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REVIEW

Therapeutic potential of natural antisense transcripts and various mechanisms involved for clinical applications and disease prevention

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ABSTRACT

Antisense transcription, a prevalent occurrence in mammalian genomes, gives rise to natural antisense transcripts (NATs) as RNA molecules. These NATs serve as agents of diverse transcriptional and post-transcriptional regulatory mechanisms, playing crucial roles in various biological processes vital for cell function and immune response. However, when their normal functions are disrupted, they can contribute to human diseases. This comprehensive review aims to establish the molecular foundation linking NATs to the development of disorders like cancer, neurodegenerative conditions, and cardiovascular ailments. Additionally, we evaluate the potential of oligonucleotide-based therapies targeting NATs, presenting both their advantages and limitations, while also highlighting the latest advancements in this promising realm of clinical investigation.

Abbreviations: NATs- Natural antisense transcripts, PRC1- Polycomb Repressive Complex 1, PRC2- Polycomb Repressive Complex 2, ADARs- Adenosine deaminases acting on RNA, BDNF-AS- Brain-derived neurotrophic factor antisense transcript, ASOs- Antisense oligonucleotides, SINEUPs- Inverted SINEB2 sequence-mediated upregulating molecules, PTBP1- Polypyrimidine tract binding protein-1, HNRNPK- heterogeneous nuclear ribonucleoprotein K, MAPT-AS1- microtubule-associated protein tau antisense 1, KCNQ1OT- (KCNQ1 opposite strand/antisense transcript 1, ERK- extracellular signal-regulated kinase 1, USP14- ubiquitin-specific protease 14, EGF- Epidermal growth factor, LSD1- Lysine Specific Demethylase 1, ANRIL- Antisense Noncoding RNA in the INK4 Locus, BWS- Beckwith-Wiedemann syndrome, VEGFA- Vascular Endothelial Growth component A

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Introduction

Formation of natural antisense transcripts (NATs) is the result of antisense transcription in most of the genes [1]. The overlapping of NATs and regulatory genes is very common phenomenon. Interestingly the characteristics of NATs are very unique and distinct regarding their functioning and working as compared to their coding regions [2]. Most of NATs lack polyadenylation, show lower expression as compared to coding regions and they frequently exhibit pronounced specific expression patterns [3]. The diversity of antisense transcripts have sparked extensive research efforts aimed at unravelling their roles in biological processes [4]. Initially, an investigation into 797 evolutionarily conserved NATs via knockdown experiments unveiled regulatory connections between complementary transcripts [5]. NATs exert their influence by two ways either enhancing or suppressing the activity of their effector genes. While this regulation predominantly occurs in proximity to the antisense transcript's origin and impacts its sense counterpart, there are also instances of trans-acting mechanisms targeting transcripts from different genomic regions.

NATs distinguish themselves from other non-coding RNAs in terms of their origin, function, mechanism of action, and regulatory roles. Unlike other non-coding RNAs that may arise from introns, intergenic regions, or enhancer regions, NATs originate from the opposite strands of protein-coding genes. While NATs primarily function through RNA-RNA interactions, other non-coding RNAs exert their effects through interactions with DNA, proteins, and additional RNA molecules. NATs often exhibit overlap with protein-coding genes, whereas other non-coding RNAs are typically transcribed from regions situated between genes or originating from enhancer RNAs [2].

Mechanistically, NATs modulate the processing and expression of other RNAs through various mechanisms (Figure 1). A significant mechanism involves engaging the Polycomb Repressive Complex 2 (PRC2), known for gene silencing, resulting in H3K27me3 trimethylation and transcriptional inactivity at specific genomic locations (Figure 1a). This represents discordant regulation, where increased NAT expression impedes gene activity in the sense direction. On the other hand, interaction of sense and antisense transcripts occur through specific RNA: RNA interactions, impacting alternative splicing by concealing splice sites and splicing signals. (Figure 1b). Additionally, RNA duplex formation can induce editing by providing double-stranded

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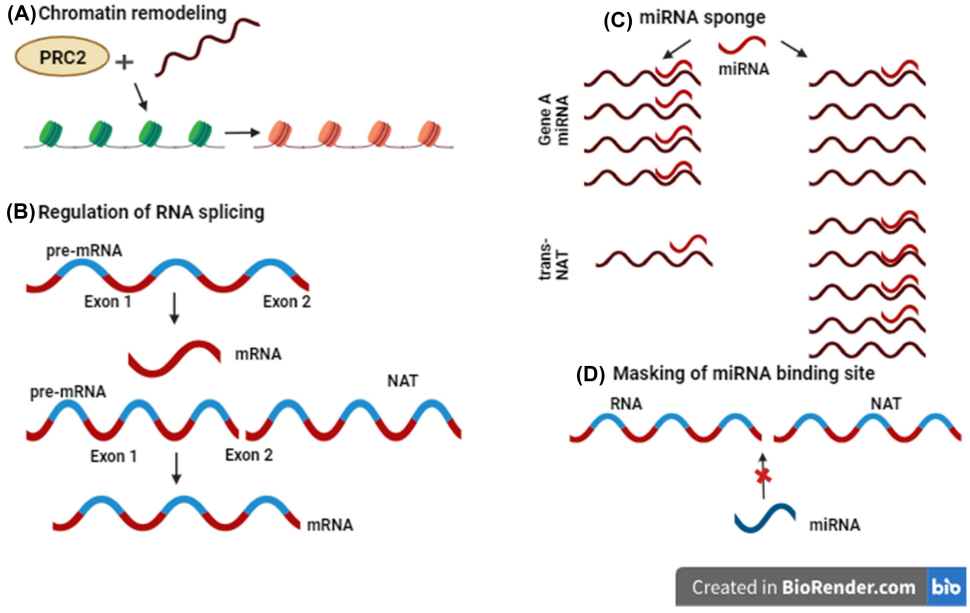

Figure 1. Functions of NATs. (a) NATs play a role in chromatin remodeling by facilitating the recruitment of the PRC1 or PRC2 complexes. (b) NATs are involved in the regulation of alternative splicing through RNA: RNA interactions. (c) NATs can act as miRNA sponges, creating a situation where an increased quantity of trans-NATs possesses miRNA target sites. (d) Another mechanism involving NATs is the masking of miRNA binding sites.

RNA substrates to adenosine deaminases acting on RNA (ADARs).

Further mechanisms encompass influencing stability of transcript by directing transcripts for protein-coding towards degradation pathways interfering with miRNA functions through the concealment of miRNA binding sites in the 3' untranslated regions (Figure 1d). Some NATs also serve as microRNA sponges, harbouring multiple binding sites that sequester specific miRNAs, preventing them from interacting with other RNAs (Figure 1c). Recognized NAT-mediated actions extend to transcriptional interference and interactions with splicing factors, among other mechanisms [6].

In the following sections, we delve into specific modes of action, with a focus on those relevant to human disease aetiology or significant for human health. Our primary emphasis remains on cis-NATs, those with sense partners located within the same genomic locus. Subsequently, we outline current approaches aimed at harnessing the unexplored regulatory potential of NATs for treating human diseases.

NATs serve as precise regulators of gene expression, perform their functions effectively even at lower expression levels, a phenomenon referred to as leaky transcription of NATs. The word 'leaky transcription' is not frequently employed in all articles; instead, a more basic term low expression is often used in most articles. Essentially, NATs has the capability to effectively fulfil their function even at lower levels, making them a highly advantageous feature. A study conducted by Zhao *et al.* discovered that the reduced production of NATs led to a decrease in the expression of genes that promote cancer [7]. A further investigation carried out by Santos et al. revealed that NATs have demonstrated a reduction in cancer occurrence when expressed at low levels [8].

NATs exhibit distinct functions in various tissues. The BDNF gene, also known as Brain-Derived Neurotrophic Factor, has an antisense transcript called BDNF-AS. BDNF is essential for the survival of neurons and the capacity of synapses to undergo changes and adaptability (synaptic plasticity). BDNF-AS, an RNA molecule that is transcribed in the opposite direction of BDNF, has been observed to regulate the level of BDNF gene expression. The function of the brain may vary across different regions. Antisense transcripts have been detected for the TP53 (p53) tumour suppressor gene. An example of a transcript that functions in the opposite direction is Wrap53, which is referred to as an antisense transcript. It plays a role in regulating the expression of p53 and has been linked to the development of cancer. Wrap53 May demonstrate heterogeneity in expression and functionality among several types of cancer tissues [8].

Types of antisense transcripts

Antisense transcripts come in various types, each with distinct characteristics. Two such categories are enhancer and PROMPTs, transcription of both of these is bidirectional [9]. Enhancer regions give rise to Enhancer RNAs, while promoter regions give rise to PROMPTs. Protein coding genes in the opposite DNA strands are used in the production of NATs (intronic and exonic). These genes are regulated by promoters which can be bidirectional. Another type, known as SINEUPs, is encoded on the antisense strand of the mRNA [10].

Antisense transcripts can be classified based on their positional relationship with the sense transcript falling into categories such as embedded, tail-to-tail and head-to-head [11]. Some NATs undergo post-transcriptional modifications like 5′-capping and the addition of polyA tails. Additionally, they may undergo alternative splicing. These modifications contribute to the diversity and regulation of NATs [7]. Types of natural antisense transcripts have been shown in the Figure 2.

