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Review Article

SINEUPs: a novel toolbox for RNA therapeutics

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RNA molecules have emerged as a new class of promising therapeutics to expand the range of druggable targets in the genome. In addition to 'canonical' protein-coding mRNAs, the emerging richness of sense and antisense long non-coding RNAs (lncRNAs) provides a new reservoir of molecular tools for RNA-based drugs. LncRNAs are composed of modular structural domains with specific activities involving the recruitment of protein cofactors or directly interacting with nucleic acids. A single therapeutic RNA transcript can then be assembled combining domains with defined secondary structures and functions, and antisense sequences specific for the RNA/DNA target of interest.

As the first representative molecules of this new pharmacology, we have identified SINEUPs, a new functional class of natural antisense lncRNAs that increase the translation of partially overlapping mRNAs. Their activity is based on the combination of two domains: an embedded mouse inverted SINEB2 element that enhances mRNA translation (effector domain) and an overlapping antisense region that provides specificity for the target sense transcript (binding domain). By genetic engineering, synthetic SINEUPs can potentially target any mRNA of interest increasing translation and therefore the endogenous level of the encoded protein.

In this review, we describe the state-of-the-art knowledge of SINEUPs and discuss recent publications showing their potential application in diseases where a physiological increase of endogenous protein expression can be therapeutic.

The richness of sense and antisense long non-coding RNAs transcriptome

Large-scale projects such as FANTOM (Functional ANnoTation Of the Mammalian genome) [1] and ENCODE (The Encyclopedia of DNA Elements) [2] have led to the discovery that a great majority of the transcriptome is composed of a diversified class of long non-coding RNAs (lncRNAs). These transcripts are longer than 200 nucleotides with poor protein-coding potential [3] and originate from intergenic, intronic or intronic/exonic DNA regions, sense (S) or antisense (AS) to protein-coding genes [4]. This large amount of untranslated RNAs has important, yet under-characterized, regulatory functions in the gene expression program of a cell [5], and has significantly contributed to the evolution of complex life and species divergence [6]. Indeed, the relative abundance of lncRNAs in an animal genome correlates with its biological complexity and phenotypic diversity [6,7]. They can function both as structural and regulatory RNAs. In the latter, lncRNAs can play a key role in gene expression control, acting as decoy, enhancers and splicing modulators [8–10].

The expression pattern of lncRNAs is highly specific and dynamic in terms of cell type, development stage, and subcellular localization [3,11], thus allowing temporal- and lineage-restricted regulation [12]. Their regulatory function can be exerted both *in cis* and *trans* [13–21].

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Natural AS transcripts are RNAs that are transcribed from the opposite DNA strand to S transcripts forming partial or total overlapped S/AS pairs. AS transcription is a common feature of genomes from bacteria to mammals [1], and S/AS pairs cover more than 70% of the whole mammalian transcriptome [4,22,23]. AS lncRNAs control the output of the protein-encoding transcriptome acting at distinct regulatory levels *in cis* including the establishment of epigenetic marks [24], transcription [25–28], splicing [29] and RNA stability [30]. AS lncRNAs contribute to tumorigenesis, genetic and neurodegenerative diseases [31–34].

lncRNAs may consist of modular structural domains [32–34], which display signs of evolutionary selection, although experimental proof of this organization is only limited to few cases [1]. Such structures seem to be more conserved than their primary sequence [35–37], supporting their functional roles in the recruitment of protein co-factors [32,38] or direct interaction with nucleic acids. A single transcript can combine distinct modular domains, partner with different proteins and target specific DNA/RNA motifs.

Transposable elements (TEs) have been proposed as candidate domains that determine the functionality of lncRNAs [39–42]. Previously considered as 'junk', TEs are now known to play pivotal roles in shaping genome diversity [43], and interestingly, comprise a significant proportion, 40% on average, of the lncRNAs nucleotide sequence [44,45]. TEs may exert their function through specific protein interaction networks, as for the preferential binding of Alu sequences in the inverted orientation to hnRNPC, TDP-43 and ILF3 [46–48].

Modularity, restricted expression profile and functionality *in trans*, represent important assets for the therapeutic exploitation of lncRNAs.

Natural SINEUPs: the discovery of a novel class of AS lncRNAs that activate translation

In 2012, we identified Anti-Sense to Ubiquitin carboxyterminal hydrolase L1 (AS Uchl1 or Uchl1os) as the first lncRNA that activates translation of its S protein-coding gene. Its activity is based on the combination of two domains: an embedded mouse inverted SINE (Short Interspersed Nuclear Element) B2 repeat enhancing mRNA translation (effector domain, ED) and an overlapping AS region providing specificity for the target sense transcript (binding domain, BD) [39,42,49]. This evidence led to the original hypothesis that embedded TEs may represent functional domains within lncRNA genes [37,40,50]. Its modular structure and activity are shared with other natural lncRNAs antisense to protein-coding genes in the mouse genome, thus suggesting AS Uchl1 to be the representative transcript of a new class of regulatory RNAs. We then directed its translation-enhancing activity to endogenous genes by artificially engineering BDs AS to the target mRNAs of interest. In light of the abovementioned consideration, we defined this class of natural and synthetic RNAs as SINEUPs since through the activity of a SINEB2 sequence they can UP-regulate the translation of a target mRNAs [42,49] (Figure 1).

AS Uchl1 RNA is transcribed in a divergent orientation from the *Uchl1* gene, a process that initiates at its second exon and ends, at the 3′, with a non-overlapping sequence. The BD starts from +32 nucleotides (nts) ending −40 nts to AUG (A is +1) in the 5′Un-Translated Region (UTR) of Uchl1 mRNA and is indicated as −40/+32.

