

## Biological functions of natural antisense transcripts

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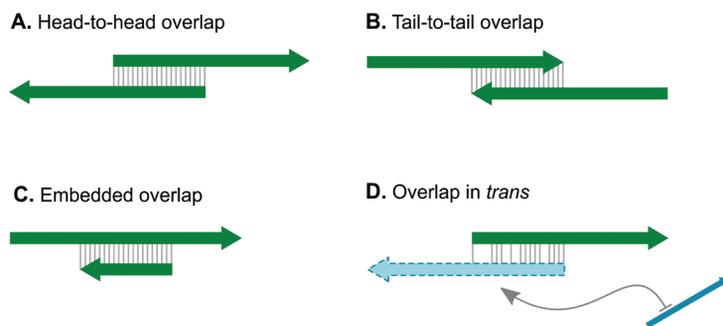
**Natural antisense transcripts (NATs) are RNA molecules that originate from opposite DNA strands of the same genomic locus (*cis*-NAT) or unlinked genomic loci (*trans*-NAT). NATs may play various regulatory functions at the transcriptional level *via* transcriptional interference. NATs may also regulate gene expression levels post-transcriptionally *via* induction of epigenetic changes or double-stranded RNA formation, which may lead to endogenous RNA interference, RNA editing or RNA masking. The true biological significance of the natural antisense transcripts remains controversial despite many years of research. Here, we summarize the current state of knowledge and discuss the sense-antisense overlap regulatory mechanisms and their potential.**

**Key words:** Natural antisense transcripts, antisense transcription, expression level regulation

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### INTRODUCTION

Natural antisense transcripts (NATs) are separated into two main categories, *cis* or *trans*, depending on their genomic origin. The most popular definition of *cis*-NATs describes these molecules as RNA sequences



**Figure 1. Types of natural antisense transcript overlap.** (A) Head-to-head overlap in *cis*; (B) Tail-to-tail overlap in *cis*; (C) Embedded overlap in *cis*; (D) Overlap in *trans*, green and blue arrows represent transcripts originated from different genomic loci, and forming double-stranded RNA. Full or partial complementarity between transcripts is indicated by a regularly spaced or disrupted “ladder” of grey vertical lines within the overlap region of *cis* and *trans* overlapping transcripts, respectively.

that originate from the opposite DNA strand of the same genomic locus, such that they physically share some genetic sequence. The overlap may be complete (Fig. 1C) or partial (Fig. 1A, B), and it may also be described as head-to-head (Fig. 1A), tail-to-tail (Fig. 1B) or embedded (Fig. 1C) (Makałowska *et al.*, 2005). The sequences of *cis*-NATs within the overlap region are perfectly com-

plementary between the sense and antisense RNAs, unlike *trans*-NATs, where transcripts originate from different genomic loci (Fig. 1D) (Vanhée-Brossollet & Vaquerro, 1998).

Almost half of the century has passed since the first natural antisense RNAs were found within the b2 region of coliphage  $\lambda$  (Bovre & Szybalski, 1969). Bovre and Szybalski concluded that double-stranded RNA (dsRNA) may be produced from overlapping transcripts under some conditions. They also suggested that RNA polymerases transcribing opposite strands in the b2 region may collide with each other, which would lead to premature transcription termination (Bovre & Szybalski 1969). Today this is known as polymerase collision, one of the transcriptional interference scenarios (Shearwin *et al.*, 2005). The golden age of natural antisense transcripts was far ahead despite this early discovery. Only 11 pairs of protein-coding genes with non-protein coding natural antisense counterparts were described until the late 1980s, all in viral genomes (Inouye, 1988). These studies consolidated the hypotheses that this type of genomic architecture was rather rare and probably limited to bacterial and viral genomes (Barrell *et al.*, 1976; Sanger *et al.*, 1977; Szekely, 1977). However, the first discoveries of overlapping genes in eukaryotic genomes were published in 1986. Henikoff and co-workers found that the *pupal cuticle protein (Pcp)* gene of *Drosophila melanogaster* was embedded on the opposite DNA strand of the *Gart* gene within its first intron (Henikoff *et al.*, 1986). Further discoveries in fruit fly (Spencer *et al.*, 1986) and mouse (Williams & Fried, 1986) were identified in the same year. The first overlapping pairs were found in humans and yeast three years later (van Duin *et al.*, 1989).

