affecting translation are expected to be present in the cytoplasm).

Single-cell studies

Single-molecule fluorescence *in situ* hybridization approaches[115] allow individual RNA molecules to be measured. As these methods are applied to single cells, they are also informative about transcriptional noise. Analyses of transcriptional noise will be further supported by the refinement of single-cell transcriptomics approaches.

Chromatin binding of ncRNAs

Genome-wide mapping of chromatin-binding sites of ncRNA molecules[27,28] will help to expand the identification of their targets and to characterize their modes of action.

CpG islands
Genomic regions that contain a high frequency of CG dinucleotides; they are often associated with mammalian promoters and are targets of cytosine methylation.

Gene imprinting
An epigenetic process by which the expression of each allele of a gene depends on its parent of origin; for example, on whether it is the paternal or maternal allele.

and this approach has only succeeded in isolated examples[29,69]. The fact that most antisense transcripts function in *cis* also makes it difficult to use genome-wide approaches, such as systematic knockdown[56] and overexpression, to identify putative functions of these transcripts. Approaches that are based on guilt-by-association, in which transcripts are linked to specific biological processes on the basis of common expression patterns across cell types and tissues[10,68], are difficult to apply to antisense transcripts because their expression can be affected by the sense transcript[29]. To attempt to disentangle the causes and consequences of antisense transcription, the dynamic analysis of transcription following environmental perturbations has proven promising[38,71]. These studies involve measuring genome-wide gene expression levels at different time points and comparing the transcriptional responses of cells with and without the key components that are involved in gene regulation by antisense transcription (for example, the histone methyltransferases SET1 (REF. 71) and SET3 (REF. 38); see below) to determine the function of these key components. Another interesting approach is to use allele-specific measurement of gene expression to determine whether an antisense transcript can function in *trans* to affect a distant sense allele[72]. At the moment, however, the detailed molecular dissection of specific cases has been the most fruitful way of understanding the functional consequences of gene regulation by antisense transcription.

Mechanisms of gene regulation

The orientation, stability, subcellular localization and inherent features, such as sequence or secondary structure, of antisense transcripts can all affect their mechanisms of action. Antisense transcripts, or the act of their transcription, can affect almost all stages of the gene expression process. Here, we discuss the different steps of gene expression that antisense expression affects, including transcriptional initiation, co-transcriptional processes and post-transcriptional processes (TABLE 1). In addition, it should be noted that, similarly to how siRNAs repress gene expression in both the nucleus and the cytoplasm (reviewed in REFS 9,12), antisense transcripts can also simultaneously function at different stages of the gene expression process. For example, the antisense transcript to the *Ty1* retrotransposon in budding yeast silences *Ty1* transcription in *trans* through chromatin modification[70] and simultaneously controls its retrotransposition post-transcriptionally[73].

Effects on transcription initiation. Antisense expression can affect transcription initiation through transcriptional interference, in which one act of transcription negatively affects a second one in *cis*[74]. This has been shown to occur by promoter competition (that is, when the assembly of the transcription machinery at one promoter physically prevents the assembly at the second one), by occlusion of binding sites due to the passage of RNA polymerase or even by chromatin or DNA modifications[74].

In particular, antisense expression has been shown to regulate transcription initiation by affecting DNA methylation — the process by which specific cytosines are methylated, for example, at CpG islands in mammalian promoters, which usually leads to their long-term repression[75]. One example is the repression of the haemoglobin α1 gene (*HBA1*) in patients with a class of α-thalassemia[76]. In this case, an aberrant *LUC7L* (putative RNA-binding protein Luc7-like) transcript runs antisense into the *HBA1* locus and methylates its promoter CpG island, which silences *HBA1* expression and consequently causes disease (FIG. 1a). Antisense transcription has also been implicated in gene imprinting in mice[77], in which the transcription of an antisense transcript

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Table 1 | **Examples of functional antisense transcription across all kingdoms of life**

Mechanism of action	Antisense locus	Effects	Species	Refs
DNA methylation	*LUC7L*	Methylates *HBA1* promoter CpG island, which represses its expression	Humans	76
	Airn	Regulates *Igf2r* imprinting by DNA methylation	Mice	77,78
Chromatin modifications	*XIST* and *TSIX*	Inactivates X chromosome gene expression	Mammals	2
	ANRIL	Represses the tumour suppressor locus *CDKN2B–CDKN2A* by both histone H3 lysine 27 (H3K27) methylation and DNA methylation	Humans	57, 80
	BDNF-AS	Represses *BDNF* by histone modification	Mammals	81
	HOTAIR	Silences the *HOXD* locus in *trans* by the recruitment of Polycomb proteins	Humans	3
	COOLAIR	Represses *FLC* sense gene by H3K4 demethylation and recruits Polycomb proteins, which increase H3K27me3 levels	Plants	85, 86
	COLDAIR	Antisense to *COOLAIR*; represses *FLC* sense gene by the recruitment of Polycomb proteins	Plants	88
	AS to *PHO84*	Represses *PHO84* by histone deacetylation both in *cis* and in *trans*	*S. cerevisiae*	4,69
	RTL	Silences transcription of the *Ty1* retrotransposon in *trans* through chromatin modification and post-transcriptionally controls its retrotransposition	*S. cerevisiae*	70, 73
Transcriptional interference	*RME2*	Represses *IME4* by transcriptional interference in *cis* and functions after transcription initiation of *IME4*	*S. cerevisiae*	5,99
Isoform variation	*ZEB2-AS*	Induces exon skipping in *ZEB2*, which produces an alternative isoform that has increased translation efficiency	Humans	7
Translation efficiency	AS to *Uchl1*	Increases translation efficiency of *Uchl1* using a SINEB2 domain	Mice	33
	SymR	Decreases translation efficiency of *SymE* by competing with binding of the 30S ribosome	Enterobacteria	6
RNA stability	*BACE1-AS*	Increases stability of *BACE1* by masking an microRNA-binding site	Humans	105, 106
	WDR83 and *DHPS*	Increase their mutual stability by forming a duplex within their 3′ untranslated regions	Humans	53

