RNA-BASED THERAPIES

OPINION

Non-coding RNAs as drug targets

Masayuki Matsui and David R. Corey

Abstract | Most of the human genome encodes RNAs that do not code for proteins. These non-coding RNAs (ncRNAs) may affect normal gene expression and disease progression, making them a new class of targets for drug discovery. Because their mechanisms of action are often novel, developing drugs to target ncRNAs will involve equally novel challenges. However, many potential problems may already have been solved during the development of technologies to target mRNA. Here, we discuss the growing field of ncRNA — including microRNA, intronic RNA, repetitive RNA and long non-coding RNA — and assess the potential and challenges in their therapeutic exploitation.

The world of RNA inside mammalian cells has been expanding for decades[1]. Each new discovery adds a new, and often surprising, layer to biological regulation and function. Experience has taught us not to dismiss a novel, unexpected, RNA-mediated activity. RNA species beyond mRNA include intronic RNAs, microRNAs (miRNAs)[2], long non-coding RNAs (lncRNAs)[3], circular RNAs (circRNAs)[4], and extracellular RNAs[5]. Collectively, these are known as non-coding RNAs (ncRNAs), because they lack clear potential to encode proteins or peptides.

Although ncRNAs lack the potential to encode proteins, they can affect the expression of other genes through a variety of mechanisms. In some cases, their mechanisms of action are well known and strategies for controlling their activity are well established. In other cases, their mechanisms are obscure or controversial. For example, the mechanism used by oligonucleotides that bind to miRNAs to block their action and inhibit gene expression is proven[6], whereas the many possible mechanisms for using lncRNAs to manipulate expression are only beginning to be understood[3].

The ability of ncRNAs to control gene expression makes them potential targets for drug development. However, the drug discovery process is never easy. Uncertainty about how ncRNAs function (and even whether they have a function) makes lead identification and development even more challenging.

This article first considers the lessons learned from nucleic acid-based targeting of mRNA — including issues associated with target specificity and toxicity — that are applicable to ncRNA-based drug discovery. We then highlight recent advances involving the targeting of miRNAs, intronic RNA and exon skipping, and repetitive RNAs, before focusing on lncRNAs — a novel and challenging class of potential drug targets.

Targeting mRNA: principles and lessons

Basic principles

mRNA has been experimentally pursued as a potential therapeutic target since the late 1970s (REF. 7). The concept is simple: identify an mRNA involved in disease, use the mRNA sequence to design a complementary oligonucleotide and introduce the oligonucleotide into cells so that, on binding to the mRNA, it disrupts the expression of a protein and the symptoms of the disease are alleviated[8,9].

One advantage of this approach is that the synthesis of complementary oligonucleotides is straightforward. It will almost always be much easier to design an oligonucleotide to bind an mRNA and inhibit protein production, than to identify a small molecule that can inhibit the function of the protein directly. Furthermore, rather than requiring months or years, lead compound identification can usually be accomplished in just a few weeks.

Any RNA can be targeted by complementary base-pairing recognition, and inhibitory oligonucleotides are chemically similar to one another. Oligonucleotides may vary in sequence, but they have similar lengths and similar chemical modifications, which are necessary to improve their stability, efficacy and pharmacokinetic properties[10]. Owing to these similarities, preclinical or clinical experiences with compounds now approved or in the pipeline are likely to have substantial predictive value for future trials of oligonucleotides that target other mRNAs, which will probably reduce the risk relative to programmes that are testing novel drug scaffolds.

Another advantage of synthetic nucleic acids is that they may be used to modulate activity of targets that appear to be 'undruggable' by small molecules[11,12]. Examples include protein–protein interactions, complex proteins for which it might be difficult to block all key functions by a single molecule, or proteins that are so closely related to others that it is impossible to achieve adequate selectivity[11,12].