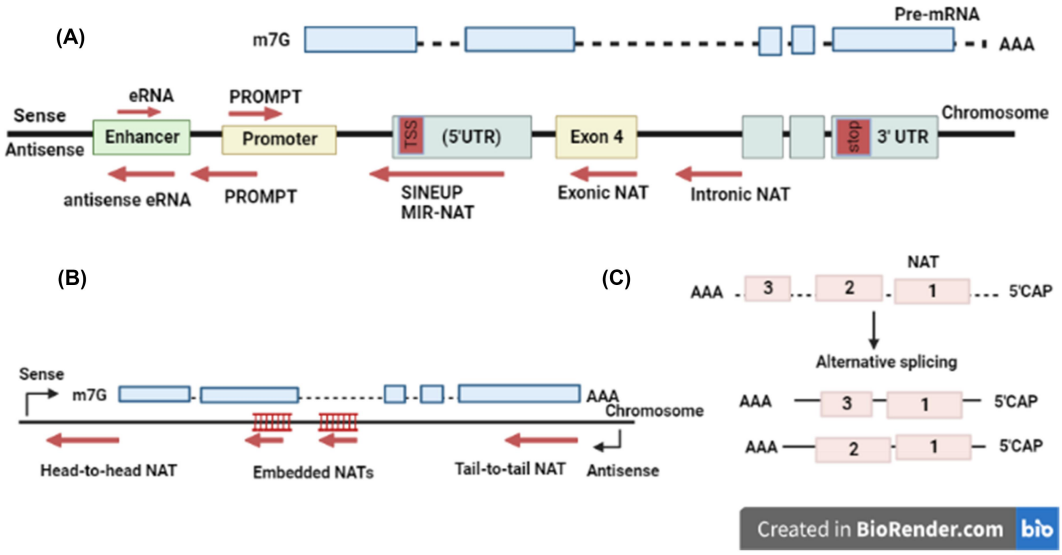

Figure 2. (a) enhancer RNAs, PROMPTs, intronic, exonic NATs and SINEUPs, NATs. (b) Embedded, tail-to-tail, and head-to-head NATs. (c) Some NATs undergo post-transcriptional modifications like 5′-capping and the addition of polyA tails, and they may also undergo alternative splicing. These modifications contribute to the diversity and regulation of NATs.

Natural antisense transcripts and immune response

According to recent studies, it has been found that NATs which are long non-coding RNAs (lncRNAs) have a substantial impact on the regulation of immune cell development and activation. This includes immune cells such as macrophages, dendritic cells (DCs), as well as adaptive immunity cells like *T*- and B-lymphocytes [12]. Several high-throughput sequencing studies have elucidated the regulatory influence of lncRNAs on immune responses subsequent to infection or stimulation mediated by pattern recognition receptors (PRRs), both in vitro and in vivo [12]. Considering the functional conservation observed in immune signalling pathways across many infection models, it is imperative to investigate and analyse the role of lncRNAs in the modulation of immune responses. Moreover, there is a necessity for comprehensive examinations of their functions in obligatory intracellular bacterial infections.

The findings reported by Walther and Schulte provide evidence that the eLncRNA IL-1β and lncRNA IL-1β-RBT46 serve as enhancers of IL1β expression in response to TLR4-MyD88-NFκB activation induced by LPS [12]. The nuclear long non-coding RNA PACER functions as a positive feedback regulator by acting as a decoy for the NF-κB p50 subunit, thereby promoting the development of the NF-κB p50/p65 heterodimer. The findings of Krawczyk and Emerson reveal that this process stimulates immunological gene expression in human macrophages through a TLR-4 dependent mechanism [13]. Moreover, it has been observed that the lncRNAs CARLR is upregulated following lipopolysaccharide (LPS) stimulation. This upregulated CARLR then interacts with the p65 component of the nuclear factor kappa B (NF-κB), consequently facilitating the transcriptional activation of pro-inflammatory genes [14]. As mentioned earlier, IL7-AS serves as a chromatin modifier through its interaction with p300 and the SWI/SNF complex, leading to the upregulation of CCL2 and IL-6 in the TLR-MAPK/NF-κB signalling pathway [12]. According to the research conducted by Ma *et al.* it has been observed that LincRNA-TNFAIP3 interacts with the chromatin regulator HMGB1, resulting in the formation of an HMGB1/NF-κB complex [15]. This complex plays a crucial role in enabling the binding of NFκB to the promoter region of IL-6. In addition, it has been observed that long non-coding RNA EPAV functions as a decoy molecule, effectively sequestering the SFPQ protein away from the p65 promoter of NF-κB, thus exerting regulatory control over NF-κB activation in murine models [12].

The LincRNA-Cox2 exhibits two unique pathways by which it exerts dual regulatory roles on NF-κB. The degradation of IκBα is promoted, resulting in the translocation of NF-κB into the nucleus and subsequent production of pro-inflammatory cytokines. In contrast, it exerts a negative regulatory effect on the expression of TLR-induced ISG CCL5 by interacting with hnRNPA/B and A2/B1 within the nucleus, thereby impeding the recruitment of RNA polymerase II to the CCL5 promoter in cells stimulated with Pam3CSK4 [16]. In vitro experiments further demonstrate a physical correlation between NF-kB subunits (RelA and p50) and the SWI/SNF complex in lipopolysaccharide (LPS)-stimulated murine macrophages. The study conducted by Hu *et al.* demonstrates that the use of siRNA to suppress lincRNA-Cox2 leads to a reduction in the interaction between NF-kB subunits and the SWI/SNF complex [17]. This suggests that lincRNA-Cox2 binds to the SWI/SNF complex in order to regulate chromatin remodelling and the NF-kB dependent transcription of CCL5.

Moreover, it has been observed that long non-coding RNA IL1α-AS, when triggered by Toll-like receptor (TLR) activation by lipopolysaccharide (LPS), Pam3CSK4, or poly (I:C), facilitates the recruitment of RNA polymerase II,

hence leading to the upregulation of IL1α expression [18]. The induction of lncRNA FIRRE occurs in macrophages following treatment with lipopolysaccharide (LPS), hence facilitating the transcriptional stabilization of IL-1β, IL-12β, and VCAM1 in a manner dependent on heterogeneous nuclear ribonucleoprotein U (hnRNPU) [19]. In contrast, TMC3-AS1 has a negative regulatory effect on IL-10 through its interaction with p65 within the nucleus, thereby impeding the binding of p65 to the NF-κB site within the promoter region of IL-10 in macrophages and intestinal epithelial cell lines [20].

NATs targeted therapeutics and control of biological processes

Regulation of transcription

NATs play crucial roles within the complex regulatory networks that finely tune gene expression during transcription [21]. This form of regulation significantly impacts subsequent post-transcriptional events by dictating the mRNA's sequence, chemical modifications, as well as its movement, cellular positioning, and proficiency in translation [22].

NATs primarily function by directing the orchestrated formation of transcriptional complexes at precise loci [23]. Concentrating on the NATs of these elements offers a promising avenue for crafting notably gene-tailored Nucleic Acid-Based Therapeutics (NBTs). Conversely, directing interventions towards the broadly applicable protein constituents of these transcriptional complexes could lead to diverse unintended effects across the genome [24].

The process of X chromosome inactivation is of utmost importance as it enables the attainment of equitable gene expression from the X chromosome. Dosage compensation is accomplished by the establishment of heterochromatin on the X chromosome designated for inactivation. The process in question is coordinated by the X chromosome inactivation centre, which depends on the transcription of two lengthy non-protein-coding genes known as XIST (X-inactive specific transcript) and TSIX (X [inactive]-specific transcript, antisense). The transcripts originating from the X chromosome inactivation centre, known as sense and antisense transcripts, are of significant importance in the regulation of X chromosome silencing. The efficacy of TSIX in silencing XIST has been established through its ability to change the chromatin structure in the promoter region of XIST, thereby establishing the fundamental components of this regulatory mechanism. Significantly, the X chromosome inactivation centre plays a crucial role in both the initiation and completion of X chromosomal inactivation. Empirical data has demonstrated that the premature cessation of Tsix transcription results in the removal of the inhibitory chromatin structure at the XIST promoter [25].

Genomic DNA methylation

DNA methylation, characterized by the introduction of a methyl group into genomic DNA, constitutes an enzymatic process central to numerous epigenetic mechanisms governing gene expression. These mechanisms encompass imprinting, epimutations, splicing modulation and various others [26].

Numerous mechanisms have been uncovered that explain how NATs influence DNA methylation. One approach involves antisense transcription that covers CpG islands, potentially disrupting the factors responsible for maintaining this phenomenon. Alternatively, NATs may form triplexes within promoter regions of genomic DNA [27]. This formation could either facilitate the recruitment of complexes for protein methylation or impede the activity of demethylating enzymes. These triplexes arise from regions of double-stranded DNA containing sequences of homopolypurine/homopolypyrimidine, accompanied by RNA molecules [10]. The stability of this triplex structure relies on hydrogen bonding interactions between the RNA and the double-stranded DNA.