Comprehensive discoveries of natural antisense transcripts began to emerge at the beginning of the 21st century in various species, including plants (Mol *et al.*, 1990; Quesada *et al.*, 1999; Osato *et al.*, 2003; Wang *et al.*, 2006), fungi (Steigele & Nieselt, 2005; David *et al.*, 2006), vertebrates (Lehrer *et al.*, 2002; Shendure & Church, 2002; Zhou & Blumberg, 2003; Veeramachaneni *et al.*, 2005; Ge *et al.*, 2008) and invertebrates (Misener & Walker, 2000; Lee *et al.*, 2005). Antisense tran-

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**Abbreviations:** NAT, natural antisense transcript; *cis*-NAT, natural antisense transcripts originating from the opposite DNA strands of the same genomic locus; *trans*-NAT, natural antisense transcripts originating from different genomic loci; dsRNA, double-stranded RNA; TI, transcriptional interference; RNAPII, RNA polymerase II; RNAi, RNA interference

scription is now considered a widespread phenomenon that concerns up to 30% of human and mouse genomes (Yelin *et al.*, 2003; Chen *et al.*, 2004; Katayama *et al.*, 2005; Zhang *et al.*, 2006).

Natural antisense transcripts were studied using various approaches, including *in silico* analyses of the expressed sequence tags (Shendure & Church, 2002; Chen *et al.*, 2004), large-scale sequencing of full-length complementary DNAs (Osato *et al.*, 2003; Wang *et al.*, 2005), and tiling arrays (Li *et al.*, 2006; Matsui *et al.*, 2008). Current approaches are often based on next-generation sequencing technologies, such as RNA sequencing (RNA-Seq), single-strand RNA sequencing (ssRNA-Seq) or chromatin immunoprecipitation-sequencing (ChIP-Seq), which allowed for the discoveries of natural antisense transcripts across species on a large scale (Lu *et al.*, 2012; Conley & Jordan, 2012; Li *et al.*, 2013; Luo *et al.*, 2013).

## BIOLOGICAL FUNCTIONS OF NATURAL ANTISENSE TRANSCRIPTS

The biological significance of natural antisense transcripts remains controversial despite many years of research. Some groups describe natural antisense transcripts as transcriptional noise with the potential to acquire a secondary function. Other groups posit that this latent regulatory potential is underestimated and should be considered as another level of gene expression regulation. The overlap between natural sense and antisense transcripts may regulate expression at the transcriptional level (*via* transcriptional interference) and/or post-transcriptional level. Regulation may be achieved *via* modulation of chromatin changes by NATs or the formation

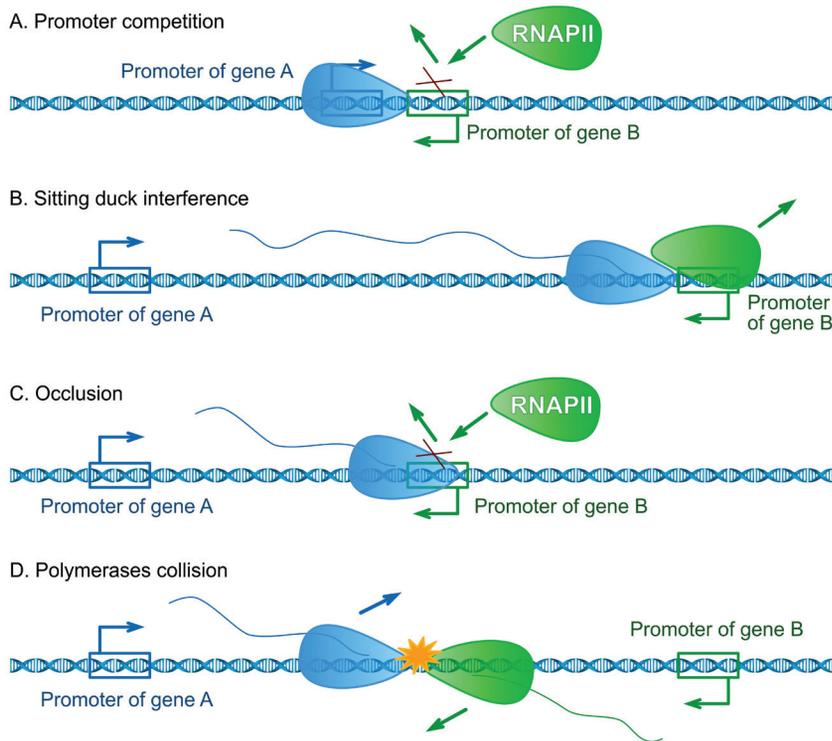
of double-stranded RNA, which leads to RNA masking, RNA interference or RNA editing (Faghihi and Wahlestedt 2009, Lu *et al.*, 2012, Celton *et al.*, 2014). Expression level regulation in *cis*, described in sections 1 and 2, may occur at the transcriptional and post-transcriptional levels. In contrast, regulation in *trans*, discussed in section 3, may act only post-transcriptionally (Vanhée-Brossollet & Vaquero, 1998).