However, although the therapeutic potential of oligonucleotides has been recognized for more than 40 years, few drugs have received approval. In this regard, Vitravene (fomivirsen; CIBA Vision Corporation/Ionis Pharmaceuticals) was approved in 1998 to treat cytomegalovirus (CMV) retinitis (now discontinued), Macugen (pegaptanib sodium injection; Pfizer/Valeant Pharmaceuticals) received approval in 2004 to treat macular degeneration and Kynamro (mipomersen sodium; Kastle Therapeutics/Ionis Pharmaceuticals) was approved in 2013 to treat familial hypercholesterolemia. Vitravene and Kynamro are antisense oligonucleotides (ASOs) that target mRNA, and Macugen is an aptamer that binds to vascular endothelial growth factor (VEGF). Vitravene and Macugen are delivered by intraocular administration, whereas Kynamro is delivered systemically.

This small number of approved agents reflects the obstacles that have been faced when attempting to target mRNA using synthetic oligonucleotides. Most drugs are hydrophobic small molecules, under 500 Da in molecular weight with fewer than five

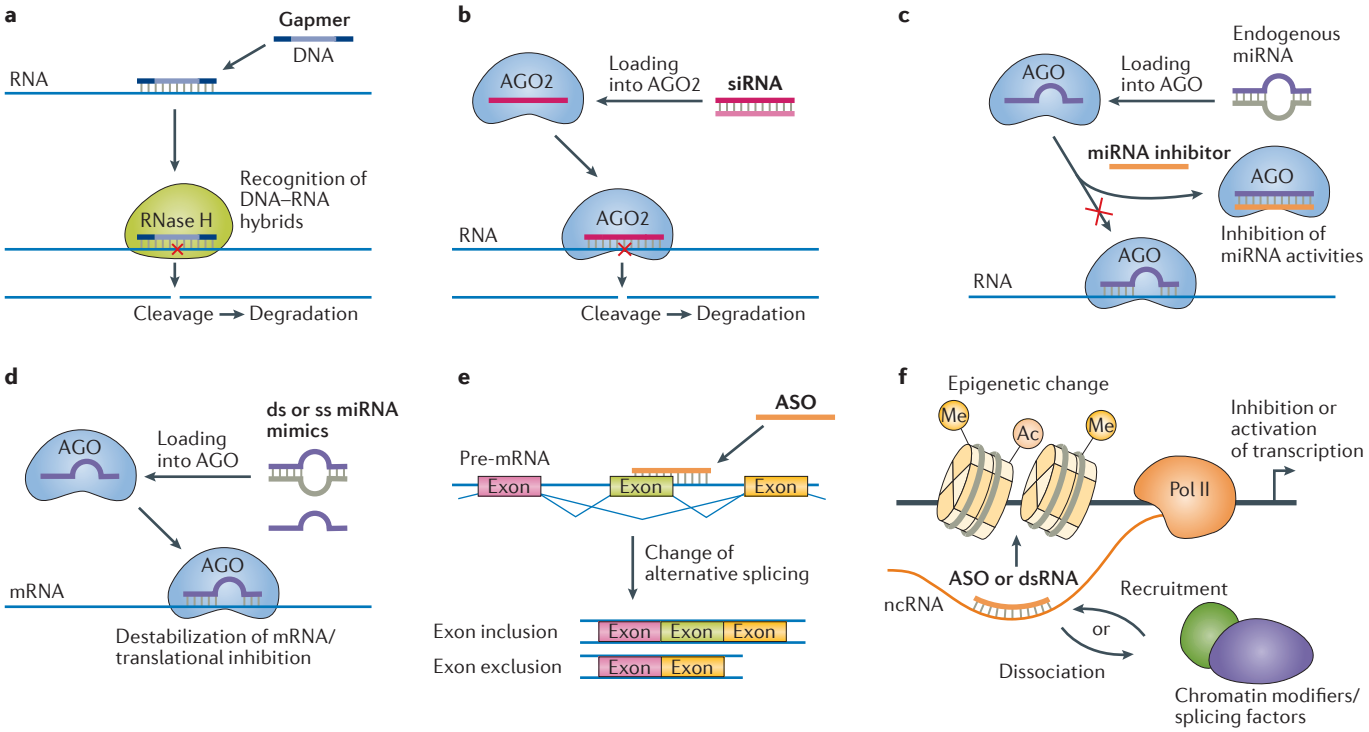

Figure 1 | **Regulating RNA levels or splicing with ASOs and duplex RNAs. a** | Reduction of cellular RNAs by antisense oligonucleotide (ASO) gapmers. RNase H recognizes the DNA–RNA duplex and cleaves the target. **b** | Reduction of cellular RNAs by small interfering RNAs (siRNAs). One strand (guide strand) is loaded into argonaute 2 (AGO2) and an active RNA-induced silencing complex (RISC) is formed. The complex binds to the complementary sequence on a target RNA and cleaves it. **c** | MicroRNA (miRNA) inhibitors complementary to specific endogenous miRNAs bind to specific miRNAs and inactivate them. **d** | Gene expression regulation by miRNA mimics. Double-stranded (ds) or chemically modified single-stranded (ss) miRNA mimics reduce target gene expression by destabilizing target RNAs and/or inhibiting translation. **e** | Regulation of alternative splicing by ASOs targeting regions close to intron-exon junctions. **f** | Modulation of transcription and epigenetic status by ASOs or dsRNAs targeting promoter-associated RNAs. Pol II, RNA polymerase II.

hydrogen bond donors or acceptors[13]. By contrast, oligonucleotides have masses in the thousands of daltons, containing many hydrogen bond donors and acceptors, and have multiple negative charges[14]. Unlike antibodies, which represent another class of large and chemically complex therapeutics, oligonucleotides that target cellular RNA must enter cells to be active.

Because oligonucleotides are so unlike traditional small-molecule drugs, three decades of intense effort have been invested in developing them as plausible therapeutic candidates. Landmark achievements in the therapeutic development of oligonucleotides have included: the ability to synthesize kilogram quantities of oligonucleotide at costs that are compatible with clinical application[15]; the demonstration that chemical modifications can reduce off-target effects (see below), lower toxicity and improve pharmacokinetic properties[14,16]; the demonstration that synthetic nucleic acids can be administered systemically and knock down their intended targets *in vivo*[14,16]; and the US Food and Drug Administration (FDA) approval of two synthetic oligonucleotides designed to target mRNA and numerous ongoing trials[17–19].