Histone modification

NATs also play a role as platforms for complexes of epigenetic histone modifiers, influencing the expression of their corresponding sense partners either in a localized manner (cis) or on a broader scale, affecting a cluster of genes (trans) [6]. For example, the increased expression of the BDNF-AS (brain-derived neurotrophic factor antisense transcript) has the effect of suppressing the expression of the BDNF gene [28]. This heightened expression of BDNF-AS leads to an increased recruitment of the histone methyltransferase EZH2, which is a component of the enhancer of zeste 2 polycomb repressive complex 2 [29]. As a result, this recruitment leads to the addition of repressive H3K27me3 marks at regulatory regions within the BDNF gene.

To manipulate this process, a specific type of antisense oligonucleotides (ASOs) known as 'AntagoNATs' has been designed to target BDNF-AS [30]. In experimental settings, these AntagoNATs have been successful in effectively increasing BDNF expression in a gene-specific manner, both in cell cultures and in living organisms [31]. When the BDNF AntagoNAT is delivered using the MIND technique, it results in a widespread increase in BDNF protein levels in the brains of rats. Notably, the MIND technique is a patient-friendly method and provide a precise dosing for delivering new therapeutic agents to the brain. Augmenting BDNF expression holds promise for addressing various neurodegenerative conditions, including Parkinson's and Alzheimer's disease [32]. Various mechanisms by which NATs exert their control on biological processes have been shown in the Figure 3.

Splicing manipulation

Removing intronic regions from pre-mRNAs activates another regulatory mechanism that greatly affects the particular sequence and, in turn, the biological function of the eventual protein [33]. Currently, it is believed that RNA splicing is a tightly regulated co-transcriptional system that, from a single pre-mRNA/genomic DNA sequence, produces several sets of mRNA isoforms, many of which have unique roles [10].

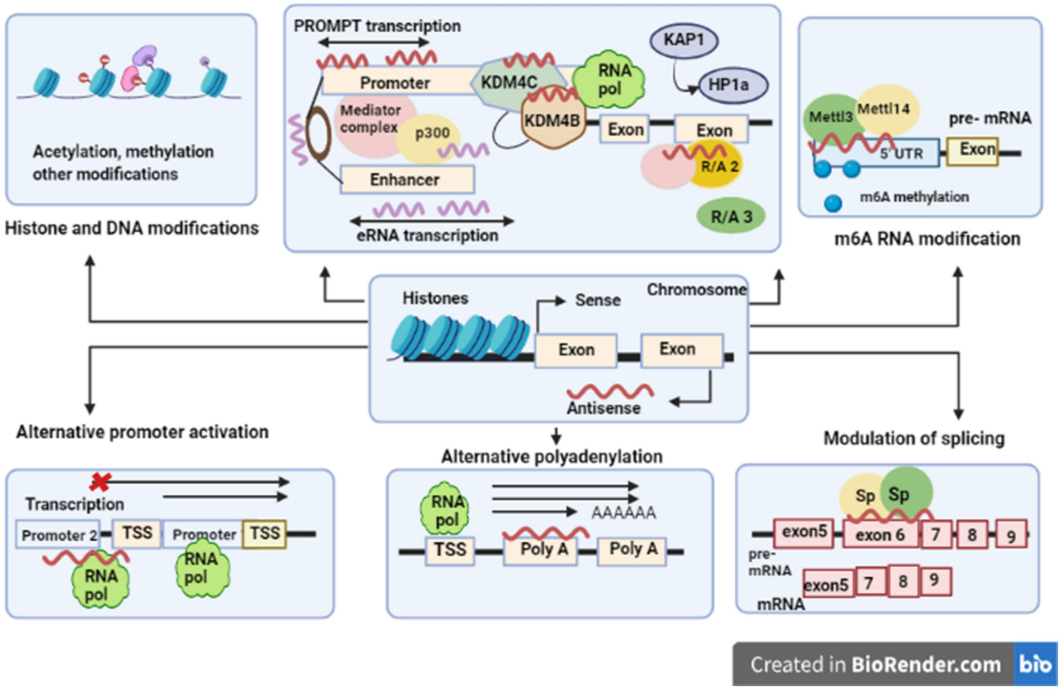

Figure 3. Nats exert control over several essential biological processes through various mechanisms. Firstly, they interact with specific DNA loci, where they serve as scaffolds for epigenetic regulatory enzymes, including DNA methyltransferases (DNMT) and histone modifiers. Secondly, NATs participate in scaffolding 3D chromosomal interactions, particularly in maintaining an active chromatin state. Thirdly, NATs are involved in PROMPT-mediated promoter-proximal pause release. Fourthly, NATs participate in imprinting processes by acting as scaffolds, decoys, or blockers for repressor and activator proteins, ultimately regulating transcription repression of specific parental alleles. Additionally, NATs play a role in DNA damage repair by interacting with proteins involved in double-stranded DNA break repair. They also mediate m6A RNA modification by recruiting regulators or enzymes associated with methyltransferase complexes (Mettl3/Mettl14) or m6A demethylases (ALKBH5), thereby influencing the availability and processing of RNA molecules. Furthermore, NATs can alter the transcription rate of specific isoforms by either antagonizing or activating alternative promoters. They also have the capacity to repress or activate polyadenylation signals, thereby affecting the transcription of alternative isoforms. Finally, NATs modulate splicing by influencing the binding and activity of splicing-promoting or -inhibiting factors (sp), which in turn facilitates the production of specific RNA isoforms.

The different ways that the 5′ (donor) and 3′ (acceptor) splice sites are used within the pre-mRNA sequence dictate the organization of each isoform [34]. These splice site sequences are distinguished by having an initial intron 5′ region that is specified by the GU dinucleotide [35]. Then comes a pyrimidine-rich region (composed of C and U, called the polypyrimidine tract) located downstream, a 'branch point' sequence indicated by an A, and a splice acceptor site indicated by an AG sequence that indicates the end of the intron at the 3′ end. LncRNAs and protein splicing factors that identify particular sequence patterns in the intronic or exonic regions around the splice site can both affect the selection of these splice sites [36]. The tissue type and developmental stage influence the expression of these lncRNAs and protein factors.

Regulation occurring after transcription

NATs also participate in the direct control of translation. While NBTs aimed at targeting these regulatory mechanisms have not yet reached clinical testing, there is substantial ongoing research and development in this area. Below, we delve into several illustrative examples.

Inverted SINEB2 sequence-mediated upregulating molecules (SINEUPs)

A novel category of NATs called SINEUPs, which are characterized by their ability to up regulate gene expression via the mediation of inverted SINEB2 sequences, has been identified in recent studies [37]. The SINEUPs are encoded on the antisense strand of the target mRNA's 5′ end and exhibit a distinctive capability to augment translation without concomitant elevation of mRNA levels [38]. SINEUP NATs possess unique characteristics, characterized by the presence of specialized binding and effector domains. The binding domain consists of an antisense region that coincides with the start codon of the target mRNA, therefore assuring specificity for the protein-coding transcript [39]. Effector domains located at the 3′ end of a SINEUP include transposable element sequences that are embedded within them. These sequences include inverted short interspersed nuclear element B2 (invSINEB2), Alu, or MIR elements [10]. The translation process may be augmented by the binding of effector domains to activating protein complexes.

The successful operation of SINEUPs relies on the proper localization of both the SINEUP protein and its mRNA target inside the cytoplasm [40]. The subcellular localization of SINEUPs and the assembly of translational initiation

