# Naturally occurring antisense RNA: function and mechanisms of action

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**Purpose of review**
Natural antisense transcripts have recently emerged as important regulators of gene expression. The transcription of an antisense RNA can influence the output of the specific gene locus on a posttranscriptional level but may also help to establish a local epigenetic imprint.

**Recent findings**
Recent advances in transcriptome sequencing have revealed widespread expression of complementary sense–antisense transcript pairs. The naturally occurring antisense transcripts can modulate the expression level of the sense transcripts or influence the sense mRNA processing. Given that both sense and antisense transcriptomes show tissue-specific regulation, these mechanisms may contribute to the physiological tuning of specific genes. An additional level of gene regulation by antisense transcripts has recently emerged: coexpressed sense and antisense transcripts can be cleaved and processed into single-stranded short RNAs (endo-siRNAs). Evidence suggests that these endo-siRNAs are linked to transcriptional silencing of the complementary transcripts. The impact of natural antisense transcripts may, therefore, not only feed forward to the protein level but also back to the genomic level.

**Summary**
Natural antisense transcripts add a further level of regulation to gene expression. The novel insights into the biology of natural antisense transcripts and endo-siRNAs may lead to improved gene silencing strategies in biomedical research with subsequent use in clinical applications.

**Keywords**
endo-siRNA, epigenetics, gene regulation, Na/phosphate cotransport, natural antisense transcript

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## Introduction

Natural antisense transcripts (NATs) are, in most general terms, long RNAs that derive from the opposite strand of annotated, protein-coding sense transcripts. NATs may contain potential open reading frames but the majority of NATs seem to be noncoding [1]. Genome-wide transcriptional analyses have revealed extensive antisense transcription not only in repetitive regions but also in protein-coding areas [2••]. The function of the majority of these NATs remains obscure. A certain proportion of NATs may constitute transcriptional noise; however, there are clear indications that some specific NATs have a gene regulatory impact [3]. Furthermore, investigations into whole antisense transcriptomes have identified common structural characteristics of some NATs pointing towards conserved themes in gene regulation by antisense transcripts [4–6]. A gene that is predominantly expressed in kidney and key to maintaining phosphate homeostasis (*Slc34a*) has helped to investigate the hypothesized paradigms of antisense transcription.

## Natural antisense transcript transcriptome

The initial investigations into antisense transcription were related to either large-scale cDNA/transcriptome sequencing projects or entailed public data set mining [5,7]. Both strategies depended crucially on a correct orientation of sequences to be analysed. Evidence of mRNA processing such as capping, splicing or polyadenylation was used to curate the relevant datasets. NATs identified along these studies can therefore be perceived as processed mRNA-like transcripts. Up to 72% of murine (and probably human) genomic loci showed evidence of antisense transcription [1,2••]. A hallmark of these processed NATs is a significant underrepresentation on the mammalian X chromosome [4,5].

Novel tag-based sequencing strategies [8••,9••], tiling arrays [10], an expansion of the focus on nonpolyadenylated transcripts [11] as well as cellular models with impaired RNA quality control [12] have increased the complexity of the antisense transcriptome dramatically. Many of the events involving NATs at this scale may represent transcriptional noise or stochastic events to maintain the epigenetic state of chromatin. Importantly, however, these studies also confirm genic antisense transcription, the formation of sense antisense hybrids and subsequent processing into short RNAs [8••,10].

NATs are often subdivided into *cis*-NATs and *trans*-NATs with reference to their locus of origin and the gene(s) they potentially regulate. This nomenclature is misleading in most cases because it assumes details of a potential regulatory mechanism that are simply unknown. This review will focus on NATs that have documented or hypothesized impact on the expression of the corresponding sense transcript and would therefore be considered as *cis*-NATs.