## TRANSCRIPTIONAL LEVEL OF REGULATION

### Transcriptional interference

Natural antisense transcripts may regulate expression at the level of transcription *via* transcriptional interference (TI). This term describes a down-regulatory influence of the two ongoing transcription processes in a relatively close proximity (Shearwin *et al.*, 2005). TI may result in transcriptional downturn, transcriptional inhibition or early transcription termination. Four main mechanisms of transcriptional interference were proposed. The first mechanism, promoter competition, is a mechanism in which promoter regions overlap, and transcription may start only at one of them at a time (Fig. 2A). The second mechanism is called “sitting duck interference”, and it describes a situation in which RNA polymerase II (RNAPII) progresses too slowly to the elongation phase, and it is dislodged by another RNA polymerase II (Fig. 2B). The third scenario, occlusion, involves the temporary blocking of transcription initiation at a particular promoter region by the ongoing elongation of RNAPII originating from a different promoter (Fig. 2C). The last mechanism, polymerase collision, occurs when two RNAPIIs, which are transcribing genes in opposite directions, block each other’s passage and collide in a head-to-head manner (Fig. 2D). Shearwin *et al.*, thoroughly discussed these mechanisms in a review (Shearwin *et al.*, 2005).

Transcriptional interference was intensively studied in recent years. A transcriptional collision model was inferred from human and mouse genomes analyses, which observed lower expression levels of sense-antisense transcripts in longer overlapping regions (Shearwin *et al.*, 2005; Osato *et al.*, 2007). RNAPII collisions were described in yeast in *in vitro* and *in vivo* models. RNAPIIs were temporarily suspended during transcriptional collision events, but the elongation complexes were stable, which extended the half-life of the RNAPII involved in this process (Hobson *et al.*, 2012). Notably, the RNAPII collision was also linked with the “off-targeting” of the *activation-induced cytidine deaminase* (AID), which initiates somatic hypermutations (SHM) and



**Figure 2. Mechanisms of transcriptional interference.**

(A) Promoter competition; (B) Sitting duck interference; (C) Occlusion; (D) Polymerase collision; RNAPII – RNA polymerase II; Blue/green boxes with arrows indicate the transcription direction and promoter regions of genes A and B, respectively. Arrows next to RNAPIIs indicate the premature end of transcription of a particular RNAPII. Based on Shearwin *et al.*, 2005.

immunoglobulin (Ig) heavy chain class switch recombination (CSR) in B cells (Muramatsu *et al.*, 2000; Meng *et al.*, 2014; Pefanis *et al.*, 2014). AID initiated on non-Ig targets is related to human B cell lymphomas (Alt *et al.*, 2013). TI was also linked with Huntington's disease, in which CAG repeat expansion within the first exon of the *huntingtin* (*HTT*) gene is associated with the disease pathology (DiFiglia *et al.*, 1997; Chung *et al.*, 2011). *HTT* expression level is down-regulated by the *huntingtin* antisense transcript *HTTAS* via transcriptional interference and/or a Dicer-dependent mechanism. Growing CAG repeat expansion in *huntingtin* weakens the *HTTAS* promoter strength and antisense expression level, which results in the up-regulation of *HTT* in Huntington's disease patients (Chung *et al.*, 2011). TI regulates the expression level of the *frequency* (*frq*) gene in *Neurospora crassa*, which is a central component of the circadian clock (Xue *et al.*, 2014; Cha *et al.*, 2015). The expression of the antisense non-protein coding gene *qrf* leads to the premature transcription termination of the *frq* gene via transcriptional interference and mediation of chromatin modifications (Xue *et al.*, 2014). The core circadian clock of animals is regulated in a similar manner (Koike *et al.*, 2012; Menet *et al.*, 2012; Vollmers *et al.*, 2012).