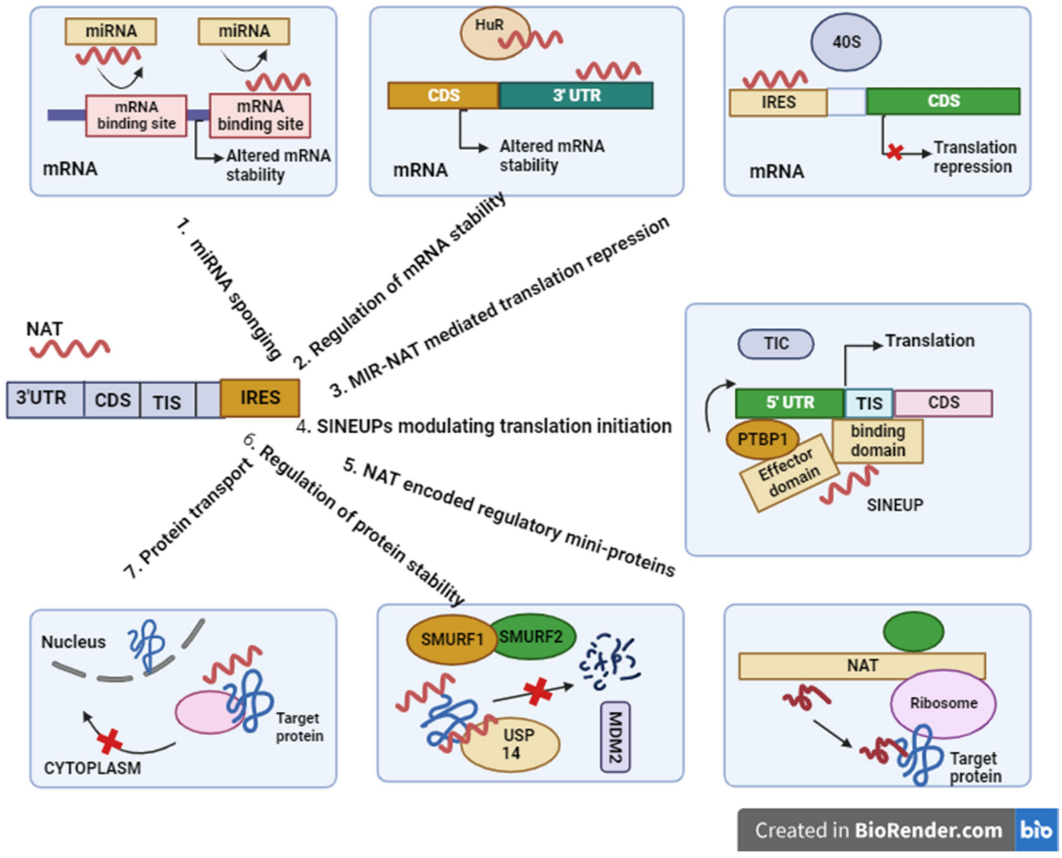

Figure 4. Nats play a pivotal role in the regulation of post-transcriptional processes. Firstly, they are involved in miRNA sponging, thereby influencing mRNA degradation. Secondly, NATs regulate mRNA stability by acting as sponges for proteins that control mRNA degradation and stability. Thirdly, a specific subtype of NATs called MIR-NATs, which overlap with 5′ untranslated regions (5′UTRs) in a head-to-head manner, compete with internal ribosome entry sites (IRES) for the 40S ribosome subunit, leading to repression of translation. Fourthly, NATs known as SINEUPs modulate translation initiation to facilitate the assembly of the translation initiation complex (TIC), resulting in the up regulation of translation. Fifthly, NATs can encode mini-proteins with regulatory functions downstream, providing an additional layer of control over cellular processes. Sixthly, NATs are involved in regulating protein stability by protecting target proteins from degradation. They achieve this by either sponging proteins involved in the ubiquitin-proteasome degradation pathway, such as SMURF1/2 and MDM2, or by inhibiting their activity. Lastly, NATs have a role in modulating protein subcellular distribution, thereby influencing cellular processes related to protein localization.

complexes are enabled by RNA-binding proteins, namely PTBP1 (polypyrimidine tract binding protein-1) and HNRNPK (heterogeneous nuclear ribonucleoprotein K). SINEUPs have the ability to engage with PTBP1, hence facilitating the recruitment of ribosome subunits to the target mRNAs [41]. The recruitment process that follows results in the establishment of translational initiation complexes, which consist of EF1A1 (eukaryotic translation elongation factor 1, alpha-1).

MIR-NATs

NATs containing repeats derived from retrotransposons, specifically mammalian-wide interspersed repeats (MIR), referred to as MIRNATs, have been found to contain functional regions that impact interactions between mRNA and ribosomes (Figure 4). This category of MIR-NATs has the potential to overlap with the functions of SINEUPs.

In the realm of illnesses, an illustrative case pertains to MAPT-AS1 (microtubule-associated protein tau antisense 1), a MIR-NAT positioned in a head-to-head configuration with the MAPT 5′UTR. The area of overlap encompasses the domain 2 of MAPT-IRES, which engages in interactions with 40S ribosomes [10]. The MAPT-AS1 molecule functions by engaging in a competitive contact with the MAPT mRNA, so impeding the translation process of the MAPT gene's protein product, often referred to as protein tau, which relies on the internal ribosome entry site (IRES) mechanism [42]. The up regulation of MAPT-AS1 or its particular crucial parts, such as the MIR element, led to a translocation of MAPT mRNA from high-density to low-density polysomes and a decrease in tau protein levels in neurons produced from human induced pluripotent stem cells [43]. Engineered iterations of the minimum essential sequence of MAPT-AS, formulated as nucleic acid-based therapeutics (NBTs), may have therapeutic promise in disorders linked to tauopathies, including Alzheimer's disease and Parkinson's disease.

Regulation of miRNA activity

Numerous NATs have been demonstrated to exert control over the translation and transcription of specific gene groups or individual genes through their capacity to sequester endogenous miRNAs (Figure 4). In certain instances, the knockdown of these NATs emerges as a valuable therapeutic avenue

[44]. For instance, in the context of an in vitro model of diabetic nephropathy, the upregulation of KCNQ1OT1 (KCNQ1 opposite strand/antisense transcript 1) led to increased inflammation and apoptosis of podocytes [45]. This effect was attributed to KCNQ1OT1's ability to sponge miR-23b-3p and enhance Sema3A expression, a direct target of miR-23b-3p. Targeting KCNQ1OT1 through NAT-based interventions could offer promise for addressing diabetes and related inflammatory disorders [46].

The orchestration of biological clock components is responsible for harmonizing numerous cellular processes with the circadian rhythms of the organism as a whole [47]. Various disorders have been associated with disruptions to this synchronization. NATs, namely PER2AS and CRY1AS, are generated from the genomic loci of major circadian clock genes (PER2 and CRY1, respectively). These NATs exhibit rhythmic expression patterns that are synchronized with their corresponding coding genes, following a 24-hour cycle [48]. NATs enhance the expression of their corresponding messenger RNAs (mRNAs) by binding to and sequestering the binding sites of certain microRNAs (miRNAs) that would otherwise bind to these mRNAs [49]. This interaction ultimately affects the dynamics of the core clock genes.

mRNA stability

NATs have an intriguing capability to engage in interactions with their respective mRNA counterparts, therefore exerting an influence on mRNA stability that is independent of miRNA activity (Figure 4). The process under consideration is shown by Nqo1-AS1, which is an antisense RNA molecule associated with NAD(P)H dehydrogenase, quinone 1. It is also referred to as Fantom3_F830212L20 [10]. This molecule is mostly found in the cytoplasm of alveolar epithelial cells in mice. Furthermore, its expression is increased in response to exposure to cigarette smoke. The elevated expression of Nqo1-AS1 serves to mitigate the detrimental effects of oxidative stress by augmenting the transcriptional and translational levels of Nqo1 and Serpina1 (a member of the serpin peptidase inhibitor, clade a) by direct binding of the antisense RNA to the 3′ untranslated region (UTR) of Nqo1 [38]. The development of ASO mimics that specifically target the area where Nqo1-AS1 forms a duplex has promise as a treatment approach for chronic obstructive pulmonary disease.

In an alternative scenario with ramifications for pathological conditions, the involvement of FAM83A-AS1 in the advancement of lung adenocarcinoma is shown via its facilitation of FAM83A upregulation and subsequent activation of the ERK (extracellular signal-regulated kinase 1) signalling cascades. The process by which FAM83A-AS1 increases the stability and subsequent production of FAM83A mRNA is achieved by the creation of an RNA duplex [50]. It is worth mentioning that the inhibition of FAM83A-AS1 May be accomplished by the use of NBTs.

Protein stability

NATs extend their influence beyond mRNA stability and are also recognized as modulators of protein stability (Figure 4). One notable mechanism employed by NATs to enhance protein stability involves the prevention of proteasomal degradation [51]. An illustrative case is demonstrated by the antisense transcript KDM4A-AS1, which forms a binding partnership with the androgen receptor (AR) protein [52]. This connection facilitates the stabilization of the Androgen Receptor (AR) by facilitating the process of deubiquitination of AR via the formation of the USP14 (ubiquitin-specific protease 14)-AR complex. This mechanism efficiently inhibits the degradation of androgen receptor (AR) via the MDM2-mediated ubiquitin-proteasome pathway [50]. The enhanced up regulation of KDM4A-AS1 in castration-resistant prostate cancer highlights its functional significance. Significantly, the use of antisense oligonucleotides (ASOs) specifically engineered to target KDM4A-AS1 has shown a notable ability to effectively inhibit the development of tumours in instances when resistance to enzalutamide is seen.

Another pertinent example concerning sickness is the involvement of ARHGAP5-AS1 (NR_027263), which exerts its influence by engaging with SMAD7 (SMAD family member 7) through its PY motif [53]. The present relationship serves as an impediment to the connection between SMAD7 and the E3 ubiquitin ligases SMURF1 (SMAD-specific E3 ubiquitin protein ligase 1) and SMURF2 [54]. The outcome of this interaction manifests as the stabilization of SMAD7 protein levels, hence resulting in the suppression of migratory capabilities in breast cancer cells. The therapeutic potential of synthetic NBT mimics that precisely target the interaction domain of SMAD7 inside ARHGAP5-AS1 shows promise in the treatment of breast cancer [55].

Localization of proteins at subcellular level

NATs have unveiled their capability to influence the subcellular distribution of proteins, a facet depicted in Figure 4. However, exploration of this dimension of their activity remains relatively uncharted territory. A case that illuminates this mechanism is exemplified by panRNA-DMP1, an NAT originating from the promoter region of the dentin matrix protein-1 (DMP1) gene. Following the prompt of EGF (epidermal growth factor) stimulation, panRNA-DMP1 emerges into action. Intriguingly, the depletion of panRNA-DMP1 in this context triggered a surge in the nuclear localization of EGFR (epidermal growth factor receptor) upon EGF treatment. This effect, in turn, conferred stability to EGFR's interactions with STAT3 (signal transducer and activator of transcription 3) [56]. As a consequential outcome, this intricate interplay amplified the migratory capacity of cancer cells [57].