## A biological sense in antisense?

The scale of the regulatory potential of NATs has only recently emerged. It became clear that all organisms use RNA complementarity to modulate gene expression and a broad variety of different strategies have evolved [3]. For example, the key enzymatic components of RNA interference (RNAi) are almost ubiquitously expressed in eukaryotes with the notable exception of the baker's yeast *Saccharomyces cerevisiae* [13]. The fission yeast *Schizosaccharomyces pombe* has conserved the RNAi pathway and knocking out components of the machinery leads to defects in chromosome segregation [14]. Interestingly, plants use RNAi to fight viruses, whereas the role of RNAi in animals remains speculative [15]. These examples demonstrate that regulatory pathways based on RNA complementarity, such as gene regulation by NATs, may show various facets that are not necessarily transferable from one model organism to another. For this reason, we will discuss predominantly evidence from vertebrates with particular focus on human and mouse.

In the pregenomic area, NATs were usually investigated in the biological context of the corresponding sense transcripts. More recently, large-scale sequencing approaches have made entire genomes and transcriptomes available for data mining. This has revealed that the regulatory impact of NATs may have implications on a genome-wide scale. The seminal advances into gene regulation by NATs at both levels, single genes and entire genomes, will be discussed below.

A limited number of NATs have established roles in well described epigenetic phenomena such as parental imprinting (monoallelic expression of specific genes according to their parental origin, including the NATs *Air* and *Kcnq1ot1*) or X chromosome inactivation (including the NAT *Tsix*) [16]. The expression of these NATs is required for epigenetic silencing but the mechanism(s) involved are not fully understood. NATs from parentally imprinted gene clusters are, thus far, the only examples in which biological relevance is corroborated by knockout experiments [17]. However, the genome-wide scale of antisense transcription greatly exceeds the number of parentally imprinted genes. It remains unclear which lessons from the investigations into imprinting will be generally applicable to NATs. For this reason, we will focus on novel findings in the field of NATs and refer to recent reviews covering parental imprinting in detail [18].

## Mechanisms related to natural antisense transcripts

There are a number of mechanisms by which NATs can influence the expression of their complementary transcripts. Hybridization of the sense and antisense transcripts may be involved but other mechanisms have been documented as well. An overview of the different possibilities is given below, including a few well characterized examples [19].

### Transcriptional interference

Given that RNA polymerase II complexes progressing on opposite DNA strands cannot pass each other, transcription of a NAT will therefore impinge on the expression of the sense transcript and *vice versa* [20]. This phenomenon is best characterized in budding yeast but can also be demonstrated in mammalian cells under experimental conditions [21–23]. Whether transcriptional interference is of physiological relevance in humans (and mice) is debatable.

### RNA splicing

There is compelling evidence that the expression of NATs may interfere with the splicing of the corresponding sense RNA transcripts. For example, the expression of two splice forms of the thyroid hormone receptor (ErbAα1 and ErbAα2) is influenced by the levels of an antisense transcript (RevErbAα) that masks a relevant splice site. The effect of the NAT can be mimicked by transfection of oligonucleotides that overlap with the splice site [24]. Another example includes the effect of an antisense transcript upon Zeb2 splicing with consequences on E-cadherin expression and epithelial–mesenchymal transition [25]. Apart from a few well described examples, there is no evidence that NATs expression correlates with alternative splicing in general.

### RNA editing

Coexpressed sense and antisense transcripts may hybridize and form RNA hybrids. Double-stranded RNA is

recognized by an enzyme adenosine deaminase acting on RNA (ADAR) that converts adenosines in long perfect RNA duplexes into inosines [26]. Because inosine pairs with cytosine, the modifications will melt the RNA hybrid and eventually promote nuclear retention [27]. Alternatively, single-site modifications will cause point mutations with the potential to alter protein structure and function [28]. Most of the identified editing sites in the human transcriptome confer to intronic sequences and not to known bidirectionally transcribed loci [29]. There are, however, well documented edited transcripts of neurotransmitter receptor genes. In addition, the transcript of the ADAR2 isoform is a substrate for both ADAR1 and 2 enzymes [30].

#### Short RNAs

NAT-sense hybrids are potential substrates for the RNAi machinery. Short RNAs derived from bidirectionally transcribed regions have indeed been found recently; however, not at a scale that would be expected from the widespread expression of NATs [8••,9••]. This aspect of gene regulation by NATs will be discussed in detail below.

In summary, none of the mechanisms outlined above merit unconditional support in relation to NAT processing.