Despite these achievements, it is not yet clear whether synthetic nucleic acids can successfully compete with small-molecule drugs and follow the path of therapeutic antibodies to form a large class of widely used compounds. Given the number and relative sophistication of current clinical trials that use nucleic acids, a much better understanding of the potential of agents that target mRNA is likely within the next five years.

Notably, small molecules have also been proposed to target disease-causing nucleic acid targets and modulate their function[20]. The advantage of this approach is that it would apply the advantages of small molecules, such as greater potential for low-cost synthesis, better membrane permeability and better oral uptake to DNA and RNA 'receptors'. In practice, owing to the similarities between nucleic acids present in cells, it is difficult to identify small molecules that can both potently bind nucleic acid targets and be selective for a disease target.

Classes of lead compounds

The most common strategies for controlling mRNA expression use ASOs and duplex RNAs (FIG. 1). A typical contemporary design for an ASO consists of a central DNA 'gap' region flanked by chemically modified nucleotides that boost binding to a complementary target and increase nuclease resistance[10,21] (FIG. 1a). These 'gapmer' ASOs form a DNA–RNA hybrid with target mRNA that recruits RNase H and promotes degradation of mRNA.

A second design for ASOs has more-uniform chemical modifications and lacks a central DNA gap. These 'steric block' ASOs function by binding to target RNA sequences and blocking access of key proteins. The most common use of steric block ASOs is to obstruct binding of regulatory proteins near critical splice junctions to redirect alternative splicing[10,21].

Duplex RNAs, by contrast, function through RNA interference (RNAi)[22] (FIG. 1b). Because RNAi is a natural mechanism for gene silencing, synthetic RNA duplexes benefit from interactions with proteins that

have been optimized through evolution to promote recognition of complementary RNA. The potential for efficient recognition and silencing to be achieved in patients drives the development of duplex RNAs as therapeutics capable of competing with gapmer ASOs.

It is currently unclear whether duplex RNAs or ASOs will prove superior in the clinic. The relative value of the approaches will differ depending on the nature of the target. However, experience using both approaches provides a basis for the discovery of agents targeting ncRNAs.

Lessons learned from targeting mRNA

By the mid-1990s, it became clear that many published descriptions of the use of ASOs inside cells were unconvincing or inaccurate[23–25]. Similar concerns have been expressed for studies involving duplex RNAs[26]. A key challenge in targeting mRNA is that duplex RNAs and ASOs, like any exogenous molecule, have the potential to produce off-target effects when added to cells.