NAT-encoded peptides

One intriguing characteristic of NATs is their ability to encode concise open reading frames (ORFs) that lead to the production of peptides with unique biological functions, as seen in Figure 4. One notable instance of this phenomena pertains to HNF4A-AS1, also known as hepatocyte nuclear factor 4 alpha antisense RNA 1 [58]. This particular entity encompasses a peptide consisting of 51 amino acids, often denoted as sPEP1. The process of converting sPEP1 into

a functional entity is regulated by the interaction among HNF4A-AS1, miRNA-409-5p, and the recruitment of EIF3G, which is a component of eukaryotic translation initiation factor 3 [10]. The crucial function of sPEP1 becomes evident when it establishes a direct interaction with eEF1A1, a protein involved in the elongation phase of eukaryotic translation. This interaction enhances the binding of eEF1A1 to SMAD4, a tumour suppressor belonging to the SMAD family. Consequently, this particular interaction has a suppressive impact on the transactivation of SMAD4 [59]. The overexpression of sPEP1 has been seen to result in a decrease in the advancement of senescence produced by serum deprivation, as well as an augmentation in the proliferation and metastatic potential of neuroblastoma stem cells. Potential therapeutic applications may be found in relevant cancer scenarios via the implementation of strategies such as the suppression of HNF4A-AS1 or the inhibition of sPEP1 translation [60].

Human diseases and the molecular significance of natural antisense transcripts

Histone methylation and acetylation

The well-established regulatory role of NATs in gene expression control through the recruitment of complex epigenetic machinery has been extensively documented [61]. Polycomb Repressive Complexes 1 (PRC1) and 2 (PRC2) are well-known participants in chromatin remodelling and have been the focus of substantial study [62]. They start important processes such as monoubiquitination of histone 2A at lysine 119 (H2AK119ub) and lysine 27 (H3K27me3) methylation of histone H3, respectively. Lysine Specific Demethylase 1 (LSD1), which demethylates histone H3 at lysine 4 (H3K4), and G9a Methyltransferase, which is in charge of di- and tri-methylating histone H3 at lysine 9, are two additional important contributors to chromatin restructuring [63].

Antisense Intergenic RNA (HOTAIR) serves as a prominent illustration of a NAT that employs this regulatory mechanism [6]. HOTAIR has a pivotal role in the repression of HOX genes by facilitating their localization to certain chromosomal domains, primarily via its interactions with PRC2 and LSD1. The overexpression of HOTAIR has been associated with the progression of many forms of cancer [64]. Breast cancer, being a prevalent malignancy among women, is associated with the suppression of metastasis-inhibiting genes, including Protocadherin 10 (PCDH10), Protocadherin Beta 5 (PCDHB5), and Junctional Adhesion Molecule 2 (JAM2), via the action of HOTAIR [65].

A similar occurrence may be seen inside the domain of Antisense Noncoding RNA in the INK4 Locus (ANRIL), first identified in persons afflicted with melanoma [66]. ANRIL has been shown to be overexpressed in several human cancers, including breast, gastric, liver, lung, melanoma, and prostate carcinomas [67]. The available body of data indicates that ANRIL has the ability to modify the structure of chromatin and inhibit the tumour suppressor locus INK4b-ARF-INK4a via its interaction with Chromobox 7 (CBX7) and Suppressor of Zeste 12 Protein Homologue (SUZ12), which are key components of PRC1 and PRC2, respectively. Furthermore, have shown a notable upregulation of ANRIL expression in hepatocellular carcinoma (HCC), establishing a correlation with tumour size. In hepatocellular carcinoma (HCC), the long non-coding RNA ANRIL engages in a molecular interaction with Enhancer of Zeste Homologue 2 (EZH2), a constituent of the PRC2, specifically in the promoter region of Kruppel-Like Factor 2 (KLF2) gene [68]. This interaction ultimately results in the imposition of epigenetic modifications that lead to the transcriptional repression of the KLF2 gene.

CpG islands methylation

CpG islands, which are CG dinucleotide-rich areas, are frequently observed in gene promoters within vertebrate genomes [69]. It is noteworthy that almost 70% of annotated gene promoters in the human genome are linked to CpG islands. According to Wanowska *et al.* (2018) the process of methylation occurring in these islands can result in the inhibition of gene transcription by impeding the accessibility of DNA to the transcription machinery [6]. The regulatory process in question involves the participation of certain antisense transcripts, as evidenced in the case of α-thalassaemia, a hereditary type of anaemia resulting from disturbances in the synthesis of α-Globin [70].

Mutations in the α-Globin (HBA2) gene sequence are considered the primary factor responsible for the abnormal gene expression seen in α-thalassaemia [71]. Nevertheless, it is important to acknowledge that changes in the methylation status of α-Globin CpG islands also play a role in the progression of this disorder [72]. The observed phenomenon is linked to an antisense transcript that arises from the LUC7-Like (LUC7L) gene, which is located on the opposite DNA strand in a convergent orientation with respect to the α-Globin cluster [6]. There has been a proposition indicating that the transcription of LUC7L results in the repression of HBA2 expression by promoting the methylation of CpG islands. The notion presented is supported by studies carried out on individuals who exhibited a deletion affecting the 3' region of the α-Globin locus, while leaving the 5' segment of the HBA2 gene unaltered. The LUC7L gene is affected by the deletion event since it leads to the removal of its transcription termination signal [73]. Consequently, an extended antisense transcript is generated, which stretches towards the HBA2 promoter region. Recent research has shown that the production of this unconventional antisense transcript leads to the methylation of CpG islands in HBA2. Furthermore, the replacement of LUC7L with the Human Ubiquitin C promoter leads to the methylation of the HBA2 promoter [6,73,74,,]. This implies that the regulatory mechanism relies mostly on the antisense transcription process per se, rather than on particular activities of the atypical antisense RNA transcript.

Parent-specific gene expression

In species possessing diploid genomes, it is customary for the majority of autosomal genes to be expressed from both homologous pairs of chromosomes [75]. Nonetheless, there is a distinct fraction of genes that demonstrate parent-specific

expression, which is commonly referred to as genomic imprinting. Genomic imprinting is a biological phenomenon characterized by epigenetic alterations occurring at the DNA or histone level during the process of gametogenesis [76,77]. Imprinted genes frequently exhibit a clustered arrangement, wherein their regulation is governed by a central imprinting control centre [78].

NATs play a crucial role in the process of genomic imprinting [79]. These transcripts contribute by forming double-stranded structures with their mRNA counterparts, which can lead to RNA interference (RNAi) or the encapsulation of specific chromosomal regions [80]. This encapsulation, in turn, recruits repressive chromatin proteins to target DNA sequences, further influencing gene expression.

Beckwith-Wiedemann syndrome (BWS) serves as an example of an illness that arises due to disturbances in genomic imprinting [81]. Abnormal gene expression patterns are frequently reported in individuals diagnosed with Beckwith-Wiedemann syndrome (BWS). These aberrant patterns are often attributed to the loss of maternal-specific methylation within the KvDMR1 cluster, which is situated at the chromosomal region 11p15.5 [82]. It is noteworthy that a considerable number of persons diagnosed with Beckwith-Wiedemann syndrome (BWS) demonstrate the manifestation of the KCNQ1OT1 antisense transcript, which is generally suppressed. The utilization of mouse models has played a crucial role in understanding the mechanism of NAT action in the context of the imprinted cluster situated at the far end of chromosome [83].

Regulation of alternative splicing

The phenomenon of alternative splicing of pre-mRNA molecules during the post-transcriptional process is a mechanism that significantly amplifies the range of transcripts derived from a solitary gene. This process has a substantial role in the reported variability in both transcriptional and protein levels in eukaryotic species [84]. One example that serves as an illustration is UXT-AS1, a long noncoding RNA that exhibits an antisense orientation. The UXT-AS1 gene plays a substantial role in the modulation of alternative splicing of the Ubiquitously Expressed Prefoldin-Like Chaperone (UXT) gene, and its correlation with the progression of colorectal cancer (CRC) has been shown. The UXT gene produces two alternatively spliced transcripts, U×T1and UXT2, which have divergent expression patterns in colorectal cancer (CRC) [85]. It has been shown that the expression of U×T1is decreased, whilst the expression of U×T2is increased. Prior research has provided empirical support for the notion that the overexpression of UXT-AS1 in cells affected by colorectal cancer (CRC) induces a discernible modification in the process of splicing, particularly in the transition from U×T1to UXT2. The observed shift may be aided by the interactions between UXT-AS1 and UXT pre-mRNA, particularly via the process of base pairing [86].

Another notable example is ZEB2 Antisense RNA 1 (ZEB2-AS1), which plays a role in the regulation of alternative splicing of the Zinc Finger E-Box Binding Homeobox 2 (ZEB2) gene [87]. ZEB2 serves as a transcriptional regulator of E-Cadherin and has a robust association with the phenomenon of carcinogenesis [88]. ZEB2-AS1, similar to UXT-AS1, has an impact on splicing processes by direct interactions with RNA molecules. ZEB2-AS1 has a strong affinity for binding to the 5' splice site situated inside the principal intron of ZEB2 pre-mRNA, leading to the retention of the intron. It is noteworthy that the preserved intron under consideration harbours an internal Ribosome Entry Site (IRES), which plays a pivotal role in facilitating the heightened production of the ZEB2 protein [89]. ZEB2 isoforms lacking the intron exhibit a deficiency in encoding functional proteins [6]. As a result, the upregulation of the NAT serves as an indirect mechanism for enhancing the production of the ZEB2 protein.