How often a transcriptional interference truly controls the expression level of genes is debatable (Hobson *et al.*, 2012). This mechanism may control the vast majority of genes because recent studies discovered that antisense non-coding RNAs are counterparts of a substantial number of genes in animals (Lehner *et al.*, 2002; Yelin *et al.*, 2003; Chen *et al.*, 2004; Lapidot & Pilpel, 2006; Conley *et al.*, 2008) and plants (Yamada *et al.*, 2003; Stolc *et al.*, 2005; Li *et al.*, 2006; Matsui *et al.*, 2008; Lu *et al.*, 2012; Luo *et al.*, 2013). Models of transcriptional interference mostly suggest a negative correlation of antisense RNA expression levels (Shearwin *et al.*, 2005). However, studies indicate that overlapping transcripts generally do not exhibit expression level correlations, and these correlations tend to be positive rather than negative (Oeder *et al.*, 2007; Grigoriadis *et al.*, 2009; Conley & Jordan, 2012; Ling *et al.*, 2013). Transcriptional interference may not generally regulate the expression level of all genes, but in some cases, TI may subtly regulate the expression levels of at least some genes where regulatory functions have emerged (Brophy & Voigt, 2016).

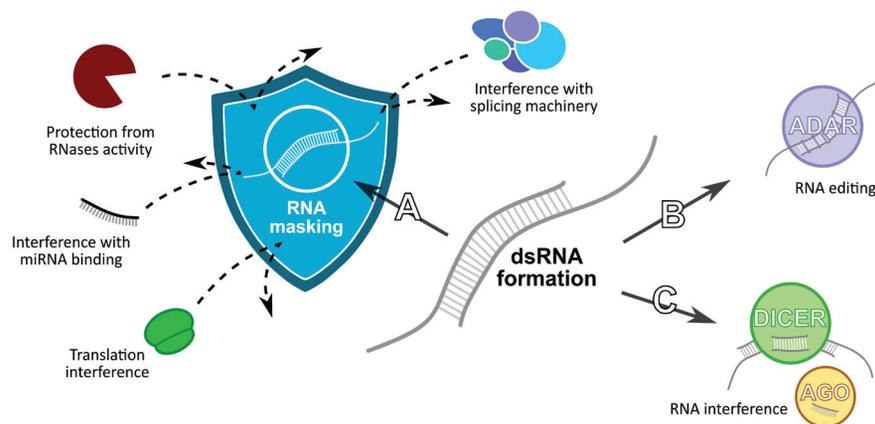
## POST-TRANSCRIPTIONAL LEVEL OF REGULATION

### RNA masking

Simultaneous transcription of antisense RNAs may lead to the formation of double-stranded RNA, which may interfere with the accessibility of the target sequences of various miRNAs (Fig. 3A). This mechanism was discovered recently for the *Sirt1* gene, which possesses a target sequence for *miR-34a*. The miRNA target sequence is located within the overlap region between the *Sirt1* gene and its antisense, *Sirt1-AS*. This positioning results in competition between *Sirt1-AS* and *miR-34a* for *Sirt1* transcript binding (Wang *et al.*, 2016). Overexpression of the *Sirt1-AS* stabilized *Sirt1* mRNA and increased its half-life from 2 to 10 hours (Wang *et al.*, 2016). Similarly, beta-secretase-1 (*BACE1*) expression level is negatively controlled by *miR-485-5p* binding and positively controlled by the formation of dsRNA by *BACE1* sense and antisense (*BACE1-AS*) transcripts. Knockdown of *BACE1-AS* exhibits the same effect as silencing of *BACE1* (Modarresi *et al.*, 2011). The imbalance of *BACE1*, *BACE1-AS* and *miR-485-5p* expression leads to an up-regulation of *BACE1*, which was linked to pathological states in patients with Alzheimer's disease (Faghihi *et al.*, 2008; Faghihi *et al.*, 2010). NATs are also involved in Parkinson's disease, where the short splice variant of *PTEN-induced putative kinase 1* (*PINK1*), called *spPINK1*, may form a dsRNA with its antisense that is complementary at almost full-length with the sense RNA. dsRNA formation leads to stabilization of the sense transcript, and the antisense knockout resulted in the loss of the *spPINK1* splice variant (Scheele *et al.*, 2007).