Airn, antisense *Igf2r* RNA; *ANRIL*, antisense non-coding RNA in the *INK4* locus; AS, antisense; *BACE1*, β-site APP-cleaving enzyme 1; *BDNF*, brain-derived neurotrophic factor; *COLDAIR*, *COLD-ASSISTED INTRONIC NON-CODING RNA*; *COOLAIR*, *COLD-INDUCED LONG ANTISENSE INTRAGENIC RNA*; *DHPS*, deoxyhypusine synthase; *FLC*, *FLOWERING LOCUS C*; *HBA1*, haemoglobin α1; *HOTAIR*, HOX transcript antisense RNA; *HOXD*, homeobox D; *Igf2r*, insulin-like growth factor 2 receptor; *IME4*, inducer of meiosis; *LUC7L*, Putative RNA-binding protein Luc7-like 1; *PHO84*, phosphate metabolism; *RME2*, regulator of meiosis 2; RTL, antisense to *LTR* (long terminal repeat); *S. cerevisiae*, *Saccharomyces cerevisiae*; *SymE*, SOS-induced *yjiW* gene with similarity to MazE; *TSIX*, *XIST* antisense RNA; *Uchl1*, ubiquitin carboxy-terminal hydrolase L1; *WDR83*, WD repeat domain 83; *XIST*, X inactive-specific transcript; *ZEB2*, zinc-finger E-box-binding homeobox 2.

Airn (antisense to insulin-like growth factor 2 receptor (*Igf2r*)), and not the *Airn* transcript itself, represses *Igf2r* by both transcriptional interference and DNA methylation[78]. However, antisense transcription can also have activating effects by protecting promoters from *de novo* methylation[18] through R-loop formation, which involves DNA–RNA hybrids, during transcription[79].

Antisense expression can also control transcription initiation by affecting histone modifications. A classic example is mammalian X chromosome inactivation, in which the long ncRNA *XIST* spreads over one copy of the X chromosome and recruits repressive chromatin-remodelling complexes, such as Polycomb repressive complex 2 (PRC2). In mice, the action of *Xist* is antagonized in *cis* by its own antisense transcript — X (inactive)-specific transcript, opposite strand (*Tsix*)[2]. Whereas *XIST* and *TSIX* affect the whole chromosome, other antisense transcripts silence specific loci. For example, *ANRIL* (antisense ncRNA in the *INK4* locus; also known as *CDKN2B-AS1*), the expression of which is increased in prostate cancer, mediates the specific repression of the tumour suppressor locus *CDKN2B–CDKN2A*[57], which encodes p15 (also known as INK4B), p14 (also known as ARF) and p16 (also known as INK4A). Specifically, the nascent antisense transcript recruits PRC2 in *cis*, which induces histone H3 lysine 27 methylation (H3K27me) and thus represses transcription from this locus[57] (FIG. 1b). Interestingly, heterochromatin formation that is induced by *ANRIL* also leads to promoter DNA methylation after cellular differentiation[80]. The specific inhibition of *cis*-repressing antisense transcripts also holds promise as a therapeutic tool to increase the expression of specific target genes[81,82]. Specifically, it has been shown in mammals that the

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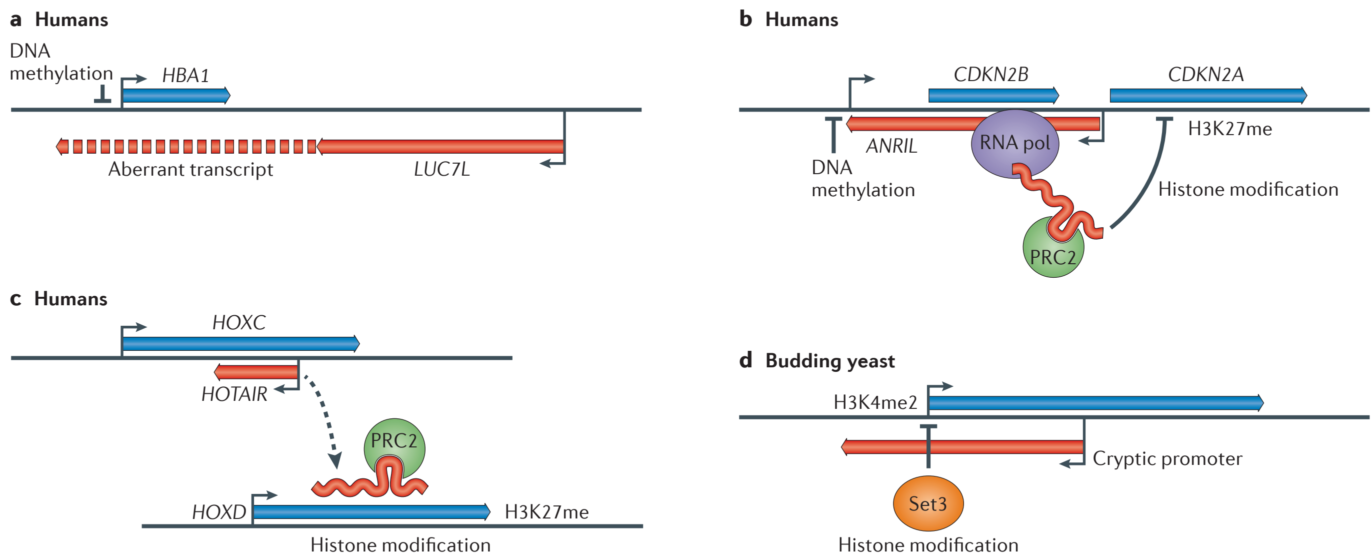

Figure 1 | **Effects of antisense expression on transcription initiation.** **a** | Aberrant transcriptional extension of the *LUC7L* (putative RNA-binding protein Luc7-like) locus produces an antisense transcript that overlaps with the haemoglobin α1 gene (*HBA1*), which methylates the *HBA1* promoter and represses its expression[76]. **b** | The nascent antisense transcript *ANRIL* (antisense non-coding RNA in the *INK4* locus) recruits Polycomb repressive complex 2 (PRC2) in *cis*, which induces histone H3 lysine 27 methylation (H3K27me). This represses transcription of the tumour suppressor locus *CDKN2B*–*CDKN2A*[57] and also leads to long-term promoter DNA methylation at this locus[80]. **c** | HOX transcript antisense RNA (*HOTAIR*) silences the homeobox D (*HOXD*) locus in *trans* through PRC2 recruitment[3]. **d** | Antisense transcripts that originate from internal cryptic promoters can modify the chromatin of their associated sense promoters by depositing H3K4me2, thereby modulating the binding of the histone deacetylase Set3 and gene expression dynamics[38].

targeted degradation of *BDNF-AS* — the antisense transcript of brain-derived neurotrophic factor (*BDNF*) — increases *BDNF* expression, probably by decreasing the repressive chromatin marks that are deposited by the antisense transcript[81].