NATs exert a significant influence on the modulation of alternative splicing in the brain, particularly in regions characterized by a substantial abundance of tissue-specific alternative splicing isoforms. An illustrative example is the 17A noncoding RNA, which has been identified as a regulator of splicing for the Gamma-Aminobutyric Acid Type B Receptor Subunit 2 (GABBR2 or GPR51). Alzheimer's disease patients exhibit a pronounced upregulation of 17A within their cerebral cortices [90]. Research conducted on neuroblastoma cells has demonstrated that this particular NAT (N-acetyltransferase) stimulates the generation of alternative splice variants of GABBR2. The disruption in the splicing process results in anomalies in intracellular signalling, which subsequently leads to an excessive generation of the neurotoxic β-Amyloid peptide. The GABBR2 protein functions as a component of a heterodimeric G-protein coupled receptor, which is accountable for the binding of gamma-aminobutyric acid (GABA) [91]. The central nervous system is primarily responsible for regulating the release of neurotransmitters.

miRNA sequestering molecules

Multiple NATs function as competitive endogenous RNAs (ceRNAs) or miRNA sponges. The ceRNAs possess several binding sites that facilitate their interaction with miRNAs, hence impeding the interaction between these miRNAs and their respective target mRNAs [92]. The complex interplay described above results in the adjustment of expression levels for both the miRNAs that are readily available and the mRNAs that they control.

The case of Phosphatase and Tensin Homologue Pseudogene 1 (PTENP1, also known as PTENpg1), The PTENpg1 locus is responsible for encoding three distinct functional transcripts, namely PTENpg1 sense, PTENpg1 antisense α, and PTENpg1 antisense β [93]. These transcripts play a collective role in the regulation of the tumour suppressor gene known as Phosphatase and Tensin Homologue (PTEN). PTENpg1 functions as a competitive inhibitor against miR-21, thereby suppressing the interaction between PTEN and miR-21, consequently hindering the advancement of cancer [94]. The function of PTENpg1 as a miRNA sponge is mediated by an interaction between RNA molecules, specifically the PTENpg1 antisense β transcript, which appends a polyA tail to the PTENpg1 sense transcript [95]. The presence of a polyA tail is essential for the effective transportation of PTENpg1 as a competitive endogenous RNA (ceRNA)

molecule from the nucleus to the cytoplasm, as well as for its proper functioning in the cytoplasm. Another antisense splice variant, known as PTENpg1 α, plays a significant role in the recruitment of chromatin remodelling complexes to the PTEN promoter [96]. This recruitment ultimately leads to the transcriptional silence of PTEN and contributes to the complicated regulatory cascade [97].

There exist numerous NATs that have the capacity to operate as competitive endogenous RNAs (ceRNAs), hence bearing significant significance in the context of cancer development and progression [98]. The tumour suppressor gene miR-150 has been found to have a notable impact on several forms of cancer, such as colorectal cancer (CRC) and osteosarcoma [99,100]. In the context of hepatocellular carcinoma (HCC), it has been observed that miR-150 has a role in the regulation of Zinc Finger E-box Binding Homeobox 1 (ZEB1), leading to the inhibition of cell invasion and metastasis. The antisense transcript known as ZFAS1 functions as a molecular sponge for miR-150 and is recognized as an oncogene in hepatocellular carcinoma [76]. The computational analysis reveals a potential role of ZFAS1 as a miR-590-3p sponge in colorectal cancer (CRC) tissues. However, it is important to note that experimental validation is required to confirm this hypothesis [101].

Another illustrative instance is TUG1, an overexpressed antisense long noncoding RNA that functions as a competitive endogenous RNA in the context of endometrial cancer. TUG1 plays a regulatory role in the expression levels of Vascular Endothelial Growth component A (VEGFA), a critical component in tumour angiogenesis in different human malignancies, by functioning as a sponge for miR-299 and miR-34a-5p [102].

Blocking miRNA binding regions

Antisense transcripts have the ability to attach to messenger RNA (mRNA) molecules, therefore obstructing the binding sites of microRNA (miRNA) [103]. This interaction facilitates their involvement in regulatory processes. The enhanced stability of mRNA molecules, which are typically susceptible to degradation by miRNAs. The process of β-Secretase 1 (BACE1) expression, which is a crucial enzyme in the development of Alzheimer's disease, has been recognized as a significant regulatory mechanism [104; 105]. BACE1 is involved in the generation of neurotoxic β-Amyloid peptides via a series of proteolytic cleavages of the amyloid precursor protein [100]. Prior studies have shown a notable elevation in both BACE1 levels and enzymatic activity inside the cerebral regions of individuals afflicted with Alzheimer's disease.

The role of BACE1 Antisense Transcript (BACE1-AS) in modulating the stability of BACE1 mRNA and its potential implication in disease pathogenesis has been shown. The deregulation of BACE1-AS and miR-485-5p has been proposed as a potential mechanism behind this phenomenon [106]. The binding location of miR-485-5p inside the BACE1 gene is located precisely at the region where BACE1-AS intersects with the sixth exon of the BACE1 gene. The aforementioned notion is substantiated by empirical evidence indicating that brains afflicted with Alzheimer's disease exhibit elevated levels of BACE1-AS and concomitantly reduced expression of miR-485-5p. It is noteworthy that the down regulation of BACE1 expression has been correlated with the concurrent up regulation of miR-485-5p and BACE1-AS [107]. Subsequent experimental investigations have corroborated the notion that the antisense transcript of BACE1 enhances its stability via RNA: RNA interactions, hence concealing a critical miRNA binding site within BACE1.

Regulatory pathways involving dicer

The c-MYC oncogene, known for its involvement in various human cancers and its role in promoting cell growth and proliferation, has been a subject of study for understanding complex gene expression regulation [108]. The intricate network of interactions involving both the c-MYC gene itself and several overlapping NATs found in its 3' distal region [89]. This comprehensive investigation unveiled a multifaceted mechanism governing c-MYC expression.

Among these NATs, one named NAT6531 was identified as forming a stem-loop structure that can be recognized by Dicer – an enzyme responsible for cleaving double-stranded RNAs, generating microRNAs or endo-siRNAs [6]. In this case, the NAT6531-derived small RNAs appear to target specific sequences within the c-MYC promoter and intron 1 [109]. Napoli and colleagues proposed that this interaction-based regulation, facilitated by the interplay between NATs and the small RNAs they generate, could contribute significantly to fine-tuning the transcriptome, extending beyond the boundaries of the c-MYC locus.

Importantly, this mode of antisense-mediated regulation doesn't solely revolve around NAT6531. Another NAT known as NAT7281 plays a role in repressing c-MYC transcription [110]. It also plays a crucial part in silencing the expression of NAT6531 and the small RNAs it produces. This added layer of complexity exemplified by NAT7281 further enriches the intricate regulatory dynamics at the c-MYC locus.

In Huntington's disease, a neurodegenerative disorder stemming from CAG trinucleotide repeat expansion within the first exon of the Huntingtin (HTT) gene, a mechanism involving Dicer has also been proposed [111]. The Huntingtin Natural Antisense Transcript (HTTAS) emerges as a potential key player in modulating HTT gene activity. Combination of mouse Dicer-null and wild-type embryonic stem cells along with various HTTAS constructs to delve into the potential influence of Dicer on HTT silencing [112].

Consistent with prior research conducted on human embryonic kidney cells, heightened levels of a specific HTTAS isoform referred to as HTTASv1 were linked to diminished HTT expression in wild-type mouse embryonic cells [113]. Notably, this relationship took a divergent turn in Dicer-null cell lines. In the absence of functional Dicer, the presence of HTTAS_v1 did not lead to the anticipated reduction in HTT expression [6]. These observations collectively underscore the significance of Dicer's role in orchestrating the regulatory effects of NATs on the HTT gene.

Other associations with diseases

While some NATs have well-established mechanisms of action in diseases, the precise molecular functions of many remain elusive. This is attributed to both a lack of experimental evidence and the intricate nature of the underlying processes. This complexity often leads to ambiguities in result interpretation, particularly since numerous NATs are believed to be involved in multiple modes of action, as exemplified earlier [114]. Another instance of such an antisense noncoding RNA is TRAF3IP2-AS1, which has been associated with at least two distinct mechanisms [43].

Firstly, TRAF3IP2-AS1 May play a role in epigenetically modulating the TRAF3 Interacting Protein 2 gene (TRAF3IP2) by influencing chromatin state alterations [115]. This is in line with findings demonstrating that heightened TRAF3IP2-AS1 expression correlates with significantly reduced expression of at least one TRAF3IP2 transcript [43]. Secondly, an alternate proposition is that TRAF3IP2-AS1 might exert posttranscriptional control over TRAF3IP2 by forming RNA: RNA duplexes. Notably, TRAF3IP2-AS1 overlaps with the second exon of the TRAF3IP2 gene, where the start codon of one of its protein-coding isoforms resides [116]. Enhanced understanding of TRAF3IP2-AS1's exclusive nuclear presence, in contrast to TRAF3IP2's global cellular distribution, further underscores its potential regulatory functions. Despite these proposed mechanisms, the precise mode of action for TRAF3IP2-AS1 remains to be definitively resolved.