AS Uchl1 transcription has a more restricted tissue distribution, as compared with the sense Uchl1 gene. The expression of S and AS Uchl1 is mostly co-regulated and no AS Uchl1 is expressed in the absence of Uchl1 mRNA [39,51]. This transcription pattern suggests a regulatory role for the AS versus its S counterpart [52]. After transcription, AS RNAs are enriched in the nucleus, where they accumulate, and their function remains unknown [39]. Interestingly, under conditions of cellular stress, as upon the inhibition of mTORC1 signaling pathway elicited by rapamycin treatment, AS Uchl1 is rapidly relocated to the cytoplasm [39]. Cytoplasmic AS Uchl1 RNA is accompanied by a shift of Uchl1 mRNA to heavy polysomes and an increase in translation, while its transcript level remains unaltered. Importantly, mRNA association to heavy polysomes requires AS Uchl1 expression and is sufficient to account for the increase in UCHL1 protein levels. As rapamycin treatment attenuates CAP-dependent translation, the enhancement of protein synthesis mediated by natural SINEUPs should involve an alternative eIF4F-independent mechanism [39].

The translation machinery is extremely energy-consuming for the cell [53], which explains the evolutionary optimization of the usage of its limited resources. Furthermore, its regulation is a means to rapidly and efficiently influence protein levels. This can occur by acting at the level of both translation initiation and elongation [54,55]. Such regulation includes the global repression of protein synthesis during cellular stress and the parallel activation of translation of stress-response proteins [56,57], achieved through different mechanisms such as Internal Ribosome Entry Sites (IRESs) [57,58], upstream open reading frames (uORFs) [59], and other cis-acting features present in the UTRs of mRNAs. While inhibition of mRNA translation *in trans* may be achieved by miRNA [60], mechanisms for protein-specific enhancement are much less known [56,57,61,62]. In this scenario, SINEUPs represent a new class of regulatory lncRNAs acting at the translational level to enhance protein synthesis *in trans*. Regulation through a

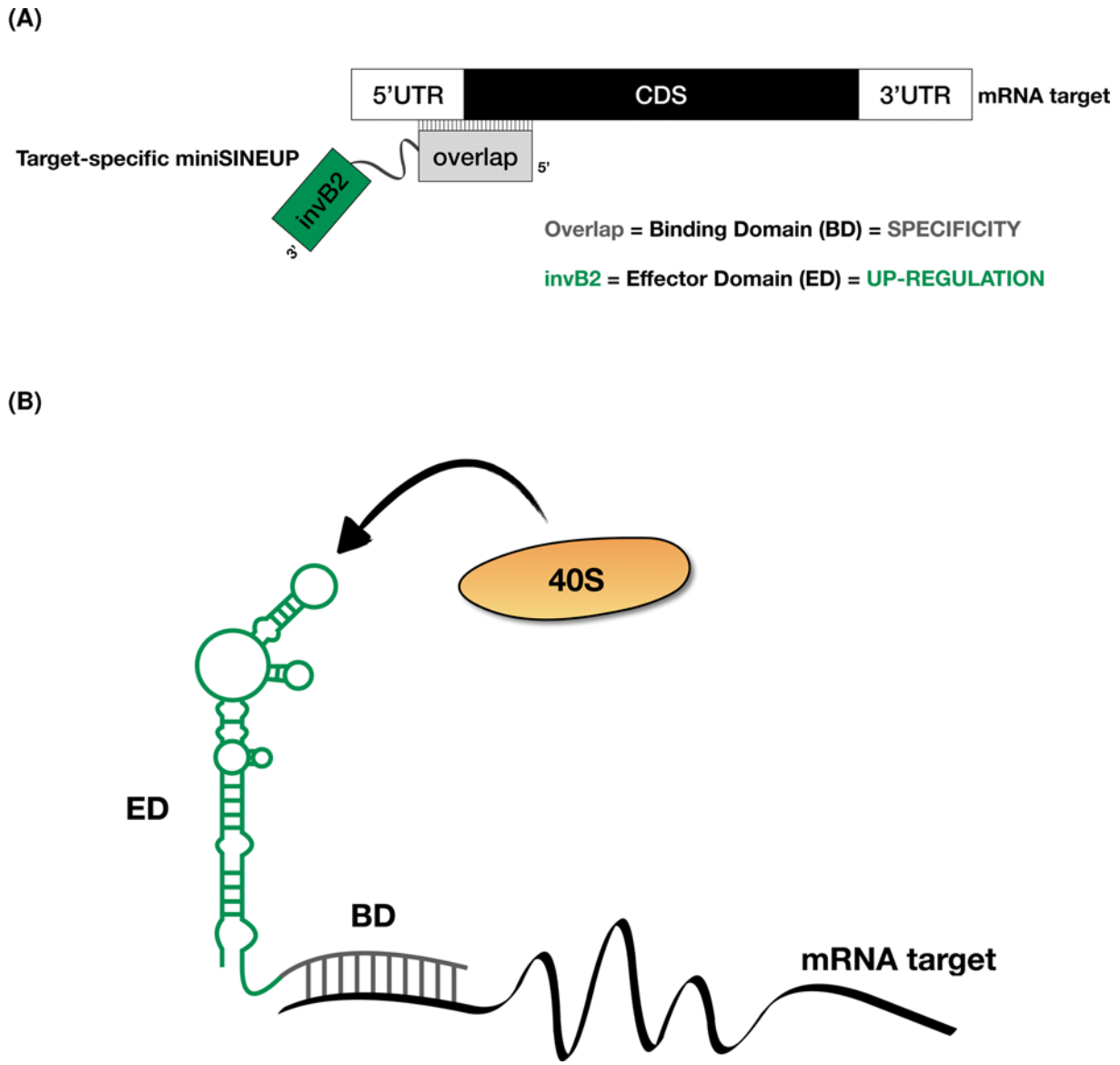

Figure 1. SINEUPs

(**A**) Schematic representation of SINEUPs functional domains. The binding domain (BD, gray) provides SINEUP specificity and it is in antisense orientation to the sense protein-coding mRNA (Target mRNA). The inverted SINEB2 element (invB2) is the effector domain (ED, green) and confers enhancement of protein synthesis. 5′ to 3′ orientation of sense and antisense RNA molecules is indicated. Structural elements of target mRNA are shown: 5′ untranslated region (5′UTR, white), coding sequence (CDS, black) and 3′ untranslated region (3′UTR, white). The scheme is not drawn in scale. (**B**) Mechanisms of SINEUP-mediated *in trans* enhancing of protein synthesis. Scheme showing S/AS 5′head-to-head divergent pairing between SINEUP and targeted mRNA.