Antisense transcripts can also mediate chromatin modifications in *trans*. The best-known example is HOX transcript antisense RNA (*HOTAIR*) in mammals, which is an antisense transcript to the homeobox C (*HOXC*) locus and a predictor of both metastasis and death when it is expressed at high levels in primary breast tumours[83]. *HOTAIR* silences the *HOXD* locus in *trans* through the recruitment of Polycomb proteins[3] (FIG. 1c). In fact, guidance of Polycomb proteins by antisense transcripts is likely to be common because PRC2 directly interacts with more than 3,000 antisense transcripts[28], which could target Polycomb proteins to specific genomic locations where they could subsequently function[27,28]. Thus, antisense transcripts can provide sequence specificity by interacting either with DNA or with the nascent transcript of the sense strand and by serving as a scaffold for the chromatin-modifying machinery[10,11,84]. This is similar to how siRNAs in *Schizosaccharomyces pombe* use nascent transcripts that originate near the centrosome as assembly platforms to guide heterochromatin formation[12].

Chromatin modifications mediated by antisense transcripts that suppress transcription initiation are not restricted to animals. In plants, *COLD-ASSISTED INTRONIC NON-CODING RNA* (*COOLAIR*), which is a set of antisense transcripts to *FLOWERING LOCUS C* (*FLC*), uses two chromatin modification pathways to repress *FLC* expression. One of these pathways involves the use of a proximal polyadenylation site upon cold treatment, which increases H3K27me3 levels through the function of Polycomb proteins[85]. The other pathway involves specific RNA-binding proteins that promote the use of the proximal polyadenylation site, which causes local histone H3K4 demethylation[86]. In this case, multiple ncRNA-based mechanisms interact, in that *COOLAIR* transcription is limited by the specific stabilization of an R-loop over its promoter[87] and that another transcript, *COLD-INDUCED LONG ANTISENSE INTRAGENIC RNA* (*COLDAIR*; antisense to *COOLAIR*), can also recruit Polycomb proteins over the *FLC* locus[88]. In budding yeast, the antisense transcript to *PHO84* (which encodes a phosphate transporter) is induced in response to chronological ageing, which causes repression of the sense gene by histone deacetylation both in *cis* and in *trans*[69]. Moreover, in budding yeast, the silencing of the *GAL1*–*GAL10* locus (the protein products of which are involved in galactose metabolism) by antisense transcription and the resulting activity of the histone methyltransferase Set1 have been extensively studied[89,90]. This mechanism has been shown to operate genome wide[48] — hundreds of XUTs have been shown to silence their sense counterparts through Set1 (REF. 48). However, despite a few clear examples of *trans*-acting XUTs, recent work suggests that XRN1 could also function as a transcription factor in the nucleus[91]. These and other well-characterized examples suggest that antisense-mediated repression of sense transcription through histone modification is common (reviewed in REFS 9,84).

Nascent transcript
An RNA molecule that results from ongoing transcription and that is still associated with DNA through the RNA polymerase.

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The act of antisense transcription, rather than the produced transcript, has also been shown to induce chromatin modifications, which are deposited during transcription and subsequently regulate the expression of the modified regions[38,71,92]. For example, in budding yeast, transcription of antisense units that arise from internal cryptic promoters directly modifies the chromatin of the associated sense genes, which delays their transcription initiation[38,89,90] (FIG. 1d).

A less investigated mechanism for the repression of transcription initiation is the formation of triple helices between transcripts and DNA[93]. We currently lack reliable estimates of the prevalence of this mechanism in antisense-mediated regulation, but several cases have been described for sense transcripts. For example, the human dihydrofolate reductase gene (*DHFR*) produces a sense transcript that overlaps the promoter and 5′ region of *DHFR*. Consequently, this RNA represses the main promoter both in *cis* and in *trans* by forming a stable triple RNA–DNA helix[94].

Co-transcriptional effects of antisense expression. Antisense expression can regulate gene expression after transcription initiation by transcriptional interference that occurs co-transcriptionally. This effect can be mediated by direct collision of RNA polymerases, by 'sitting-duck' interference (that is, when an elongating polymerase removes another that is already bound to its promoter) or by one RNA polymerase acting as a 'roadblock' for other incoming elongating polymerases[74].

If a DNA region is simultaneously transcribed in both directions, this leads to a collision of the transcription machinery (FIG. 2a). Although phage polymerases that transcribe opposite DNA strands are able to bypass each other *in vitro*[95], this is not the case for more complex bacterial[96] or eukaryotic[97] RNA polymerases. Transcriptional interference by direct polymerase collision is most likely when there are two strong convergent transcription units, as it is unlikely for two weak transcription units to be simultaneously transcribed. However, polymerase pausing can increase transcriptional interference, even for weakly transcribed units, by extending the time of polymerase occupancy[98]. An example of transcriptional interference that functions after transcription initiation is the repression of the *IME4* locus (which encodes a key regulator of meiosis) in budding yeast by its antisense transcript regulator of meiosis 2 (*RME2*)[5,99]. In this case, a 450-bp internal region of the *IME4* gene is necessary for antisense-mediated repression, which suggests that antisense-mediated transcriptional interference blocks the elongation, but not the initiation, of the *IME4* transcript[99] (FIG. 2b).

Antisense transcription can also regulate which transcript isoforms are produced by the sense gene. For example, antisense expression can affect mRNA splicing by masking specific splice sites and preventing their processing. A well-known example in humans is the zinc-finger E-box-binding homeobox 2 gene (*ZEB2*), which encodes a transcriptional repressor of E-cadherin. Its antisense transcript prevents the processing of a large 5′ intron that contains an internal ribosome entry site on the *ZEB2* mRNA[7]. This does not change the abundance of the *ZEB2* mRNA but increases its translation efficiency (FIG. 2c). Throughout metazoan evolution, genes that produce multiple spliced isoforms are associated with antisense transcription, which indicates that antisense-mediated regulation could be a common mechanism to control alternative splicing[100]. Antisense-mediated exon skipping has also been exploited therapeutically to change the levels of alternatively spliced isoforms or to restore disrupted open reading frames[101].