The interactions between proteins and NATs are another area of unresolved instances [117]. High-mobility group box 2 (Hmgb2) is a multifunctional protein that has been linked to interactions with the antisense transcript LPR1-AS, which is especially pertinent in relation to Alzheimer's disease [6]. It is well-known for its impact on gene transcription through influencing the activity of transcription factors. The gene Low Density Lipoprotein Receptor-Related Protein 1 (LRP1) is the source of LPR1-AS [118]. LRP1 is a multifunctional endocytic receptor that plays a role in several physiological processes, including the elimination of β-Amyloid. It has been established that LPR1-AS binds to Hmgb2, and Hmgb2 interacts with the transcription factor Sterol Regulatory Element-Binding Protein 1a (Srebp1a), which is connected to LRP1. In the end, this interaction results in the suppression of Srebp1a's transcriptional activity, which lowers LRP1 expression. It's interesting to note that Lrp1 mRNA and Lrp1-AS RNA bind base-pairingly, blocking the contact between Lrp1-AS and Hmgb2. The intricacy of comprehending the roles of NATs in diverse biological contexts is increased by this complicated interaction [44].

While many NATs' underlying functions are still unknown, a sizable portion of them show potential as molecular indicators for diseases in humans [119]. Their function as diagnostic and prognostic markers in a wide range of malignancies is especially remarkable. A good example is HOTAIR, which is significantly overexpressed in several tumours, such as colorectal, pancreatic, hepatocellular, and breast carcinomas. An adverse prognosis in patients is correlated with this increased expression. Importantly, ideal indicators should not only be dysregulated in illness conditions but also stable and simple to identify in biological fluids [120].

Prostate Cancer Associated 3 (PCA3) offers a convincing scenario. Differentiating prostate cancer (PC) from prostate-specific antigen (PSA), a widely recognized predictor of PC risk, is the elevated levels of PCA3 [121]. Interestingly, the PCA3 test is a non-invasive technique with improved specificity that allows PCA3 expression to be measured from patient urine samples.

ASO based therapies

ASOs, or antisense oligonucleotides, provide a variety of structural and functional diversity [122]. The most notable of them are gapmers, which are made up of chemically altered nucleotides on either side of a DNA gap. These changes increase their resistance to nucleases and increase their binding affinity to complementary RNAs [123]. As substrates for RNase H1, a non-sequence-specific endonuclease that cleaves the RNA strand, gapmers create heteroduplexes with target RNAs (Figure 5a). A different family of ASOs binds with RNA molecules, preventing splicing factors from accessing the molecule and affecting RNA splicing patternsThese ASOs do not have a central gap [124].

On the other hand, duplex RNAs work similarly to endogenous siRNAs through RNA interference (RNAi) mechanisms, cleaving the targeted RNA molecules (Figure 5b). Duplex RNAs, however, have other modes of activity. Similar to ASOs, they have the ability to target promoter-associated RNAs in order to alter chromatin and control transcription (Figure 5c). The target antisense transcripts cellular location frequently influences the decision between ASOs and duplex RNAs. When it comes to regulating the expression of nuclear antisense RNAs, ASOs are generally more efficient in the nucleus [125]. On the other hand, it is assumed that the target operates inside the cytoplasm, duplex RNAs would be more appropriate.

While most therapeutic oligonucleotides have been designed to target mRNAs and miRNAs and have shown clinical benefits, using oligonucleotides to target antisense lncRNAs presents unique challenges [126]. One significant challenge is the potential for off-target effects, where unintended interactions occur, leading to undesired outcomes. These off-target effects can occur through various mechanisms, including binding to proteins and triggering non-specific reactions like the interferon response. Additionally, oligonucleotides may exhibit partial complementarity to unintended targets, leading to various toxicological challenges such as proinflammatory responses, nephrotoxicity, thrombocytopenia, and hepatotoxicity [127]. Strategies exist to mitigate off-target effects, such as comprehensive bioinformatics analysis to identify potential targets displaying both full and partial complementarity.

Understanding the mechanisms of action of antisense lncRNAs is crucial for successful therapeutic development [128]. Clinical trials have shown that targeting NATs without a clear understanding of their mechanisms can lead to unexpected results. Functional studies are essential to comprehend how oligonucleotides and NATs interact before embarking on clinical trials [129]. Additionally, it is vital

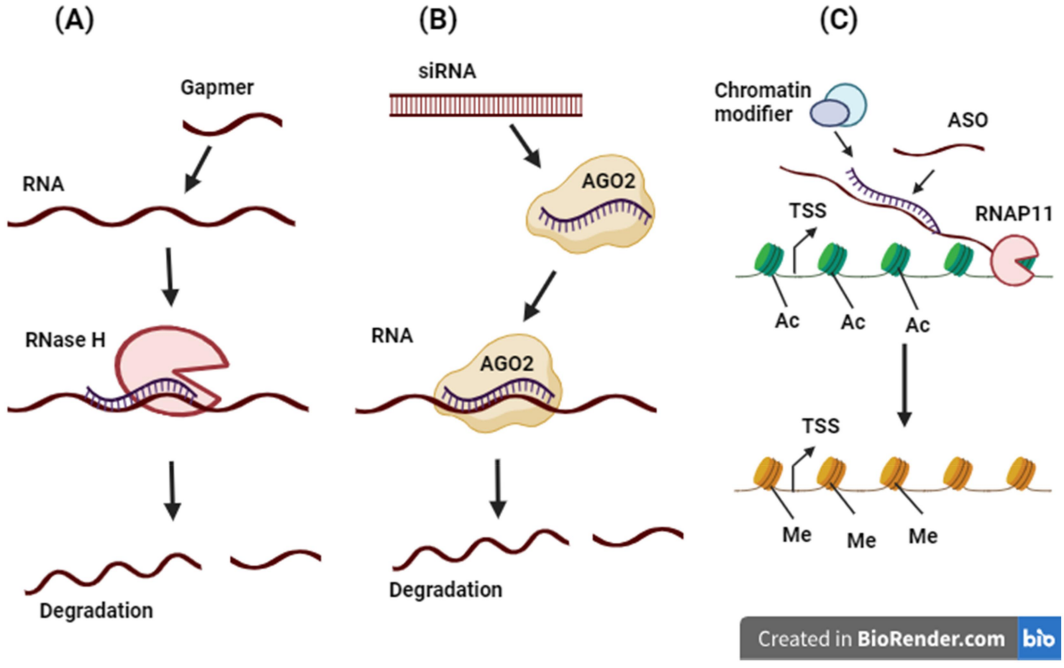

Figure 5. (a) ASO gapmers facilitate the downregulation of RNA levels. These ASOs form a duplex with the target RNA molecule. This duplex is then recognized by an enzyme called RNase H, which cleaves the RNA molecule, leading to its degradation. (b) Another method for downregulating RNA levels is through small interfering RNA (siRNA). When siRNA molecules are introduced, they are incorporated into a complex known as the RNA-induced silencing complex, along with the argonaute-2 protein (AGO2). This complex guides the siRNA to the target RNA, where it induces cleavage and subsequent degradation of the RNA within the RNA interference (RNAi) pathway. (c) ASOs can also be utilized to modulate repressive epigenetic changes. In this case, chromatin modifiers recognize the duplex formed by the ASO and the target RNA. This recognition can lead to alterations in repressive epigenetic marks, such as H3K27me3 histone modification, potentially influencing gene expression. These changes occur in proximity to the transcription start site (TSS) and can include the addition or removal of histone modifications like H3K27ac.

to determine whether the NATs of interest primarily act in the nucleus or the cytoplasm, as this knowledge informs targeting strategies.

A significant challenge in implementing oligonucleotide-based therapies lies in their limited ability to effectively reach target organs and tissues following systemic administration, with less than 1% of oligonucleotides typically reaching the intended cellular compartments [130]. Overcoming this challenge requires a deeper understanding of cellular uptake, transport, and metabolism mechanisms. Administering higher intravenous doses is constrained by potential toxicity, and natural tissue barriers, such as the blood-brain barrier, hinder systemic distribution [95]. Direct delivery methods to specific sites, such as intracerebroventricular or intrathecal injections for the central nervous system, can be effective but are costly and require specialized expertise.

Despite the challenges outlined earlier in the development of NAT-based therapies, notable advancements have been made in recent times. These advances can be attributed to the evolution of chemistry and technology, coupled with improved insights into the biological intricacies of antisense transcription and its associated RNA molecules. Consequently, a number of fruitful research endeavours have been undertaken, and certain therapies have progressed to clinical stages. Specific instances are now under meticulous investigation and hold promising prospects for the effective treatment of previously untreatable disorders. Several of these examples are elaborated below.