natural SINEUP lncRNA provides a rapid enhancement of target protein level since SINEUP RNA is stored in the nucleus and rapidly moves to the cytoplasm as needed [39].

Systematic computational screening in the mouse transcriptome identified with high confidence 31 other antisense transcripts containing an inverted SINEB2 with a similar architecture to S/AS Uchl1 pair, therefore appearing to have the potential to exhibit SINEUP activity [39]. In the same study, the antisense transcript to *Uxt* gene confirmed the expected activity of transcription-independent stimulation of translation of its target. Thereafter, additional AS transcripts have been identified having the same activity (i.e. AS-eln, AS to the elastin gene [63]) expanding the list of validated, natural mouse SINEUPs.

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Importantly, two natural SINEUPs were identified and experimentally validated, in the human genome [64]. The first one is an AS transcript to human protein phosphatase 1 regulatory subunit 12A (PPP1R12A), named R12A-AS1, which contains, at its 3′ end, a short Free Right Alu Monomer repeat element (FRAM), which replaces the mouse SINEB2 domain with the same translation-enhancing functionality. The second, an AS to Integrin-Alpha FG-GAP Repeat-Containing Protein 2 (ITFG2), also showed SINEUP activity, mediated by an ED containing an inverted MIRb TE [64]. 129 potential human natural SINEUPs were computationally identified as part of S/AS pairs with a protein-coding gene in a head-to-head configuration and presenting an embedded TE of these families in the non-overlapping region [64]. This evidence suggests that SINEUPs could be a general and widespread mechanism for gene expression regulation in eukaryotes. Importantly, we have recently shown that, despite their lack of primary sequence homology, both SINEB2 and FRAM bind the dsRNA-binding protein ILF3, suggesting possible converging evolution of embedded TEs [48].

Synthetic SINEUPs: functional domains and design optimization

By swapping BD sequences, synthetic AS lncRNAs can be designed re-directing AS Uchl1 activity to target ectopically expressed transcripts, such as those encoding for GFP [39], or endogenous genes (Figure 1). Using the BD to provide specificity, SINEUP technology is therefore scalable. Synthetic SINEUPs have shown efficacy in targeting a number of specific mRNAs including FLAG-tagged proteins and secreted recombinant antibodies and cytokines [49,63,65,66]. Most importantly, synthetic SINEUPs can act on endogenous mRNAs both *in vitro* and *in vivo*, as first demonstrated by specific SINEUPs designed to target genes associated with neurodegeneration (PARK7/DJ-1) [49] (Figure 2). Although the extent of the increase in protein may differ, SINEUPs have been shown to work in cells lines of mouse, hamster, monkey and human origin, thus providing a molecular tool with wide applicability in *in vitro* experimental settings. Interestingly, our preliminary data also show synthetic SINEUPs are active in *Drosophila* cells, proving they are hijacking an evolutionary conserved cellular machinery (Tettey Matey A., et al., unpublished). It is of note that the increase of the endogenous expression of the target gene is between 1.5- and 3-fold. This feature makes SINEUPs an ideal tool to perturb gene expression *in vivo* within a physiological range.

Both BDs and EDs have been extensively analyzed in an effort to understand the relationship between structure and activity and to gain deeper insights into the mechanism of action of naturally occurring SINEUPs. This has led to an optimized design of artificial SINEUPs, and the identification of the minimal structural features needed for the activity [67].

The first generation of synthetic SINEUPs derived from natural AS Uchl1 and were about 1200 nts long with the BD of 72 nts, the ED of 170 nts, in addition to intervening sequences, a partial Alu element (73 nts) and a 3′ tail [39]. Deletion analysis of AS Uchl1 RNA proved that only two sequences were required for SINEUP activity: the BD and the ED. This led to the synthesis of active miniSINEUPs RNAs that were obtained with the exclusive combination of BD and ED giving rise to a ≈250 nt long transcript [2]. Robust data have been accumulated for miniSINEUP proving the ability to increase protein levels for several targets including GFP [3] and DJ-1 [3].

While the natural anatomy of the antisense sequence found in AS Uchl1 has been considered the model for BD design in synthetic SINEUPs, additional BDs have been successfully tested for several mRNA targets. These have been generally obtained by trimming sequences at both ends with respect to the sense A (+1) of the translation initiation site AUG. So far, we have identified -40/+4, -40/0, -14/+4 and -14/0 antisense sequences as the most probable effective BD variants ([68,69] and unpublished data). It is of note that a BD as short as 14 nts may still confer strong SINEUP activity [68,70]. They may present substantial differences in their activity for a single mRNA or by comparing them for different targets. Interestingly, examples have been found where highly effective SINEUPs contained a BD which pairs with an internal AUG sequence within the ORF of the target mRNA [70]. It remains to be determined whether BDs should be designed exclusively as AS to regions adjacent or comprising the translation initiation site or also against homology regions located far from the AUG within the 5′UTR, in the open reading frame or in the 3′UTR sequences.