Formation of dsRNA may also increase stability of the involved RNA molecules via protection from digestion by ribonucleases aimed at single-stranded RNA degradation, which was demonstrated in cyanobacterium *Prochlorococcus* sp. RNase E (Stazic *et al.*, 2011). Sense-antisense duplexes may protect RNA molecules from entering into nonsense-mediated decay (NMD), which was demonstrated in yeasts (Wery *et al.*, 2016). Protection against single-stranded RNases was also demonstrated in *nds-2a*, which is a stable, naturally occurring human dsRNA of sense-antisense transcription origin. Knockdown of *nds-2a* dsRNA using strand-specific locked nucleic acid (LNA) gapmers resulted in numerous mitotic-related effects which suggests a functional role of these RNA duplexes (Portal *et al.*, 2015).

dsRNA formation may influence alternative splicing of the rat and human  $\alpha$ -thyroid hormone receptor (*TRa*) gene, which encodes two splice variants, *TRa1* (active, hormone-binding variant) and *TRa2* (inactive, non-hormone-binding variant). NAT only binds to the longer, non-hormone-binding *TRa2* splice variant. *TRa2*-NAT dsRNA formation is presumably responsible for the negative regulation of *TRa2* expression level, which pre-



**Figure 3. Regulatory roles of double-stranded RNA (dsRNA) formation.**

It may lead to: (A) RNA masking, that may cause the transcript's protection from an RNase activity, interference with translation and splicing machinery, or interference with miRNA binding sites' accessibility; (B) dsRNA editing by the adenosine deaminase acting on RNA (ADAR); (C) RNA interference by Dicer-dependent post-processing of dsRNA to short siRNA, followed by Argonaute (AGO) loading into the RNA-induced silencing complex.

vents the inactive variant expression (Munroe & Lazar, 1991; Hastings *et al.*, 2000). Munroe recently demonstrated that the *TRA2* variant was not present in marsupials and platypus, and comparative analysis revealed that only *TRA2* was adopted as a *TRA* expression level regulator in eutherian lineage (Munroe *et al.*, 2015).

Another example involves regulation of the *E-cadherin* – protein complex which plays a key role in cellular adhesion. Dysfunctions of this complex are associated with increased tumor metastasis (Beavon, 2000). *Zeb2* is a transcriptional repressor of *E-cadherin*. Expression of the *Zeb2* NAT induces an alternative splicing of *Zeb2*, which results in the presence of an intron containing an internal ribosome entry site (IRES) that is necessary for the *Zeb2* protein synthesis (Beltran *et al.*, 2008).

RNA masking may also inhibit expression at the translational level, which was demonstrated in the *BCMA* gene. The amount of *BCMA* mRNA in cells is not altered by the expression level of antisense (normal or increased expression). However, increased expression of *BCMA*-antisense RNA results in a lower amount of the *BCMA* protein (Hatzoglou *et al.*, 2002). The functional significance of NATs at the translational level was demonstrated in more detail for *PU.1* transcription factor expression regulation, where the *PU.1* antisense interferes with the formation of the *PU.1* elongating complex (*eEF1A*-mRNA) (Ebraldze *et al.*, 2008).

### RNA editing (A-to-I)

Natural antisense transcripts that form double-stranded RNA could become a target for the adenosine deaminase acting on RNA (ADAR) in a process called RNA editing (Fig. 3B). ADAR editing leads to adenosine (A) deamination into inosine (I), which is further interpreted as guanosine (G) by the cellular translational and splicing machinery (Nigita *et al.*, 2015). Peters and co-workers investigated the 162-nt long overlap region of the *4f-rnp* and *sas-10* overlapping gene sequences in *Drosophila melanogaster* and discovered that approximately 20% of the *4f-rnp* and *sas-10* transcripts show marks of RNA editing at random positions (Peters *et al.*, 2003). The extent to which RNA editing plays a biologically significant role in natural antisense transcripts is not fully understood (Wight and Werner 2013). Nevertheless, reports of thousands of human, mouse and fly RNA editing sites are reported in different contexts (Laganà *et al.*, 2012; Wang *et al.*, 2013; Ramaswami & Li, 2014; Zhang *et al.*, 2016). Therefore, the discovery of the links of these sites with NATs on a broader scale is likely a matter of time.