Targeting inhibitory NATs with oligonucleotide-based techniques to increase gene expression shows promise in treating disorders like Angelman syndrome that result from haploinsufficiency [131]. The lack of a functional maternal UBE3A gene, which encodes E3 Ubiquitin Protein Ligase, is the cause of Angelman syndrome, a neurodevelopmental condition for which there is no known cure [132]. These mutations, which are usually deletions, disrupt the gene. The reason for this absence is that the paternal allele lacks an active gene copy due to epigenetically suppressed by its antisense transcript [133].

The reactivation of the parental gene is anticipated to occur via the inhibition of the antisense transcript's function, therefore compensating for the absence of maternal allele expression [134]. The upregulation of the paternal allele Ube3a is significantly enhanced in mice by the reduction of antisense RNA Ube3a-ATS, achieved by using two ASO gapmers. The administration of ASO therapy demonstrated a modest improvement in mice exhibiting cognitive deficits [135]. Significantly, there was an absence of alteration in the transcription of supplementary genes originating from the same chromosomal region, namely Snorpn, Snord115, and Snord116.

An illustrative case illustrating the involvement of an antisense transcript in gene regulation may be noticed in the context of apolipoprotein A1 (APOA1), a crucial constituent of high-density lipoprotein (HDL) in plasma. The APOA1 gene locus contains an antisense transcript called APOA1-AS, which serves as a negative regulator of APOA1 at both the cellular and organismal levels [136]. As a consequence, the inhibition of APOA1-AS in cultured cells leads to an upregulation of APOA1 expression [137]. The findings derived from

chromatin immuno precipitation investigations indicate that APOA1-AS has the potential to modulate histone methylation patterns, hence exerting an influence on the transcriptional activity of the APOA1 gene.

Moreover, utilizing ASOs to target APOA1-AS results in elevated APOA1 expression in liver cells, both in humans and monkeys [82]. Taken together, these discoveries offer a potential strategy for regulating cholesterol levels, which is particularly significant as low HDL cholesterol levels are associated with an increased risk of cardiovascular diseases. Pharmacologically increasing HDL levels remains a proposed approach to mitigating the risk of cardiovascular diseases [138].

Another instance of discordant regulation can be observed in the context of the SCN1A gene. When the expression of the antisense transcript of SCN1A, known as SCN1ANAT or AC010127.3, is reduced or its interactions with epigenetic proteins are disrupted using oligonucleotides, it leads to an elevation in the expression of the protein-coding SCN1A gene [6]. Studies have shown that the application of ASOs can partially ameliorate disease-related characteristics in a mouse model of Dravet syndrome.

BDNF (brain-derived neurotrophic factor) is a member of the neurotrophin family, a group of growth factors crucial for various aspects of neuronal biology, such as growth, differentiation, and maintenance [139]. Disruptions in the expression of neurotrophins have been linked to certain psychiatric and neurodegenerative disorders, implying that increasing their levels might have potential benefits in specific conditions [140]. BDNF has an antagonist in the form of an antisense transcript called BDNF-AS or BDNFOS, which negatively regulates BDNF expression both in vivo and in vitro, as demonstrated in the three scenarios mentioned earlier. Furthermore, targeted inhibition of BDNF-AS transcripts using antisense oligonucleotides (ASOs) has led to the specific enhancement of BDNF expression (Figure 2a).

The peptide-conjugated PNAs 1 and 2 (PPNA1 and PPNA2), which are classified as antisense oligonucleotides (ASOs), have been specifically engineered to exhibit complementarity with distinct segments of the filamentous temperature-sensitive protein Z (ftsZ) gene. This gene is of paramount importance for the replication process of methicillin-resistant Staphylococcus aureus (MRSA). Both PPNA1 ASO and PPNA2 ASO had bactericidal properties, significantly inhibiting the growth of methicillin-resistant *Staphylococcus aureus* (MRSA) in cell culture. A different ASO, known as peptide-conjugated LNA (PLNA787 ASO), has been designed to specifically target the mRNA of ftsZ in *Staphylococcus aureus*. The administration of PLNA787 ASO shown a notable suppression of methicillin-resistant *Staphylococcus aureus* proliferation in cellular cultures. Moreover, the therapeutic effectiveness of the treatment was observed in in vivo experiments, as demonstrated by an elevated survival rate in mice that were infected with the Mu50 strain of S. aureus [141].

The CPP-PNA, specifically referred to as rpoA-PNA ASO, has exhibited successful targeting of the RNA polymerase α subunit (rpoA) in *Listeria monocytogenes*, which is a crucial component involved in transcription activities. The growth of *L. monocytogenes* in broth culture was significantly reduced by the rpoA-PNA ASO, which effectively inhibited bacterial DNA transcription. The introduction of rpoA-PNA to *Caenorhabditis elegans* nematodes that were infected with L. *monocytogenes* resulted in a significant decrease of 72% in their growth [142].

A unique ASO known as 3'-SLT PPMO was developed with the purpose of targeting the 3′ stem-loop (3′SLT) region present in the Dengue viral genome. This region plays a critical role in the translation and synthesis of viral RNA. The 3'-sialyllactose-conjugated peptide-conjugated phosphorodiamidate morpholino oligomer (3′SLT PPMO) shown notable efficiency in Baby Hamster Kidney fibroblasts (BHK) cells, resulting in a substantial decrease in viral RNA levels by more than 450-fold. The observed decrease effectively impeded the process of viral translation and RNA production. Additionally, Vivo-MO-1, an alternative ASO, demonstrated significant efficacy in its ability to specifically target the 3′ stem-loop (3′ SLT) located within the 3′ untranslated region (UTR) of the Dengue viral genome. Dendritic cells that were exposed to ASO Vivo-MO-1 demonstrated inhibition of Dengue infection, leading to a significant reduction in viral RNA levels exceeding 1000-fold [143].

Promising results were noted in the therapeutic intervention of Ebola infection in murine subjects by employing a peptide-conjugated PMO antisense oligonucleotide (PPMO) incorporating an arginine-rich peptide. The PPMO ASO under consideration was specifically engineered to selectively target the VP24 mRNA, thereby inhibiting viral replication in an efficient manner. The injection of VP24-AUG PPMO at doses of 50 μg and 5 μg resulted in total protection (100%) and significant protection (90%), respectively, against deadly Ebola infection in mice [144].

The Beta-Site Cleavage Enzyme 1 (BACE1) and its corresponding antisense transcript (BACE1-AS) provide an example of concordant regulation [145]. In this scenario, the antisense transcript boosts the stability of BACE1 by concealing microRNA binding sites. Both BACE1 and BACE1-AS are elevated in Alzheimer's disease (AD) patients [146]. Given BACE1's role in producing the pathogenic β-Amyloid peptide fragment in AD, it represents a significant target for potential pharmaceutical interventions. However, conventional drug-based inhibition of BACE1 activity has been associated with undesired effects, likely due to the enzymes additional molecular functions.

Notably, research has indicated that the introduction of siRNAs targeting BACE1-AS into mouse brains results in reduced levels of both BACE1-AS and its sense counterpart, BACE1, at both the mRNA and protein levels. Consequently, a decrease in β-amyloid aggregation and soluble levels was observed in vivo. This indirect approach to reducing BACE1 levels by targeting its antisense transcript offers a potential pharmacological strategy for mitigating AD-associated β-Amyloid aggregation when traditional methods prove insufficient.

The most cutting-edge and promising method for treating disorders linked to NATs is through oligonucleotide-based therapeutics; however, other strategies, such the CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeats-associated nuclease 9) are also being explored.

According to recent research, CRISPR-Cas9 components can improve the disease phenotype and fix gene mutations in animal models of Duchenne muscular dystrophy [147]. However, issues including immunogenicity of CRISPR-Cas9 components, off-target effects, and decreased fitness of altered cells must be resolved [148]. Since cis-NATs and their corresponding genes share genomic regions, it may be even more challenging to apply the CRISPR-Cas9 system to the field of antisense RNAs and prevent off-target effects.

Conclusion

NATs have emerged as essential components of the human transcriptome, although our understanding of their biological roles and regulatory mechanisms is still in its early stages. They possess a dual role in both normal cellular processes and the development of human diseases, making them valuable resources for medical research and potential clinical applications. NATs hold promise as disease biomarkers due to their functional activity at the RNA level, offering more precise disease indicators compared to protein-coding transcripts. Moreover, they present exciting opportunities for innovative therapeutic approaches.

Pharmaceutical companies are interested in NATs and other long noncoding RNAs that can be successfully manipulated utilizing oligonucleotide technology in both living species and laboratory settings. Some pharmaceutical companies are working on developing a CURNA platform that suppresses the function of disease-associated NATs by using antagoNATs (antisense oligonucleotides targeting NAT transcripts). They are searching for proteins that are predominantly linked to orphan diseases by preclinical research, and it is actively screening over 400 gene targets. These are uncommon illnesses, as defined by the FDA's Orphan Drug Act, affecting fewer than 200,000 Americans. These illnesses include conditions such as mucopolysaccharidosis I, Rett syndrome, and Dravet syndrome.

Despite the technical challenges mentioned earlier, it is foreseeable that NATs will soon become central to the development of noncoding RNA-based strategies to combat a wide range of human ailments. These conditions encompass various categories, including cancer, cardiovascular diseases, neurological disorders, and muscular diseases.

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Author contributions

AA, ZS and AK developed the idea, AA wrote the manuscript, BM and QT helped in formatting the manuscript.

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