Importantly, to design the appropriate BD, the precise knowledge of the real transcription start site (TSS) of the target mRNA is crucial. In our experience, we have found that the annotation of the reference sequence is often not representative of the cell-type-specific usage of TSSs and of the 5'UTRs of endogenous mRNAs. To build specific SINEUPs, we are taking advantage of the FANTOM5 collection of Cap Analysis of Gene Expression (CAGE) datasets, which represents the widest catalogue of annotated promoters and TSSs in mammalian samples [71]. Using the ZENBU Genome Browser Tool for data visualization [72], we typically monitor the TSS usage at the gene of interest in the tissue where we aim to increase its protein expression.

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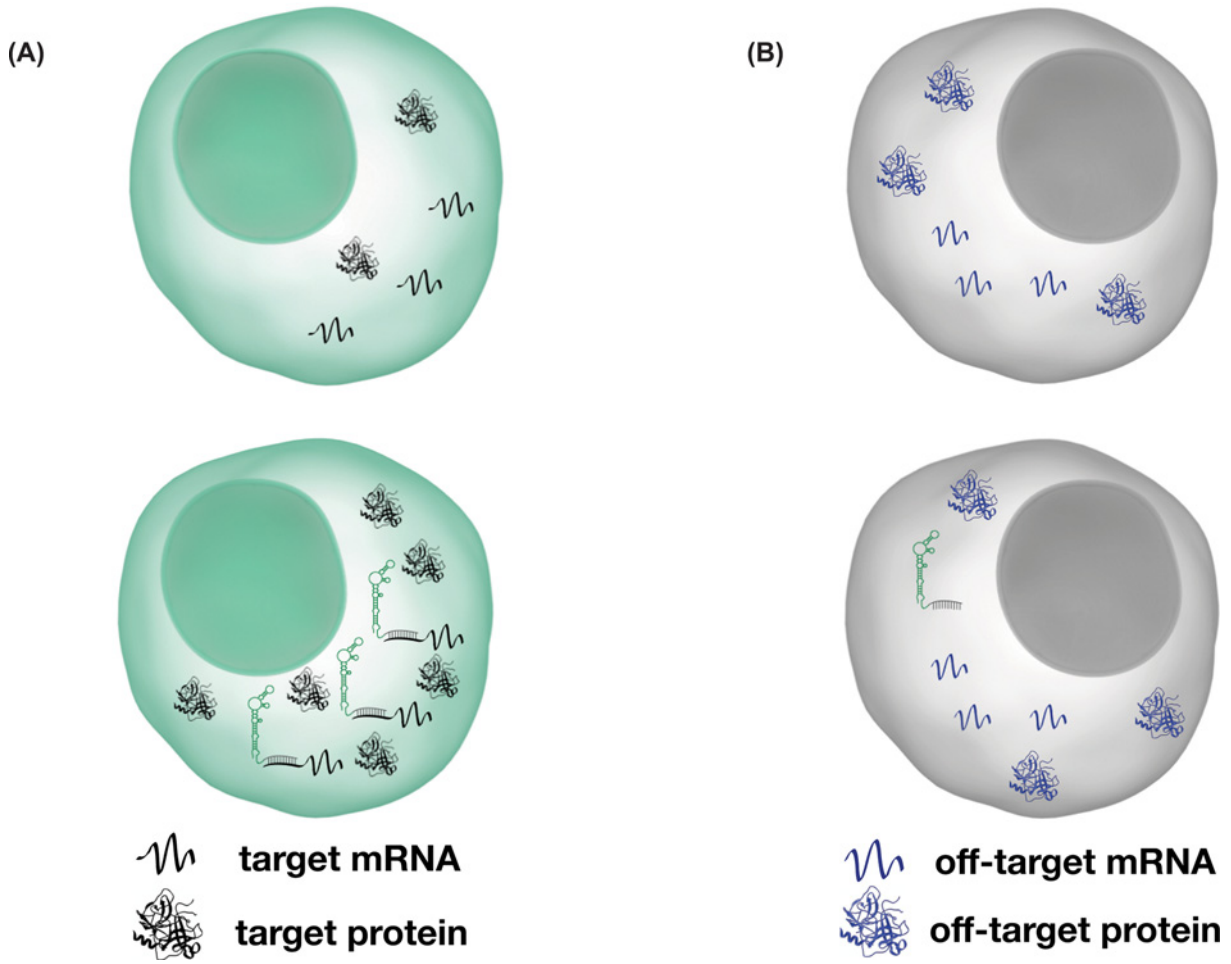

Figure 2. Specificity of SINEUP effect

SINEUPs act on endogenous mRNA and since the BD sequence is designed to be specific only for the target mRNA, off-target activity is virtually absent. (**A**) SINEUP is expressed in a cell that contains the target mRNA, leading to the increase of protein levels. (**B**) If SINEUP is expressed in a cell that does not contain the target mRNA, there is no effect on the translation of other mRNAs.

The invSINEB2 sequence from AS Uchl1 is the ED in all synthetic SINEUPs we have designed and experimentally validated so far. To elucidate its molecular mechanism and to optimize its use in therapeutic SINEUPs, we have studied its secondary structure by chemical footprinting, NMR and experimental analysis of RNA mutations [73]. By chemical footprinting, we unveiled the presence of four Internal Loops (IL) and three Stem Loop (SL) elements (Figure 3). Interestingly, when the terminal hairpin structure was disrupted by deleting nucleotides 68–77 of the invSINEB2 sequence from the full length AS Uchl1 (ΔSL1 mutant), SINEUP ability to up-regulate UchL1 protein levels was completely abolished proving a crucial role of SL1 in the activity. The structure of the key hairpin was further refined by NMR studies of the fragment in solution, showing an A-type helical stem terminated by a triloop structure [73]. By combining experimental data (nuclear overhauser effect, NOE) and molecular dynamics simulations, it was possible to obtain a minimal set of four conformations for the SL1 which are compatible with the experimental data [74].