Antisense expression can also lead to alternative transcript isoforms by mechanisms that are independent of splicing. For example, transcriptional interference by antisense enhancer RNAs that are expressed in mouse embryonic stem cells during specific differentiation stages can lead to the appearance of shorter sense transcript isoforms with alternative termination sites[102] (FIG. 2d). In bacteria, a similar mechanism called transcription attenuation has been shown to affect the length of sense mRNAs. This phenomenon induces premature termination of the sense transcript[103] through the interaction between sense and antisense transcripts, which also allows differential regulation of genes in a single operon[103].

Post-transcriptional effects of antisense expression. Finally, antisense expression can regulate the post-transcriptional 'life' of a sense mRNA (FIG. 3). This effect can be indirectly exerted, as in the case of *ZEB2* above, in which antisense expression controls translation efficiency by affecting the produced transcript isoform[7] (FIG. 3c). In this section, however, we focus on direct post-transcriptional effects of antisense transcripts. These effects are potentially faster than the mechanisms described above, as they act on mRNA molecules that are already present in the cell and are not affected by the lag between a change in transcription rate and the establishment of a new mRNA concentration level. One limitation of direct post-transcriptional regulation is that it requires both the sense and the antisense RNA molecules to be simultaneously present in the same cell. This is a limitation in organisms such as yeast, in which genes are expressed, on average, at a level of only one mRNA molecule per gene per cell[104] and in which levels of antisense transcripts are even lower. Notably, when antisense transcription affects sense expression through the chromatin-mediated mechanisms that are discussed above, it is not necessary for the antisense and sense transcripts to be present in the same cell at the same time; steady state levels of antisense transcripts also become irrelevant, as such effects are maintained by chromatin modifications. The direct post-transcriptional effects of antisense–sense transcript interactions that have been described are diverse and include both positive and negative effects on translation and mRNA stability.

An example of an activating effect on translation is the mouse ubiquitin carboxy-terminal hydrolase L1 (*Uchl1*) antisense transcript, which increases the translation of *Uchl1* (REF. 33). Specifically, the antisense transcript binds to the 5′ region of the sense transcript, and

Polymerase pausing
A process in which an RNA polymerase temporarily halts elongation while remaining associated with DNA. It is associated with transcriptional regulation after initiation and is particularly frequent in metazoans.

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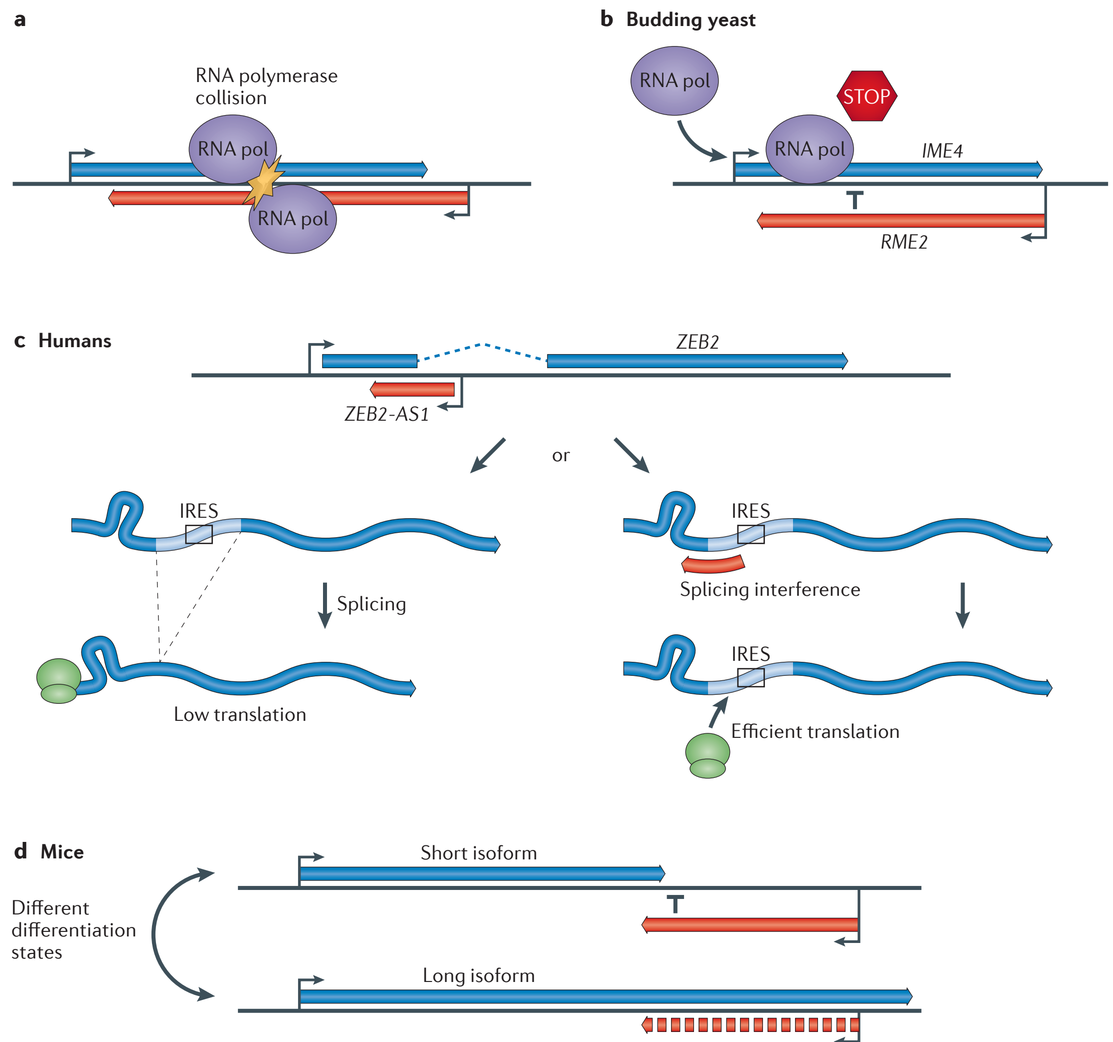

Figure 2 | **Co-transcriptional effects of antisense transcription.** **a** | Head-to-head transcription can lead to RNA polymerase collision[96,97]. **b** | Transcriptional interference of *IME4* (which encodes a key regulator of meiosis) by its antisense gene regulator of meiosis 2 (*RME2*)[5] requires the presence of an internal sequence in a specific orientation. This supports a model in which *RME2* blocks polymerase elongation at the *IME4* locus but not initiation of its transcription[99]. **c** | The transcription of ZEB2 antisense RNA 1 (*ZEB2-AS1*) prevents the processing of a 5′ intron that contains an internal ribosome entry site (IRES)[7]. A sequence in the 5′ untranslated region of the *ZEB2* mRNA limits ribosome scanning, such that only the presence of this IRES in the final product allows efficient *ZEB2* translation. **d** | Transcriptional interference by antisense transcripts can limit the length of the sense transcript and lead to the production of shorter sense transcript isoforms[102]. By contrast, when the level of antisense transcript expression is low (dashed line), the long sense isoform can be produced.