## Has every gene its own agenda?

Traditionally, NATs are investigated in the physiological context of their corresponding sense transcripts. An interesting interplay between sense and antisense transcript expression during developmental, physiological and pathophysiological processes has been demonstrated for specific genes [3]. A very restricted selection of examples is given below.

#### Msx1

The expression of the transcription factor Msx1 and the corresponding antisense transcript have been thoroughly studied during mouse embryonic development [31]. Both transcripts are expressed in the mandibular region from about 8 days postfertilization onwards. The expression pattern, revealed by in-situ hybridization, changes from reciprocal during earlier phases (around day 11) to overlapping (around day 16) [32]. Cell culture studies suggest that the antisense transcript negatively affected the sense RNA at the posttranscriptional level, whereas the Msx1 RNA or protein seemed to stimulate antisense expression [33].

#### β-Secretase-1

Amyloid precursor protein undergoes a sequential cleavage process to form protein fragments (Aβ 1–40, Aβ 1–42) that are linked to the pathophysiology of Alzheimer's disease. β-Secretase-1 is a key enzyme in this process and its expression is concordantly regulated by a processed and spliced antisense transcript [34•]. Neuroblastoma-derived (SH-SY5Y) cells upregulated the level of the antisense transcript and also β-secretase-1 protein in response to various stressors, including Aβ 1–42. The importance of the antisense transcript was corroborated by the finding that humans with Alzheimer's disease also showed an elevated level of antisense RNA. It was concluded that the β-secretase-1 antisense transcript was central to a feed forward response in the cascade of amyloid-β formation [34•].

#### Hypoxia-inducible factor 1α

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that coordinates gene expression in response to oxygen restriction. The gene encoding one component of the heterodimeric factor, HIF-1α, is transcribed in both directions. The antisense transcript overlaps the 3′ noncoding region of the sense transcript in a tail-to-tail arrangement. Expression of the antisense transcript antagonizes HIF-1 expression in response to prolonged hypoxia [35]. It is hypothesized that the antisense transcript exposes an AU-rich element in the 3′ untranslated region of the sense transcript, enabling the binding of regulatory factors, which reduce the half-life of the transcript. Increased expression levels of the antisense transcript correlated with the progression and poor prognosis of breast carcinomas [36], thus corroborating the relevance of the NAT.

#### Erythropoietin receptor

The expression of the erythropoietin receptor (Epo-R) gene (in dog) is significantly upregulated in growing left lung after right pneumonectomy. Increased levels of mRNA and protein are paralleled by the expression of an antisense Epo-R transcript and all three molecules localize to the same cells *in vivo*. The Epo-R antisense transcript contains two open reading frames, one of which fully overlaps with the Epo-R-coding region. Coexpression of the complementary transcripts in HEK-293 cells revealed a complex regulatory pattern that involved both regulation of mRNAs and the translated proteins [37•].

There is a rapidly increasing number of NATs with documented physiological implications. Further examples are given in a recent detailed review by Beiter *et al.* [3], in which NATs are regarded as regulators of the sense-encoded protein. Given the functional diversity of these NAT-regulated proteins, careful investigation will be required in order to establish the rules for the regulatory impact of NATs.

## A common sense in antisense?

An alternative view of NATs and their regulatory impact on gene expression has recently been presented [38••]. The case is originally based on the observation that