Recently, NMR 'fingerprints' were used as sensitive probes to divide the full-length inverted SINEB2 sequence into minimal units that retain the original structure and function. One dynamic domain and two discrete structured domains (named C and M domains) were thus identified [75]. The 31–199 nts fragment, largely corresponding to the C domain, showed an identical fold and retained 80% of the SINEUP function of the full length inverted SINEB2 sequence.

These data are instrumental for the identification of key structural determinants for ED function, to allow further miniaturization of the molecule and to introduce mutations aimed at increasing the stability and activity of SINEUPs.

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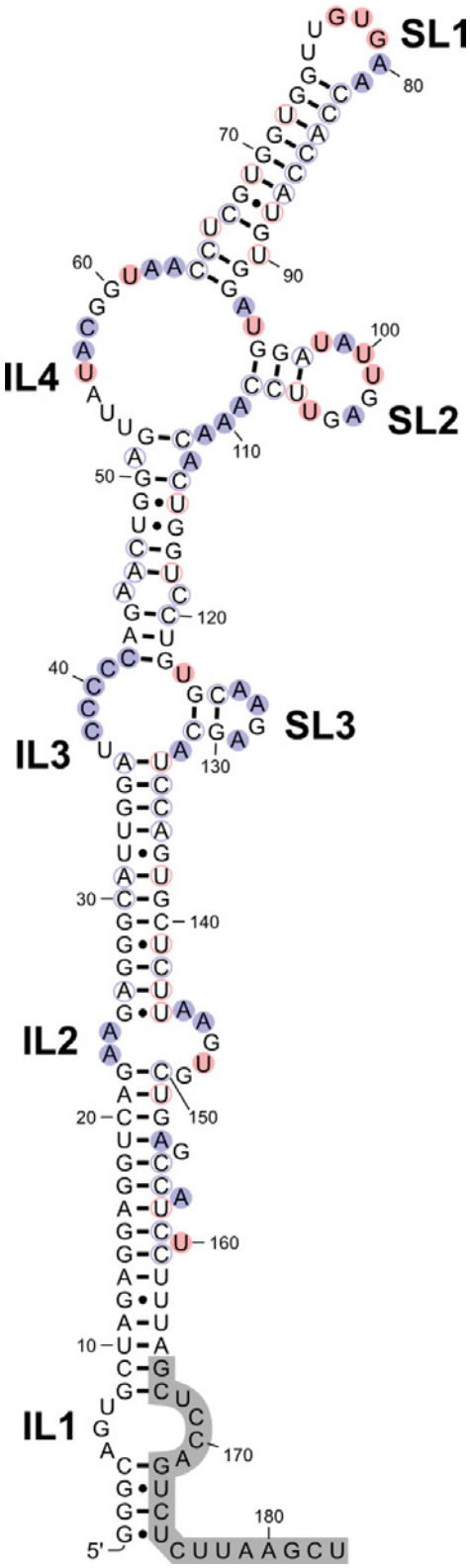

Figure 3. Secondary structure of the invSINEB2 ED of AS Uchl1

Dimethyl sulfate (DMS) and 1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMCT) were used as methylating agents. DMS and CMCT reactive nucleotides are shaded in blue and red, respectively. Internal loops and stem-loops are labelled as ILx and SLx, respectively. Non-reactive nucleotides are only circled. The segment shaded in grey corresponds to the DNA primer hybridization site. Reproduction from Figure 1 in [73] (reused with permission).

Furthermore, it is a starting point for future comparative studies of EDs in other natural SINEUPs to identify common structural features.

By using a phage display-based approach to screen RNA-protein interactions *in vitro* [76], we identified the double-stranded RNA-binding protein (RBPs) interleukin enhancer-binding factor 3 (ILF3) as a protein partner of AS Uchl1 RNA [48]. Initially isolated as a transcription factor in the IL-2 promoter-binding complex, ILF3 may regulate splicing and translation and could be involved in RNA metabolism, including transport, localization and stability [77]. Interestingly, ILF3 can also bind FRAM sequences, the embedded TE acting as an effector domain in human natural SINEUPs. While SINEB2 and FRAM do not present extensive homology at the primary sequence and there is no clear consensus sequence for ILF3 binding, these results suggest they form conserved secondary structures that can bind common interacting partners. This is relevant under the hypothesis that embedded TEs can act as evolutionary convergent functional domains. Experimental evidence also suggests that ILF3/invSINEB2 interaction can moderately influence AS Uchl1 nuclear retention [48].

SINEUP RNA also interacts with RBPs such as PTBP1 (polypyrimidine tract binding protein-1) and HNRNPK (heterogeneous nuclear ribonucleoprotein K) [78]. The formation of the complex between SINEUP RNA and these proteins was essential for RNA localization and translational initiation assembly. While SINEUP-GFP RNA was retained in the nucleus in the absence of target, its co-expression with EGFP drove SINEUP RNA shuttling to the cytoplasm. By knocking down or overexpressing PTBP1 and HNRNPK proteins, SINEUP nucleocytoplasmic shuttling and activity were modified proving the relevance of S/AS RNA complex formation with RBPs in SINEUP dynamics and function [78].