the SINEB2 domain on the antisense molecule then increases *Uchl1*-translation efficiency (FIG. 3a). In addition, nuclear–cytoplasmic shuttling of the antisense transcript regulates the efficiency of *Uchl1* translation[33]. Although this is currently an isolated example, it suggests a modular mechanism of antisense transcript function, in which one element recognizes the target mRNA molecule and other elements (in this case, the SINEB2 domain) affect its post-transcriptional behaviour. Antisense transcripts can mediate not only activating effects but also repressive effects on translation, as in the case of *SymE*, which encodes an enterobacterial toxin that is induced by SOS (that is, an inducible DNA repair system)[6]. Its antisense transcript *SymR* binds to the 5′ end of the *SymE* transcript, where it blocks the binding site of the 30S ribosomal subunit, thereby inhibiting *SymE* translation (FIG. 3b).

Antisense expression can also affect the stability of target mRNAs. Antisense transcripts have been shown to increase the stability of their target sense mRNAs by masking specific sites that would otherwise lead to mRNA degradation. One example, which comes from humans, is that of the antisense transcript to the β-site APP-cleaving enzyme 1 gene (*BACE1*), which encodes β-secretase 1 — an enzyme that has a central role in the progression of Alzheimer's disease. The antisense transcript forms an RNA duplex with the sense mRNA[105], and this duplex masks a binding site for the miRNA

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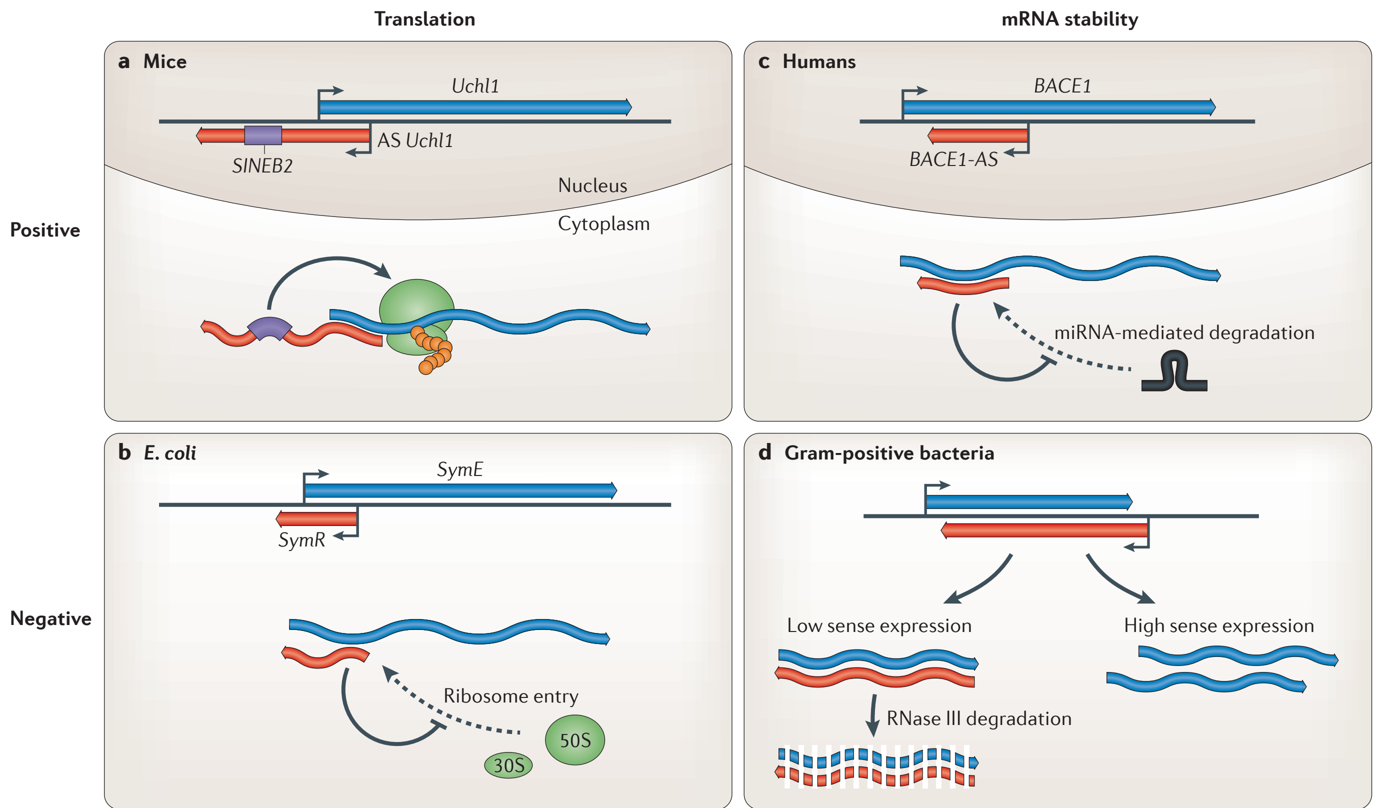

Figure 3 | **Post-transcriptional effects of antisense transcription.** **a** | The 5′ region of an antisense transcript to the ubiquitin carboxy-terminal hydrolase L1 gene (AS *Uchl1*) recognizes its sense transcript and increases the translation efficiency of *Uchl1* — an effect that depends on its SINEB2 domain[33]. **b** | In *Escherichia coli*, the antisense *SymR* transcript competes with the 30S ribosome subunit for the binding to *SymE* (the sense transcript), consequently decreasing the translation efficiency of *SymE*[6]. **c** | An antisense transcript to the β-site APP-cleaving enzyme 1 gene (*BACE1-AS*) forms an RNA duplex with the sense transcript (*BACE1*); this masks the binding site of the microRNA miR-485-5p and, consequently, suppresses the miRNA-mediated degradation and translational repression of *BACE1* (REF. 106). **d** | Spurious low-abundance sense transcripts (blue) produced in Gram-positive bacteria can form double-stranded RNAs by pairing with their corresponding antisense transcripts (red), which are also constitutively expressed at low levels. These double-stranded RNAs are consequently processed by RNase III to produce pervasive short RNAs[108]. Only highly expressed sense transcripts surpass the threshold of antisense-mediated degradation.