Several factors have combined to make off-target effects a recurring problem for research using nucleic acids to modulate gene expression (BOX 1). Owing to the simplicity of the concept, researchers have often assumed that any effect observed is due to recognition of the expected target, especially when the effect fits the hypothesis. Duplex RNAs or ASOs are complex molecules that can cause off-target effects through multiple mechanisms[27,28] (BOXES 1,2). They are often delivered into cultured cells using cationic lipid[29], which, although an excellent delivery tool, can compound the observation of off-target effects. Nucleic acids can also exert effects that are sequence dependent but independent of interactions with the intended RNA target.

How can an 'on-target' mechanism be established? Fortunately, it is relatively simple to build a strong case for an on-target mechanism if diligent control experiments are designed and performed[30,31]. When screening for ASOs or duplex RNAs, the goal is to identify at least two or three potent compounds that are capable of knocking down gene expression and that are complementary to the target RNA. By identifying multiple compounds that share complementarity to the target and produce a common phenotype, the possibility that the phenotype is due to nonspecific binding of the compounds to unintended targets is reduced. A nucleotide BLAST (Basic Local Alignment Search Tool), using databases of genomic DNA and transcript sequences, would help to remove candidates having significant potential of off-target interactions.

After identifying these lead compounds, one then designs multiple controls that are either 'scrambled' (groups of bases swapped within the control compounds relative to the active parent compound) or 'mismatched' (mismatched bases introduced relative to target, for example within a putative seed sequence of a duplex RNA). It is important to include control compounds that maintain groups of bases in the same order, in case small motifs are responsible for phenotypes independent of the overall complementarity to the target. For example, the CpG dinucleotide has the potential to exert potent immunostimulatory effects independent of the surrounding sequence or intended target[32].

Experiments using positive or negative control nucleic acids are relatively rapid and inexpensive, and they involve lipid-mediated transfection of nucleic acids into cultured cells[29]. The concentration of nucleic acid needed to produce an effect depends on the identity of the target and the type of cell culture dish used. For example, an experiment in a 96-well plate will produce different values relative to experiments performed in 6-well plates because the overall amounts of nucleic acid used per cell are likely to differ. Cell growth and distributions sometimes change depending on plate formats, affecting transfection efficiency. Experimental conditions affect relative efficiencies; therefore, comparisons become problematic when experiments are not run in parallel using similar protocols.

The combination of lipid and ASO or duplex RNA will invariably cause toxicity and cell death when used at higher concentrations, which can confound experimental interpretation. This problem is especially troublesome when an anti-proliferative phenotype is being examined. 'Gymnotic' delivery — in which the oligonucleotide is delivered directly in saline solution — may minimize toxicity and therefore be useful in studies examining proliferation or cell cycle changes, or analysis that require cells to be repeatedly treated over time[33,34]. However, gymnotic delivery requires 10–50 times as much ASO and cannot be used for delivery of duplex RNA.

Emerging classes of RNA targets

Beyond mRNA, emerging classes of RNA targets include miRNAs, intron–exon junctions, repetitive RNA, and lncRNAs. Each class of RNA performs different endogenous functions, providing a variety of opportunities and challenges for drug discovery.

Box 1 | Sources for off-target effects and strategies to limit them

Irreproducible research and misguided interpretation of results are important roadblocks to biomedical science[130,131]. Irreproducible research is especially troubling for gene silencing because nucleic acid-based drugs can provoke off-target effects by multiple mechanisms:

Protein binding. Nucleic acids can bind to proteins on the surface of cells or inside cells. Such binding can cause many changes, including induction of the interferon response. This outcome is so potent that oligonucleotides designed to provoke the immune response have been developed as antiproliferatives and vaccine adjuvants[32].

Partial complementarity to unintended targets. Even if designed to be fully complementary to only their intended target, nucleic acids will also have partial complementarity to many other targets. The potential magnitude of this problem is emphasized by the realization that as low as seven-base complementarity (seed match) between a microRNA and its target is adequate for mediating expression.