Synthetic SINEUPs: a platform to confront unmet clinical needs

SINEUP technology is well-positioned to tap into a very promising landscape for nucleic acid-based therapies. In the last decade, gene therapy has greatly expanded, registering significant improvements in terms of safety and efficacy while showing big promises to target untreatable diseases [79,80]. Different strategies have been developed to express medicinal molecules *in vivo* including (i) viral delivery of genes, (ii) the use of synthetic antisense oligonucleotides (ASOs) and (iii) RNA-based systems (reviewed in [79,81,82]). SINEUPs may represent the technology of choice when each of these strategies encounters unsurmountable roadblocks and conceptual limitations. In gene therapy, Adeno-Associated Virus (AAV) vectors have a relatively small cargo capacity. This constrain reduces the list of genes that can be delivered [83]. Transgene expression may reach levels well beyond the physiological range, representing a potential issue in terms of safety. The lack of specific promoters for every cell type gives rise to ectopic expression of the transgene in unwanted cells. ASOs are designed to inhibit the expression of regulatory AS lncRNAs (Natural Antisense Transcripts, AntagoNATs), thus indirectly increasing endogenous S mRNA levels. However, based on current knowledge, the scarcity of inhibitory natural AS lncRNAs to genes of interest limits the apparent breadth of applicability of this technology. For RNA-based therapies, in the case of *in vitro* synthesized, chemically modified mRNAs, so successful in SARS-CoV-2 vaccination, ectopic overexpression is largely beyond the physiological range and occurs within a short timeframe, conflicting with the requirement to mirror the endogenous expression of the gene of interest. Similar drawbacks in terms of specificity reside in small activating RNAs (saRNAs) that are used to trigger transcriptional activation of the gene targets. Furthermore, heterochromatin regions may be out of reach and therefore insensitive to the treatment, severely limiting its applications.

At present, two strategies can be pursued to deliver SINEUPs molecules to the patients. The first approach takes advantage of AAV delivery where a single SINEUP molecule can be chronically expressed *in vivo*. This approach is essential when the increase should be in a physiological range and ectopic expression in unwanted cells should be avoided. It does not require the use of a cell-type specific promoter since the expression of the target mRNA is necessary for the SINEUP activity. It extends the repertory of therapeutic genes since it can increase the expression of a target protein when its cDNA is too large for AAV cargo capacity.

Second, in selected cases, additional advantages can be envisioned for delivering SINEUPs as RNA molecules. The use of a chemically synthesized SINEUP would be an important advancement since RNA-based drugs do not cause stable modification to the genome, therefore reducing the risk of genotoxicity. In the last years, important progress have been made in the delivery of RNA therapeutics, especially siRNAs and ASOs, to a variety of tissues, including the central nervous system, and it can be repurposed for SINEUP RNAs. To this end, two crucial milestones should be achieved. Active SINEUPs should be shortened to less than 60 nts for economically sustainable manufacturing and efficient delivery *in vivo*. Since *in vitro* transcribed (IVT) SINEUP RNA is not active when transfected in cells, chemical modifications should be added to preserve RNA function and stability in the human body. Encouraging

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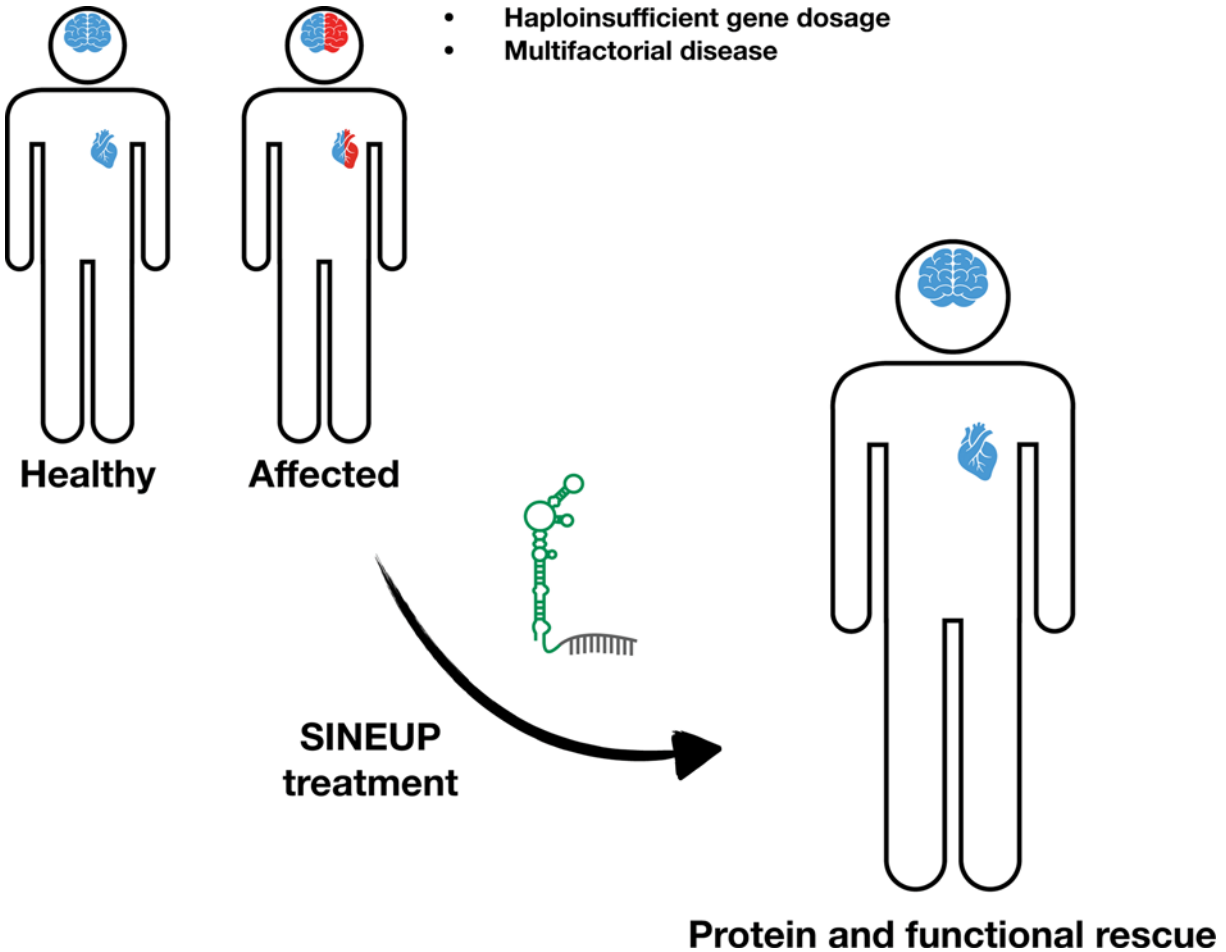