miR-485-5p, which consequently suppresses miRNA-induced decay and translational repression of *BACE1* (REF. 106) (FIG. 3c). This case is especially noteworthy, as it illustrates the competition between two different kinds of regulatory RNAs (that is, miRNAs and antisense transcripts) to 'fine-tune' gene expression levels, and suggests a role for antisense transcripts in directly binding to miRNAs and acting as miRNA sponges[107]. Regulation of gene expression by antisense expression can also occur between two convergent protein-coding RNAs. For example, in humans, the stabilities of the WD repeat domain 83 mRNA (*WDR83*) and the deoxyhypusine synthase mRNA (*DHPS*) are increased by the formation of an RNA duplex that consists of their 3′ UTRs[53].

Sense–antisense transcript pairing can also have negative effects on mRNA stability; for example, in Gram-positive bacteria, double-stranded RNAs that are formed by the genome-wide pairing of sense–antisense transcripts are degraded by RNase III[108]. In this case, it has been postulated that the presence of antisense transcripts imposes a threshold, so that only highly expressed transcripts will escape degradation, whereas transcripts that are expressed at lower levels (that is, cryptic transcripts) will pair with their antisense transcripts and will consequently be immediately targeted for degradation[108] (FIG. 3d).

miRNA sponges
RNA molecules that have multiple binding sites for specific microRNAs (miRNAs); they are therefore able to function as decoys to sequester miRNAs and prevent them from binding to their targets.

Biological relevance

So far, we have discussed different mechanisms of action for antisense transcription, which can affect different steps of the gene expression process and can even function simultaneously at multiple steps. However, the regulation of gene expression by antisense transcripts does not exist in isolation — it is integrated with other mechanisms to achieve complex regulatory effects. Here, we conceptualize different ways that cells integrate antisense transcription and focus on the biological advantages of gene regulation by antisense transcription compared with that by protein regulators.

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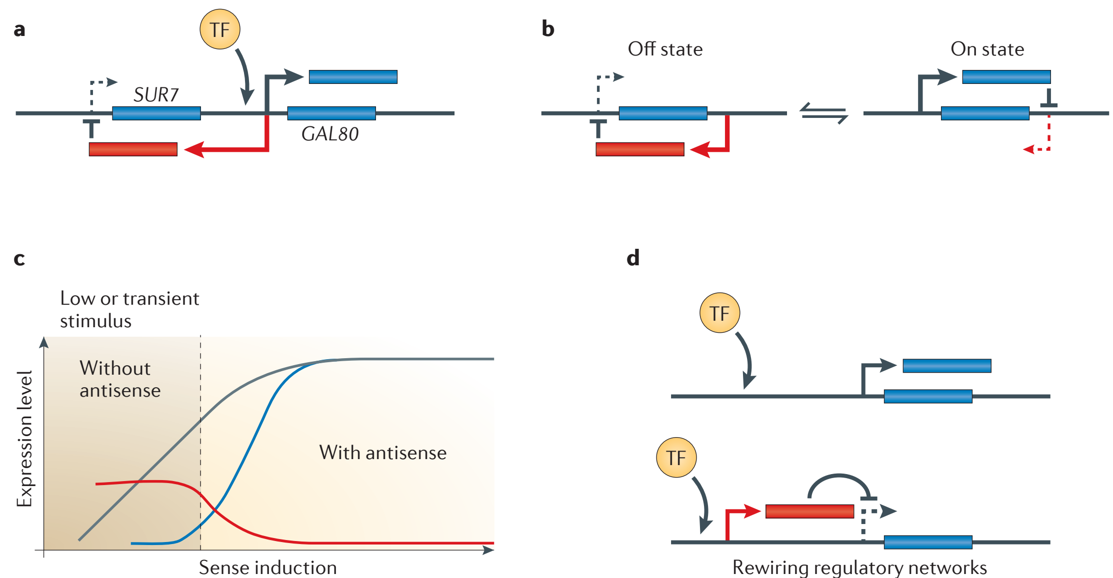

Figure 4 | **Biological implications of antisense expression.** **a** | Antisense transcripts can transmit regulatory signals to neighbouring promoters[29]. Upon galactose induction in budding yeast, the same transcription factor (TF)-mediated activation pathway induces the expression of *GAL80* and an antisense transcript that originates bidirectionally from its promoter. This antisense transcript runs upstream into the promoter of *SUR7* and represses its activity, thereby spreading *GAL80* regulatory signal to both genes. **b** | Sense–antisense pairs can be regarded as self-regulatory circuits, in which the unit can be in either an on state (that is, the sense gene (blue) is expressed (solid arrows) and the antisense gene (red) is repressed (dashed arrows)) or an off state (that is, the target sense gene is repressed and the antisense gene is expressed). **c** | The presence of antisense transcripts (red) can induce a threshold-dependent (that is, ultrasensitive[117]) on–off switch on sense-gene regulation (blue). When the gene does not have an antisense transcript, its expression follows different kinetics (grey). **d** | Antisense or non-coding RNA transcription can 'rewire' regulatory networks, which inverts the final effect of a transcription factor. A transcription factor that activates gene expression (upper panel) can also behave as a repressor (lower panel) if it activates the expression of a non-coding RNA (red) that has repressive effects on the downstream gene (blue). Part **a** is modified, with permission, from REF. 29 © (2011) Macmillan Publishers Ltd. All rights reserved.

Antisense transcripts as regulatory hubs. Owing to the ability of transcription factors to recognize short DNA sequences that are present in the promoters of their target genes, they are generally better suited than antisense transcripts for globally coordinating the expression of groups of genes. However, antisense transcription can also coordinate gene expression of multiple genes both in *cis* and in *trans*. In addition, antisense transcripts are established as hubs for gene regulation by the ability of antisense-mediated regulation to integrate diverse types of regulatory signals, including both transcriptional and post-transcriptional ones, and to function at multiple steps of the gene expression process. One of the factors that allow antisense transcripts to integrate these diverse signals is the intrinsic modular flexibility of ncRNAs[10,11]. An illustrative example is *HOTAIR*, which regulates gene expression both by binding to diverse loci across the genome[27,28] and by recruiting chromatin-modifying machinery[3]. Antisense transcripts, such as *HOTAIR*, can also function as scaffolds for chromatin-associated complexes. Most components of the chromatin-modifying machinery lack sequence specificity, but RNAs can bind to specific sequences and therefore recruit and scaffold chromatin-associated complexes into larger functional units[10–12,56,84]. In addition, ncRNAs can scaffold complexes that are bound to different regions of the genome, thus bringing them together. Therefore, another attractive hypothesis is the role of antisense transcripts in remodelling three-dimensional chromatin structure, as described for the human HOXA distal transcript antisense RNA (*HOTTIP*)[109].