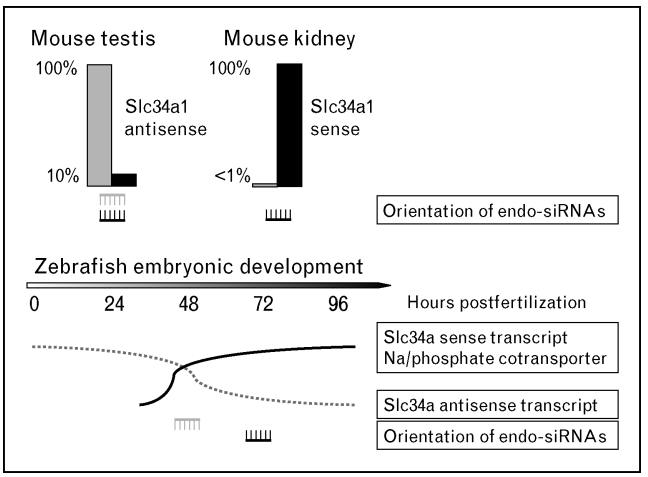
NATs with mRNA-like features and complementarity with the corresponding sense transcript are significantly underrepresented on the human and murine X chromosome [4,5]. The bias was not observed with antisense transcripts that lack an exonic overlap with the sense transcript. These key findings prompted two conclusions. First, the X chromosome bias indicated that members of that specific group of NATs feel comparable evolutionary pressure. It was, therefore, hypothesized that these NATs share a common strategy to exert a regulatory impact on gene expression. Second, the prerequisite of exonic complementarity between processed sense and antisense RNAs suggested that RNA hybridization was key to this selection process [39]. As indicated above, none of the mechanisms investigated in the context of double-stranded RNA processing applied to NATs unconditionally, though RNAi or a related process seemed the most likely option. Recent findings from large-scale short RNA sequencing approaches and also investigations into specific genes corroborate a link between the expression of antisense transcripts and the generation of so-called endo-siRNAs [8••,9••,37•,40•].

## Nonrandom orientation of endo-siRNAs

Several studies, including an investigation of the short RNA transcriptome in mouse oocytes [8••], found that a proportion of endo-siRNAs were derived from complementary regions of sense–antisense transcript pairs. The vast majority of endo-siRNAs, however, were derived from repetitive areas or pseudogenes [8••,9••]. Consequently, endo-siRNAs were suggested to mediate pseudogene silencing and organize chromatin structure [41]. Alternatively, it was argued that genic endo-siRNAs may be underrepresented as a result of a transient expression of NATs [42]. Interestingly, the orientation of the identified sequences was clearly nonrandom and mapped to the protein-coding (sense) strand.

This strand bias was also observed in two studies [38••,40•] that investigated a single gene in zebrafish (*Slc34a*) and mouse (*Slc34a1*). The gene encodes a Na-dependent inorganic phosphate transporter that is predominantly expressed in kidney [43]. The focus on a single gene allowed for a higher time and tissue-specific resolution of the expression analysis and detected endo-siRNAs that derived from the antisense strand. During zebrafish development, expression of the *Slc34a* antisense transcript preceded *Slc34a* sense transcription with a window of significant coexpression at 48 h after fertilization. Upon coexpression, endo-siRNAs with antisense orientation could be detected. Twenty-four hours later, the orientation changed and only sense-oriented endo-siRNAs were detected (Fig. 1) [40•]. In mouse, tissue-specific orientation of endo-siRNAs was reported. In the kidney, where the Na/phosphate transporter is expressed, only sense-oriented endo-siRNAs were found. In testis, both sense and antisense-derived products could be visualized on northern blots [38••].

**Figure 1 Expression of Slc34a-related transcripts and endo-siRNA production**



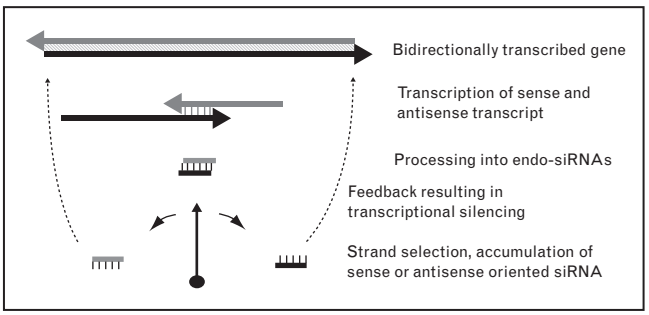

The levels of the sense transcript (black) and the antisense transcript (grey) are indicated (nonquantitative). Endo-siRNA production was assessed in different mouse tissues (upper panel) and during zebrafish embryonic development (lower panel). Significant coexpression may lead to transiently detectable levels of both endo-siRNA strands. The predominantly expressed transcript seems to dictate the orientation of the endo-siRNAs. Results are summarized from [38••,40•].