### RNA interference

Another possibility for the functional relevance of natural antisense transcripts is post-transcriptional

dsRNA-dependent RNA interference (RNAi) through endo-siRNA – endogenous small-interfering RNAs (Fig. 3C). Endo-siRNAs were reported in various species, including plants (Borsani *et al.*, 2005; Zhang *et al.*, 2012; Li *et al.*, 2013; Yu *et al.*, 2016), fungi (Lee *et al.*, 2010) and animals (Tam *et al.*, 2008; Watanabe *et al.*, 2008; Okamura *et al.*, 2011; Werner *et al.*, 2014; Ling *et al.*, 2016). The mechanism of endo-siRNA maturation from NAT-derived dsRNA that is understood the most, suggests a Dicer-dependent character of dsRNA processing to short siRNA, followed by Argonaute loading into the molecular silencing machinery, RNA-induced silencing complex (RISC) (Czech & Hannon, 2011; Kwak & Tomari, 2012). However, studies of *Neurospora crassa* suggest that siRNA is formed using an unknown Dicer-un-

related pathway, forming the so-called dicer-independent small interfering RNA (disiRNA) (Lee *et al.*, 2010). Natural antisense transcripts were also described as a source of short RNA (*Sox4\_sir3*) in mouse, which resembled piRNA rather than siRNA. *Sox4\_sir3* is 24-nt long and possesses a 5'-end-sequence bias for uridine. Comparative *in silico* analyses suggest that *Sox4\_sir3* is a central nervous system-related piRNA (Ling *et al.*, 2016).

Formation of dsRNA may protect natural antisense transcripts from single-stranded RNase activity and simultaneously limit the prevalence of NATs primarily to the nucleus. Formation or transport of a double-stranded RNA to the cytoplasm could trigger an immune response. Cellular machinery recognizes dsRNA as a viral infection and promotes the inhibition of protein synthesis and the transcriptional induction of interferon and other cytokines, which may ultimately lead to cell death (Wang & Carmichael, 2004; Kumar *et al.*, 2004; Gantier & Williams, 2007). NAT-related dsRNAs are primarily located in the nucleus, possibly to avoid the above-mentioned immune response (Faghihi & Wahlestedt, 2006; van Heesch *et al.*, 2014; Portal *et al.*, 2015). The regulatory function of NATs in the cytoplasm is limited to regulation by endo-siRNAs, which are present in the nucleus and cytoplasm (Portal *et al.*, 2015). However, stable sense-antisense duplexes that were predominantly located in the cytoplasm were also reported (Dallosso *et al.*, 2007; Michael *et al.*, 2011). Therefore, the extent to which the cellular interferon pathway is activated in response to naturally occurring dsRNAs is debatable (Wang & Carmichael, 2004).

Genome-wide analyses using RNA-Seq and single-stranded RNA-Seq protocols, strengthened by parallel analyses of small RNA or degradome sequencing, were proposed in recent years to expand our knowledge of the NATs involved in the endogenous RNA interference (Lu *et al.*, 2012; Li *et al.*, 2013; Werner *et al.*, 2014; Yu *et al.*, 2016). These studies revealed that nearly 4% of the *Arabidopsis thaliana* cis-NATs produce putative endo-siRNAs, and approximately 200 of these endo-siRNAs exhibit relatively high expression levels  $\geq 10$  RPKM (Reads Per Kilobase per Million mapped reads) (Li *et al.*, 2013). Studies in orchid (*Dendrobium officinale*) identified 63 natural antisense transcripts that produced endo-siRNAs (Yu *et al.*, 2016). A total of 2292 NATs were reported as a source of endo-siRNA in rice (*Oryza sativa*) (Lu *et al.*, 2012). A large-scale analysis of the small RNA transcriptome in human embryonic kidney cells revealed that the sense-antisense transcription of 169 RefSeq genes may lead to the formation of endo-siRNAs. These endo-siRNAs were also mostly enriched by AGO1 and RNAPII, which correlated with the actively transcribed endo-siRNA precursors (Werner *et al.*, 2014). Our understanding of the biological impact of these findings requires further study, but the use of next-generation sequencing for large-scale endo-siRNA studies has already revealed their widespread occurrence and regulatory potential.