Toxicity. Any synthetic organic molecule has the potential to affect cell proliferation if the concentration is high enough. In our investigations, with synthetic nucleic acids in cultured cells delivered by cationic lipid, the window for efficacy was between 1–50 nM for cells cultured in six-well dishes (efficacy varies as media volume to well surface area changes).

There are several important steps that should be used to promote research reliability and limit off-target effects, including:

- Identify alternative hypotheses to explain interesting results and vigorously test them
- Identify multiple active antisense oligonucleotides (ASOs) or duplex RNAs
- Test multiple negative controls (scrambled or mismatched oligomers)
- Determine the copy number of the RNA target per cell
- Validate key conclusions using multiple independent experimental approaches
- Perform key experiments multiple times in independent trials
- Investigate the mechanism sufficiently to establish a working hypothesis to explain activity

Box 2 | **Genasense — a lesson in why the mechanism matters**

The development of Genasense (oblimersen sodium; Genta Inc.)[132], a 'first-generation' antisense oligonucleotide composed of phosphorothioate DNA (PS-DNA) that targets the mRNA encoding B cell lymphoma 2 (BCL-2), provides an instructive example of the need to understand the mechanism of action of nucleic acid-based drugs. BCL-2 is overexpressed in some cancer cells, and blocking its expression was thought to make cells more susceptible to treatment with established anticancer agents and apoptosis[133,134].

PS-DNA lacks the affinity for target sequences that characterizes 'second-generation' gapmer designs, which contain various ribose modifications on the 3′ and 5′ flanks in addition to PS linkages[10]. In retrospect, initial experiments involving Genasense showed no more than modest potential. Nevertheless, Genasense moved through clinical trials that went on for more than a decade. In spite of promising initial clinical results, repeated phase III trials failed to show sufficient benefit[135,136].

While these trials went on, the Stein laboratory[136–138] showed that the pro-apoptotic effects of Genasense were independent of its sequence. Inhibiting BCL-2 expression with duplex RNA that act through RNA interference (RNAi) did not induce apoptosis, further suggesting that observed anti-proliferative effects could not be due to recognition and silencing of the intended BCL-2 target. Indeed, oligonucleotides that contain phosphorothioate modifications are known to be susceptible to off-target effects[139] and interactions with unpredicted targets therefore probably explains the effects of Genasense.

Up to one billion dollars was spent on the development of Genasense, before bankruptcy ended trials in 2012. Had mechanism been thoroughly investigated early in the drug discovery process, it is likely that these trials would never have been initiated, patients would have been spared a treatment that yielded no clear benefits, and resources would have been directed towards more fruitful efforts.

During the years of Genasense's development, and sometimes promising clinical trial results, the question was raised regarding whether understanding the mechanism of action mattered[140,141]. A patient may not care about the mechanism of a successful drug, but if investigators know the mechanism of a drug development candidate, it will make it much more likely that the drug will be successful and applied in a manner most likely to be beneficial.

When considering emerging classes of RNA, it is important not to be dogmatic with regard to terminology, as this may limit data interpretation and mechanistic understanding. For example, an intronic RNA may also contain a repetitive RNA target and therefore qualify for inclusion in both categories. Furthermore, the term lncRNA itself can be problematic, as some of these ncRNAs may encode miRNAs or peptides.

The experience that has been gained by using oligonucleotides to target mRNA has important implications for targeting other RNA species. Indeed, designed nucleic acids may bind to any RNA species to modulate activity. Lessons learned regarding control oligomers and phenotype interpretation may therefore be applicable for emerging classes of RNA.

Targeting ncRNA has the potential to offer novel therapeutic opportunities. ASOs and duplex RNAs that target mRNA act by reducing gene expression. However, targeting ncRNA may provide the opportunity to therapeutically activate gene expression. In addition, as miRNAs typically regulate many genes, blocking a single miRNA might enable the manipulation of a signalling pathway at multiple points.

miRNA

miRNAs form a major class of functional ncRNAs[35,36], and their potential to be therapeutically targeted has been well reviewed elsewhere[2,37]. Oligonucleotides that are complementary to an miRNA can block its activity, whereas duplex or chemically modified single-stranded RNAs that mimic an miRNA can trigger enhanced activity[2,6] (FIG. 1c,d).