To conclude, endo-siRNA production is possibly linked to the widespread expression of NATs. Because antisense expression shows tissue-specific and development-specific expression patterns, a large proportion of endo-siRNAs may await experimental detection [2••]. The process leading to the accumulation of either the sense-oriented or the antisense-oriented short RNA strand is regulated. Strand selection seems to be influenced by the expression level of the two complementary transcripts.

## A paradigm for gene regulation by natural antisense transcripts?

A model of how the expression of NATs may influence transcriptional output has recently been brought forward [42]. On the basis of the observation from zebrafish and mouse testis, transient coexpression of a coding sense transcript and a noncoding antisense transcript were assumed. The model suggests that some of the complementary mRNAs hybridize and initiate processing into endo-siRNAs. Alternatively, both sense and antisense transcripts would persist as processed mRNAs and influence endo-siRNA strand selection. One of the strands will eventually accumulate. The prevailing strand of the endo-siRNA would then guide a silencing response towards either the sense or the antisense strand and eventually lead to transcriptional silencing

**Figure 2 Schematic summary of hypothetical processing of sense–antisense transcript pairs into endo-siRNAs**




The balance of strand selection is influenced by the level of each of the transcripts and possibly by mutations or polymorphisms.

of the complementary gene. Under physiological conditions, the antisense transcript would eventually become silenced. The crucial steps of this paradigm are supported by evidence from various model systems [44,45]. However, compelling direct experimental proof is lacking. A putative benefit for the organism that expresses NATs at a large scale is also unclear. The production of NATs just to form endo-siRNAs that result in the silencing of the same transcripts seems a wasteful use of resources, rather than a process that merits evolutionary conservation. This led to the intriguing hypothesis that predicts that polymorphisms or mutations in general may influence strand selection [38••]. As a consequence of reversed strand selection, the sense transcript may become silenced, thus limiting damage of mutated transcripts (Fig. 2).

Intuitively, the proposed model explains the underrepresentation of NATs on the mammalian X chromosome. Owing to a possible reversal of endo-siRNA strand selection, antisense transcription may harbour the risk of silencing the protein-coding sense transcript. This may lead to a complete gene knockdown and, as a consequence, antisense transcription may be suppressed on the X chromosome. On autosomes, the same scenario would lead to monoallelic expression of the sense-encoded protein from the affected locus (random imprinting). The recently detected significant correlation between genome-wide antisense transcription and randomly imprinted genes supports this line of argument [38••].

## Consequences beyond kidney

The testis shows significantly increased expression of NATs, including the NAT related to *Slc34a1* as documented during specific stages of spermiogenesis [46]. The cells show a generalized upregulation of transcription and translational repression [47]. In parallel, widespread apoptosis of developing spermiocytes is observed [48]. It is tempting to speculate about the biological role of endo-siRNAs in this context. Skewed endo-siRNA strand selection would lead to the silencing of the protein-coding sense gene and increase selective pressure on that particular haploid cell. If mutations were to affect the orientation of the endo-siRNA, a purifying selection of the developing sperm population would be observed.

In diploid cells, antisense transcription correlates with monoallelic expression [42] and a comparable scenario may apply. The consequences for the cell would be different because the loss of one sense transcript could be compensated by the other allele. Allele specificity of the entire process could simply depend on the timing of transcription from one allele or the other. In somatic cells, sense–antisense coexpression and endo-siRNA processing could occur during specific differentiation states or, alternatively, antisense transcription could be coinduced with the sense transcript but at a lower level. There is indeed evidence for both scenarios. Promoters of sense and antisense transcripts display partly overlapping transcription factor-binding sites that would allow for coregulation [49]. On the other hand, antisense transcripts are slightly upregulated in embryonic stem cells [38••] and selected sense–antisense pairs may be coexpressed in other defined cell populations, possibly tissue-specific stem cells (H. Peters, personal communication).

## Conclusion

NATs are known to regulate the expression level of their protein-coding sense transcript counterpart, with important physiological implications. A novel level of sense–antisense transcript regulation that involves endo-siRNAs has recently been suggested, with far-reaching implications. Further understanding of this field will hopefully be gained by a comprehensive description of time-resolved and development-resolved antisense transcriptomes.

## Acknowledgements

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