Another way in which antisense transcripts can act as regulatory hubs is by locally spreading regulatory signals to neighbouring genes. This has been observed in antisense transcripts that originate from bidirectional promoters in organisms with compact genomes, such as in budding yeast[29] and fission yeast[110]. For example, upon galactose induction in budding yeast, the antisense transcript that originates bidirectionally from the *GAL80* promoter runs upstream into the promoter of *SUR7*, thereby repressing its activity. In this manner, the regulatory signals that impinge on the *GAL80* promoter are spread to the promoter of *SUR7* (REF. 29) (FIG. 4a). Such local crosstalk also occurs in bacteria, in which some protein-coding transcripts have long UTRs that silence the expression of neighbouring operons encoding opposing cellular functions[16]. Local transcriptional crosstalk is not limited to organisms with compact genomes and can also affect larger genomic regions. For example, in mice, upon growth factor stimulation,

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'ripples' of transcription that originate from a target promoter spread across 100-kb regions. This induces histone acetylation and the coordinated upregulation of both coding and non-coding neighbouring genes[111].

Sense–antisense pairs as self-regulatory circuits. The precise locus specificity that is afforded by nucleotide sequence complementarity allows antisense transcripts, or the act of their transcription, to have specific effects on their targets. As sense and antisense transcription units are reciprocally complementary, in principle, they can mutually affect one another to establish self-regulatory circuits between sense and antisense expression. In the case of mutual repression (FIG. 4b), the effects can be subtle when antisense expression slightly modulates the expression of the sense gene (that is, fine-tuning its expression). Alternatively, there might be more drastic effects, in which the pair can be in either an 'on' state (in which the sense gene is expressed and the antisense gene is repressed) or an 'off' state (in which the target sense gene is repressed and the antisense gene is expressed) — that is, the pair can form a bistable switch. It is important to note that these switches are established independently for each locus. Thus, cases in which antisense and sense expression are positively correlated[24,29] in experiments using cell populations are compatible with alternative expression from different cells or alleles.

Studies have shown that the repressive effect of antisense transcripts is sufficient to establish switch-like behaviour of the sense gene. For example, budding yeast seems to use the equilibrium between sense and antisense transcripts to increase both the dynamic range of gene expression and the cell-to-cell variability in levels of protein expression[29]. In the case of *SUR7* expression, its dynamic range is expanded in the lower range by antisense transcription that reduces basal or leaky levels of sense expression in the off state, thus increasing the range between minimum and maximum expression[29]. The ability of antisense transcription to regulate multiple levels of gene expression could further enhance this response. In fact, systems that involve both transcriptional and post-transcriptional antisense-mediated regulation have been shown to achieve more efficient gene repression, in which any transcriptional leakage is blocked post-transcriptionally[112]. Thus, antisense transcripts provide a more robust (that is, less noisy) sense-gene repression in the off state than transcription factor-based mechanisms.

In the on state, when the sense transcript is expressed, antisense-mediated regulation is expected to lead to noisier expression of the sense gene. This is due to the low level of antisense transcripts that are present in the on state, which makes them sensitive to transcriptional bursting[113]. Cell-to-cell variability in gene expression is likely to be increased in the on state, both because even low levels of antisense expression increase transcriptional noise and because sense–antisense equilibrium is established independently for each locus within individual cells[29]. Such variability, which can be achieved even at low levels of antisense transcription, could be important for adaptation. It allows cells in the same populations to respond differently to identical environmental stimuli, which could be advantageous for some cells in the population[114]. This increased variability is expected to be particularly relevant for stress-related genes, which are in fact enriched for the presence of antisense transcripts in budding yeast[29]. As recently confirmed in the case of *PHO84* in budding yeast, anticorrelated sense and antisense expression[4,69] can actually be due to exclusive expression of either transcript among single cells in a population[115], which provides an opportunity for antisense-mediated regulation to contribute to cell-to-cell phenotypic variability.

Effects on the kinetics of transcriptional regulation. Antisense expression not only alters the abundance of sense transcripts but also affects the kinetics of the transition between differential gene expression states[116]. In general, regulation by antisense transcripts is faster than that by transcription factors, especially for antisense transcripts that function post-transcriptionally[112]. Additionally, the observed equilibrium between sense and antisense transcription (FIG. 4b) supports a model in which antisense expression induces a threshold-dependent (that is, ultrasensitive[117]) on–off switch for sense expression[29] (FIG. 4c). Specifically, the activation of the sense transcript needs to be high enough to oppose the repressive (that is, buffering) effect of the antisense transcript before the equilibrium can be altered and an increase in sense expression can be produced. This antisense-dependent ultrasensitivity allows buffering against low levels of activating stimuli, such as transient spurious activation signals, and also enables non-linear gene expression responses. For example, in *Synechocystis* spp. cyanobacteria, the presence of an antisense transcript (*isrR*) allows cells to ignore transient stimuli[118]. Only upon continued stimulation does the production of the sense iron-stress chlorophyll-binding protein transcript (*isiA*) overcome the repressive degradation effect of the antisense *isrR* transcript, which results in an accumulation of the sense transcript and thereby allows the bacteria to adapt to iron stress conditions[118,119] (FIG. 4c). Additionally, upon removal of the stimulus, a system that is based on antisense regulation can rapidly recover back to its basal state, especially if the antisense transcript is quickly degraded[113].

Rewiring regulatory networks. Antisense transcripts can function in conjunction with protein regulators to modify their effects. For example, cells can use ncRNA expression to invert the final effect of other transcriptional regulators. This ncRNA-mediated regulation not only allows the coordinated expression of neighbouring genes, in which an antisense transcript originating from a bidirectional promoter can spread signals that regulate sense expression[29] (FIG. 4a), but can also function as a simple mechanism to rewire regulatory networks (FIG. 4d). An example of this rewiring is the transcription of a long ncRNA *IRT1* that overlaps the *IME1* promoter, which is a key regulator of sporulation in budding yeast[92]. The transcription factor Rme1 activates the transcription of *IRT1*, which arises from a promoter that is upstream

Dynamic range
The range of expression levels between the minimum expression level of a gene in its basal or repressed state and its maximum expression level upon full activation.