Figure 4. SINEUPs as therapeutic strategy

SINEUPs could be used as a therapeutic approach in several pathological conditions. In particular, in haploinsufficiency disorders and multifactorial disease. Genetic diseases with the lack of one functional allele for a single (haploinsufficiency) usually present a decrease of protein levels of the target gene/s leading to a pathological phenotype. The application of SINEUP may restore the physiological levels of the target protein/s and the normal phenotype in the individual affected by the genetic disease. In multifactorial diseases, the increase of pro-survival factors and enzymes may be beneficial for the patients. However, dosage and off-target distribution activity of these factors are crucial for the efficacy of these treatments. Thus, SINEUPs may be an optimal therapeutic opportunity.

recent data have proved that the incorporation of selected chemically modified ribonucleotides during IVT may restore SINEUP activity and therefore may be candidates for being included into SINEUP RNA drugs [84].

However, more studies with specific techniques (e.g. nanopore sequencing) are necessary to unveil the natural chemical modification of SINEUP RNA to better improve its efficacy *in vivo*.

In current medical practice, there are several unmet therapeutic needs to increase protein levels. As a broad classification, we can envision the use of SINEUP technology for therapeutic benefit in (i) genetic diseases with the lack of one functional allele for a single gene (haploinsufficiency) and (ii) complex diseases where the increase of a compensatory pathway may preserve or restore physiological activities (Figure 4).

Haploinsufficiencies are a wide spectrum of diseases (several hundreds) where the protein product of both alleles is required to ensure the normal phenotype, but one allele is inactive due to hereditary or germline mutations leading to lower expression of a functional protein. They are heterogeneous (each of them involving a different gene) and rare (they occur in a very limited number of patients), limiting drug development by the private sector. Importantly, recent data have shown that the overexpression of some of these target genes can be detrimental, phenocopying the disease or leading to life-threatening side-effects [85]. These worrisome results strongly support the need for new technologies able to restore the expression of the gene of interest in a physiological range. In these circumstances,

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SINEUP technology modestly increases protein expression of about 1.5–3-fold, thus restoring physiological levels in case of haploinsufficiency disorders while virtually limiting or eliminating any toxicity elicited by uncontrolled overexpression.

In many complex, multifactorial diseases, the increase of pro-survival factors and enzymes may improve the well-being of patients. As an example, exogenous delivery of neurotrophic factors has been proposed as therapeutic treatments for neurodegenerative diseases [86]. However, dosage and bioavailability issues hamper the therapeutic benefits of current delivery strategies [86]. Moreover, toxicity from off-target distribution highlights the need for tissue-specific expression (reviewed in [87]). Similarly, increasing the concentrations of transcription factors and enzymes involved in pathways whose efficiency is lowered in neurodegenerative diseases, such as autophagy [88,89] and mitochondrial biogenesis [90], can result in valuable novel therapeutic options.

Synthetic SINEUPs: proof-of-concept models for therapeutic applications

Since the initial discovery, our groups synthesized several artificial SINEUPs, targeting both endogenous and exogenous genes, proving both the functionality and the versatility of this technology for target-specific manipulation of gene expression in a reproducible manner.

As a roadmap for the use of SINEUPs in the clinic, we have recently carried out proof-of-concept experiments to demonstrate the ability of SINEUPs to revert pathological phenotypes in different relevant biological models of human diseases: (i) a medakafish model of an haploinsufficient disease; (ii) patients' cells from a genetic human disease; (iii) a mouse model of neurodegenerative disease.

A medakafish model of a human genetic disease: microphthalmia with linear skin defects syndrome

The first demonstration of SINEUP activity in an animal model of human diseases was obtained in a medakafish (*Oryzias latipes*) model of microphthalmia with linear skin defects (MLS) syndrome [91]. MLS is a X-linked, dominant, male-lethal disorder, characterized by microphthalmia, brain abnormalities and skin defects in heterozygous females [92]. MLS syndrome is caused by mutations in enzymes of the mitochondrial respiratory chain such as the holocytochrome c-type synthase (HCCS) [93] and the subunit 7B of cytochrome c oxidase (cox7b) [94]. Medakafish model of MLS displays down-regulation of cox7b, obtained through an exon-skipping frameshift mutation, resulting in microcephaly and microphtalmia [94].

A SINEUP for medakafish cox7b was designed, *in vitro* transcribed and microinjected into medakafish embryos. Strikingly, the microcephaly and microphthalmia disease phenotype was completely reverted in 50% of the embryos, due to restoration of physiological levels of cox7b, in the absence of transcriptional effects. Disease markers such as reduced cox-IV levels and enhanced programmed cell death were also rescued by *SINEUP-cox7b* [91]. Importantly, this was the first demonstration that the SINEUP molecule was able to elicit the synthesis of a functional protein *in vivo*.

Patients' cells of a human genetic disease: Friedreich's ataxia

Friedreich's ataxia (FRDA) is a fatal and presently untreatable genetic disease due to a decreased expression of frataxin (FXN), caused by the homozygous hyperexpansion of GAA triplet repeats [95]. The extent of the hyperexpansion is variable and strongly correlates with disease severity and age of onset. Larger hyperexpansions result in lower residual protein levels and a more aggressive disease phenotype [96,97]. The *FXN* gene encodes for frataxin, a small iron-binding protein localized to the mitochondria [98,99], which plays a central role in the biosynthesis of the iron-sulfur cluster (ISC) [100,101], an essential cofactor for several enzymes [102,103]. Insufficient ISC biosynthesis detrimentally affects many pathways, involving intracellular iron homeostasis, mitochondrial activity and the response to reactive oxygen species [104,105].