### Epigenetic modifications

Natural antisense transcripts may regulate expression levels of the sense genes and mediate chromatin modifications within the gene sequence, promoter or enhancer regions, the entire *locus* or even surrounding genomic *loci* (Li & Ramchandran, 2010; Halley *et al.*, 2013; Wight & Werner, 2013). For example, expression of the *HBA2* gene may be down-regulated by a repressive chromatin modification within the *HBA2* promoter regions by its

antisense transcript, *LUC7* (Tufarelli *et al.*, 2003). Another example is *brain-derived neurotrophic factor* (*BDNF*) and its antisense, *BDNF-AS*, which may play a role in the guidance, introduction and maintenance of the histone H3K27me3 modification within the *BDNF* locus (Modarresi *et al.*, 2012b). *BDNF-AS* was associated with the recruitment of *polycomb repressive complex 2* (*PCR2*), which locally induces the trimethylation of histone H3K27 within the locus, without exerting any effect on the surrounding loci (Modarresi *et al.*, 2012b). Decreased expression of *BDNF* is associated with Alzheimer's, Parkinson's, and Huntington's diseases (Bathina & Das, 2015). Knockout of *BDNF-AS* results in an up-regulation of *BDNF* expression levels, which supports the therapeutic potential of this mechanism (Modarresi *et al.*, 2012a). In contrast to *BDNF-AS*, which locally induces histone modifications, an antisense of the mouse *Kcnq1* gene, *Kcnq1ot1*, may impact the entire *Kcnq1* domain. *Kcnq1ot1* is responsible for the recruitment of *PCR2* and *G9a* methyltransferases and the induction of repressive histone modifications of the *Kcnq1* gene and several up- and down-stream localized genes (Pandey *et al.*, 2008). Notably, large-scale interactions of *Kcnq1ot1* were present in a lineage-specific manner in mouse placenta, but not fetal liver. The regulatory significance of *Kcnq1ot1* is emphasized by the insertion of a premature transcriptional stop signal and synthesis of a truncated *Kcnq1ot1* transcript, which results in an up-regulation of all genes in the *Kcnq1* domain (Mancini-Dinardo *et al.*, 2006). NAT recruitment of *PCR2* is also involved in the X-chromosome inactivation by *X-inactive specific transcript* (*Xist*) and its antisense, *Tsix* (Halley *et al.*, 2013). *Tsix* biallelic expression before the X-inactivation leads to the silencing of *Xist* on both X chromosomes via H3K27me3 histone modifications over the *Xist* promoter regions (Ohhata *et al.*, 2015). *Tsix* expression becomes monoallelic at the early stage of chromosome inactivation, which results in a de-repression of the *Xist* promoter and heterochromatinization of the chromosome X where *Tsix* was silenced (Lee *et al.*, 1999, Ohhata *et al.*, 2015). *Tsix* dysfunctions may lead to several X-linked diseases (Chaligné & Heard, 2014; Charles Richard & Ogawa, 2016).

## NAT REGULATORY FUNCTIONS IN TRANS

Natural antisense transcripts that function in a *trans* arrangements (*trans*-NAT) have not been studied as intensively as *cis*-NATs. *Trans*-NAT sequences within the "overlap" region may not be fully complementary to the target sequence because these sequences originate from different genomic loci (Vanhée-Brossollet & Vaquero, 1998). However, this partial complementarity may still lead to the formation of double-stranded RNAs. Recent studies demonstrated that thousands of human (Szcześniak & Makalowska, 2016) and plant (Szcześniak *et al.*, 2016) transcripts exhibit the potential to form lncRNA-RNA duplexes, and new functional *trans*-NATs are continuously being discovered (Roberts & Morris, 2013). Every DNA-mediated duplication and retrotransposition event is generally a source of a sequence that is complementary to the original sequence. The emerged copy possesses the potential for expression, which may lead to *trans*-NAT formation (Muro & Andrade-Navarro, 2010; Roberts & Morris, 2013). One well-studied example is the *nitric oxide* (*NO*) neurotransmitter in *Lymnaea stagnalis*, which is involved in long-term memory formation and associated with food-reward conditioning (Kemenes *et al.*, 2002). *NO* production is catalyzed by

*NO-synthase* (*NOS*), which is negatively regulated in *trans* by the *NOS* pseudogene antisense transcript (*antiNOS-2*) via dsRNA formation of *NOS* mRNA and *antiNOS-2*. Decreased *antiNOS-2* expression levels facilitate memory formation in classical conditioning (Korneev *et al.*, 1999; Korneev *et al.*, 2002; Korneev *et al.*, 2013). Pseudogenes in mouse oocytes are also a source of endo-siRNAs that originate from dsRNA that forms between parental mRNA and homologous pseudogene antisense sequences (Tam *et al.*, 2008; Watanabe *et al.*, 2008). The identified endo-siRNAs complex are Dicer-dependent and enriched with Ago2. Target sequence expression levels of the detected complex increased in Dicer and Ago2 knockout mutants (Watanabe *et al.*, 2008).