Transcriptional bursting
A stochastic process in which a promoter changes from an inactive state to an active or open state that allows the production of multiple RNAs in a short period of time, before returning to the inactive state.

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of the *IME1* promoter, but this activating upstream signal is transformed into a repressive one by the ncRNA, which silences the downstream *IME1* promoter. Although *IRT1* overlaps the *IME1* promoter on the same strand, antisense transcripts can mediate similar effects; for example, both sense and antisense transcripts that are repressed by Set3C (which is a histone deacetylase that recognizes H3K4me) regulate the expression of their neighbouring genes in a similar manner[38]. Thus, the rewiring of regulatory networks by both antisense transcripts and ncRNAs could be a general mechanism for switching transcription factor functions between activators and repressors.

Rapid evolution of antisense-mediated regulation

Regulation by antisense transcripts has potential advantages over regulation by transcription factors because it allows rapid evolution. The sequences of ncRNAs are constrained by factors such as RNA secondary structure, genomic position and expression level; collectively, the sequences that encode these ncRNAs accumulate fewer substitutions than neighbouring neutral sequences[61]. In addition, both the presence and regulation of sense–antisense units show evolutionary conservation[58–60]. However, the appearance of new ncRNAs is less evolutionarily constrained than strategies that are dependent on proteins because it does not involve the modification of protein-coding regions. This allows a rapid generation of antisense transcripts and contributes to the evolution of regulatory circuitries. In the simplest case, in which the act of transcription is the main regulatory function of an antisense transcript, no evolutionary limitations exist with respect to the RNA sequence itself or to its final abundance. Additionally, antisense-mediated regulation does not require any factors that are not already intrinsic to sense expression; thus, evolutionarily, it could have provided a regulatory mechanism that did not require the invention of new machinery.

Antisense transcripts in bacteria appear rapidly on an evolutionary timescale[62,63]. This high evolvability of ncRNAs is also seen in higher eukaryotes, as shown in a study of closely related mammals[61]. These studies have found that the gain or loss of gene loci is more frequent for non-protein-coding genes than for protein-coding ones. In fact, the rate of evolutionary turnover for antisense transcripts is similar to those reported for transcription factor-binding events[120] or for other regulatory sequences[121]. It has been suggested that rapid evolution of non-coding regulatory mechanisms allows rapid adaptation[61]. However, this rapid evolution also implies that the same antisense transcript cannot be easily studied across different species. In addition, although ncRNAs or antisense transcripts may not initially encode proteins, they can provide ‘raw material’ for evolution to act on to give rise to proteins. Therefore, although we do not know the proportion of ncRNAs with regulatory functionality, some are likely to provide fitness advantages in the future. Along these lines, both ncRNAs and antisense transcripts have been proposed to be proto-genes that generate a pool of transcripts encoding short polypeptides, which acts as a reservoir for new genes. This represents an evolutionary continuum between antisense transcripts or other ncRNAs and protein-coding genes[122].

Antisense transcripts may also participate in shaping the evolution of genome architecture. Namely, they provide an intrinsic mechanism for the regulation of gene expression, in which not only the promoter and its terminator, but also other neighbouring regions and transcripts, affect gene expression through ncRNAs that invade a gene boundary[29]. In addition, when considering groups of neighbouring loci, both the interleaved organization of the transcriptome[25,35,55] and the transcriptional crosstalk between loci[29] constrain the evolution of genome architecture. In particular, gene shuffling could be evolutionarily restricted in regions that support beneficial interactions between loci, whereas in other regions, new rearrangements could give rise to novel interaction networks that are mediated by ncRNAs and antisense transcripts.

Conclusions

Research over the past few years shows that antisense transcription is pervasive and, in many cases, it regulates gene expression at multiple stages, from the control of chromatin state to the modulation of the post-transcriptional life of mRNAs. When studying a particular gene locus, its overlapping antisense and ncRNA transcription should be taken into account to improve experimental designs that are aimed at understanding its regulation and phenotypic consequences. However, to deepen our understanding of antisense transcription and to fully characterize the different mechanisms of action used by cells to regulate gene expression, many technical and conceptual developments are required.

From a technical point of view, both detailed biochemical dissection of antisense-mediated regulation and additional genome-wide functional studies will be instrumental. To distinguish between effects caused by the act of transcription and those caused by the produced transcripts, it will be necessary to disentangle the measurement of RNA levels[35] from its transcription and degradation[37,67] (BOX 2). This, together with the application of methods such as chromatin immunoprecipitation followed by sequencing (ChIP–seq), will improve our mechanistic understanding of *cis*-acting antisense transcripts. The study of specific subcellular populations of transcripts[25] will help to differentiate among co-transcriptional, post-transcriptional or chromatin-mediated effects. Experimental designs that involve time course analyses will be instrumental for determining the dynamics of the non-coding transcriptome and will thus be critical for distinguishing between the causes and consequences of antisense expression. Furthermore, the application of new single-cell[115] and transcriptomic[55] technologies promises a clearer picture of both the function and structure of antisense transcripts, as the variability of sense–antisense expression among different cells of the same population might obscure the actual effects of antisense expression in individual cells. Carrying out comparative studies across species will help to understand how the different antisense-mediated mechanisms

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evolved and the extent to which they are conserved. Finally, the development and application of new techniques, such as the genome-wide mapping of specific binding of RNAs to chromatin[27,28] and the identification of proteins that are bound to each RNA molecule[123], will open new avenues for studying *trans* effects of ncRNAs.

As our understanding of the process of antisense expression improves, the use of antisense transcripts as biotechnological tools to control gene expression is also likely to increase. Such studies will not only offer new genetic tools, such as transcriptional gene silencing produced by convergent sense and antisense transcripts[124], but will also reveal new mechanisms that cells might be using to control gene expression.

In conclusion, we believe that the field of gene regulation by antisense transcription, and by ncRNAs more generally, will continue to expand and will provide important advances in our understanding of gene regulation, crosstalk between neighbouring loci and the evolution of genome architecture.

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Competing interests statement
The authors declare no competing interests.

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