Several SINEUPs variants for *FXN* were designed and tested to maximize SINEUP activity while maintaining the active molecule as short as possible [69]. BDs with minimal or no overlap to the gene coding sequence induced the maximal SINEUP activity. The most effective BDs were combined directly to the invSINEB2 (ED) sequence, without intervening linker sequences, thus producing active *miniSINEUP-FXNs* that were ≈250 nucleotides long. Interestingly, functional BDs were as short as 14 nts. *SINEUP-* and *miniSINEUP-FXNs* positively regulated frataxin in the range of 2-fold in FRDA-derived fibroblasts and lymphoblasts, re-establishing physiological levels of frataxin. Importantly, the absence of *off*-target effects was experimentally proved. This increase was sufficient to restore the

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physiological mitochondrial activity, a major disease-associated phenotypic trait, in patient-derived primary cell lines [69].

A mouse model of a human complex disease: Parkinson's disease

Parkinson's disease (PD) is one of the most common neurodegenerative disorders and is caused by loss of dopaminergic (DA) neurons in the Substantia Nigra pars compacta (SNpc), which underlies the motor symptoms that characterize the disease. These typically include rigidity, tremor and bradykinesia [106]. Most of the PD cases are sporadic [107], but rare familial cases (less than 5%) have provided crucial insights into the molecular pathways of neurodegeneration. Currently, PD remains incurable. Dopamine replacement strategies with the DA precursor L-DOPA is widely employed for alleviation of PD motor symptoms, but its long-term use is connected to several side effects and drug resistance [108]. Glial cell-derived neurotrophic factor (GDNF) is a well-established neurotrophic factor, promoting the survival of DA neurons [109,110] and it has been intensively studied as a potential agent to halt neurodegeneration in PD [111]. However, long-term delivery of GDNF by intra-parenchymal infusion resulted in toxicity [112] and did not show clear therapeutic effects [113], emphasizing the need for the development of alternative delivery strategies or approaches aimed at the stimulation of endogenous GDNF production within physiological levels.

We have recently shown that a synthetic *miniSINEUP-GDNF* was able to increase endogenous GDNF protein levels by approximately 2-fold. The BD was 18 nts long, targeting *gdnf* mRNA around the AUG (-14/+4). Importantly, *miniSINEUP-GDNF* was selective in enhancing GDNF protein levels, as no off-target effect was found. AAV9-mediated delivery into the striatum of WT mice led to the expression of the *miniSINEUP-GDNF* RNA, an increase of endogenous GDNF protein for at least six months and the potentiation of the function of the DA system. Mice injected with AAV9- *miniSINEUP-GDNF* were more sensitive to the locomotor effect of amphetamine and showed an increased release of DA in the striatum after amphetamine or potassium infusion in the microdialysis probe. Interestingly, the common side effects caused by the ectopic expression of GDNF, such as loss of body weight and decrease in food intake, were not observed in mice injected with AAV9-*miniSINEUP-GDNF*, consistent with the selective and moderate increase of endogenous GDNF protein. Furthermore, *miniSINEUP-GDNF* was able to ameliorate motor deficits and neurodegeneration of DA neurons in the 6-hydroxy-dopamine (6-OHDA) neurochemical mouse model of PD [68].

On the road to the clinic

SINEUPs display several features that make them innovative tools for the development of therapeutics potentially targeting a large number of presently untreatable conditions. Their unique target-specific action *in trans* at the translational level, their ability to restore physiological levels of expression, their well-studied modularity and their potential for miniaturization, makes the design of effective synthetic SINEUPs feasible. SINEUP-based therapeutics have the significant potential to fill a gap in the present repertoire of drugs for rare orphan diseases such as haploinsufficiencies and complex multifactorial diseases. Currently, several laboratories are successfully developing synthetic SINEUPs for their target gene of choice (*personal communication*) increasing knowledge and awareness of this technology within the scientific community.

Summary

- SINEUPs are a novel class of lncRNAs that selectively increase the translation of the target mRNA.
- Synthetic SINEUPs represent a versatile tool for increasing the expression of endogenous proteins of interest for therapeutic purposes.
- The understanding of the fine details of SINEUP mechanism may provide new insights to optimize SINEUP activity *in vivo* for its therapeutic purposes.

Competing Interests

S.G., P.C., H.T. and C.S. declare competing financial interests as co-founders of Transine Therapeutics, Cambridge, UK.

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Author Contribution
S.G., S.E., C.B. conceived the paper. All the authors wrote the paper.

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Abbreviations
6-OHDA, 6-hydroxy-dopamine; AAV, Adeno-Associated Virus; AS, Antisense; ASOs, Antisense oligonucleotides; DA, Dopaminergic; FRDA, Friedreich's ataxia; FXN, Frataxin; GDNF, Glial cell-derived neurotrophic factor; HCCS, Holocytochrome c-type synthase; ILF3, Interleukin enhancer-binding factor 3; IRES, Internal ribosome entry site; ISC, Iron-sulfur cluster; IVT, *In vitro* transcribed; lncRNA, Long non-coding RNA; MLS, Microphthalmia with linear skin defects; ORF, Open reading frames; PD, Parkinson's disease; RBPs, RNA-binding proteins; S, Sense; SINE, Short Interspersed Nuclear Element; TE, Transposable Element; TSS, Transcription Start Site; UchL1, Ubiquitin carboxyterminal hydrolase L1; UTR, Un-Translated Region.

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