Three potential endo-siRNA precursor regions, *esiRNA1*, *esiRNA2* and *esiRNA3* were identified in the human hepatocellular carcinoma pseudogene  $\psi$ PPM1K sequence. Endo-siRNA may arise from these precursors in two ways. The first mechanism is related with

*esiRNA3* and based on dsRNA formation by  $\psi$ PPM1K antisense and the cognate gene (*PPM1K*) transcripts. The second mechanism involves *esiRNA1* and leads to endo-siRNA maturation from the hairpin structure formed by the  $\psi$ PPM1K transcript. *esiRNA1* may down-regulate the expression of the cognate *PPM1K* (*protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup>-dependent*) gene and *NEK8* (*NIMA-related kinase 8*) gene. These effects were not observed in a  $\psi$ PPM1K mutant with deletion of the *esiRNA1* precursor region (Chan *et al.*, 2013).

*Trans*-NATs also induce chromatin epigenetic changes. Methylation of the *Oct4* gene promoter region by the recruitment of *Ezh2* methyltransferase is linked to an antisense transcript of *Oct4-pseudogene 5 - asOct4-pg5*. Separate knockdowns of *Ezh2* and *asOct4-pg5* resulted in *Oct4* up-regulation via demethylation of its promoter regions. A down-regulatory influence of the *asOct4-pg5* was also RNAi independent (Hawkins & Morris, 2010).

Natural antisense transcripts are able to regulate the sense gene expression levels in a *cis* and *trans* manner in some genomic arrangements. For example, the *DHRS4* gene cluster is composed of three highly homologous genes, *DHRS4*, *DHRS4L1* and *DHRS4L2*, and the *DHRS4* gene is regulated in *cis* by *AS1DHRS4*, a head-to-head antisense transcript. *AS1DHRS4* also regulates *DHRS4L1* and *DHRS4L2* genes in *trans*. *AS1DHRS4* controls the epigenetic silencing of all promoter regions within the *DHRS4* gene cluster via interaction with *EZH2* and *G9c* methyltransferases. Knockout of *AS1DHRS4* increases the expression of genes in the *DHRS4* gene cluster (Li *et al.*, 2012).

The number of known pseudogenes that gained a new regulatory function by antisense transcription is small, but yearly discoveries refine our understanding of the potential of *trans*-NATs to regulate gene expression on another level.

## CONCLUDING REMARKS

Natural antisense transcripts possess a great potential to regulate the sense gene expression at transcriptional and post-transcriptional levels, and their functional relevance is supported by the numerous reports of their tissue-specific expression (Lu *et al.*, 2012; Conley & Jordan, 2012; Ling *et al.*, 2013).

Notably, a growing number of studies link NATs with various human diseases, including the Fragile X syndrome (Ladd *et al.*, 2007; Khalil *et al.*, 2008), Alzheimer's (Parenti *et al.*, 2007; Faghihi *et al.*, 2008; Faghihi

*et al.*, 2010; Bathina & Das, 2015), Parkinson's (Scheele *et al.*, 2007; Bathina & Das, 2015), and Huntington's diseases (Chung *et al.*, 2011; Bathina & Das, 2015), and cancer (Dallosso *et al.*, 2007; Yu *et al.*, 2008; Pasmant *et al.*, 2011; Chaligné & Heard, 2014). NATs also play a role in hypertension, asthma or thyroid dysfunction (Michael *et al.*, 2011), and metabolic (Li *et al.*, 2012), immune (Hatzoglou *et al.*, 2002) or cardiovascular disorders (Annilo *et al.*, 2009). The medical applications of the regulatory functions exerted by NATs over their sense gene counterparts were scrutinized in comprehensive reviews by Khorkova (Khorkova *et al.*, 2014) and Halley (Halley *et al.*, 2013).

The biological significance of natural antisense transcripts will likely be debatable for a long time. However, our increasing understanding of the NAT biology sheds new light on the functional importance of antisense transcription, which may not regulate every single natural sense-antisense pair, but is surely essential for the proper functioning of all living organisms.

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