Both miRNA inhibitors and mimics are currently being developed against a variety of targets[38–41] and tested in clinical trials (TABLE 1). Examples include: mimics of miRNA-34 (miR-34) that are designed to repress oncogene expression and block tumour growth[39]; single-stranded oligonucleotides complementary to miR-122 that are being developed to treat Hepatitis C virus[40] and single-stranded oligonucleotides complementary to miR-21 being applied to treat Alport nephropathy, a chronic kidney disease[41].

Intronic RNA

Approximately 30% of the genome encodes intronic RNA, making it one of the most prevalent species of ncRNA[42]. Because steric-blocking ASOs can be designed to be complementary to key regulatory sequences, they have the potential to redirect alternative splicing, leading to the biosynthesis of different protein isoforms (FIG. 1e). The potential to 'switch' from one splice variant to another suggests that targeting key splicing control sequences within introns or exons can offer opportunities for therapeutic development[43–45]. As compounds targeting exons or introns can both affect splicing, both will be considered here. Kole *et al.*[46] first noted oligonucleotide-directed alternative splicing in 1993, using an ASO complementary to intronic RNA within β-globin RNA, that corrected splice defects found in β-thalassemia in a cell-free system.

Typically, the mechanism of splice modulation involves blocking the binding site for splice factors[45]. One alternative mechanism and an example of an unexpected pathway for oligonucleotide-directed splice modulation involves recruitment of the interleukin enhancer-binding factor 2 (ILF2)–ILF3 complex to target sites through interaction with ASOs containing 2′-fluoro modifications[47].

Perhaps because of the relative simplicity of the predominant mechanism for splice correction, substantial progress has been made in both preclinical and clinical studies[47–57] (TABLE 1). Successful splice correction has now been achieved for dystrophin (encoded by *DMD*; associated with Duchenne muscular dystrophy)[50–52,55,56], harmonin (*USH1C*; Usher syndrome)[53], survival motor neuron protein 2 (*SMN2*; spinal muscular atrophy (SMA))[54] among others. Most clinical attention has focused on drisapersen[50–52] and eteplirsen[56] for treating Duchenne muscular dystrophy and nusinersen[57] for spinal muscular atrophy.

Case study: dystrophin. Clinical studies of two compounds — drisapersen (BioMarin Pharmaceutical) and eteplirsen (Sarepta Therapeutics) — have been carried out to investigate the potential for dystrophin restoration. Drisapersen is a 2′-*O*-methyl phosphorothioate oligonucleotide, and eteplirsen is a phosphorodiamidate morpholino oligomer. Both compounds are designed to target a sequence within exon 51, induce exon skipping and generate a shorter mRNA that will produce a partially active dystrophin. This 'exon-skipped' dystrophin is produced by patients with Becker's muscular dystrophy, a condition with a better prognosis than Duchenne muscular dystrophy[43].

As of early 2016, phase III clinical trials had not demonstrated drisapersen to be sufficiently effective to win FDA approval[58].

Table 1 | **Representative oligonucleotide-based drugs targeting miRNAs, repetitive RNAs and pre-mRNAs currently in clinical trials**

Company	Drug name	Chemistry	Mechanism of action	Target	Disease	Clinical status*	Refs
Mirna	MRX-34	dsRNA (liposomal nanoparticle formulation)	miRNA mimic	miR-34 targets	Solid tumours and haematological malignancies	Phase I	39,142
Regulus	RG-101	GalNAc-conjugated	miRNA inhibitor	miR-122	HCV	Phase II	143,144
	RG-012	NA	NA	miR-21	Alport syndrome	Phase I	145
	RG-125 (AZD4076)	GalNAc-conjugated	miRNA inhibitor	miR-103/107	Nonalcoholic steatohepatitis	Phase I	146
Roche	Miravirsen (SPC3649)	LNA	miRNA inhibitor	miR-122	HCV	Phase II	147–150
miRagen	MRG-201	NA	miRNA mimic	miR-29b targets	Cutaneous and pulmonary fibrosis	Phase I	–
	MRG-106	LNA	miRNA inhibitor	miR-155	Haematological malignancies	Phase I	–
BioMarin	Drisapersen (also known as GSK-2402968 or PRO051)	2′-O-methyl phosphorothioate	Exon skipping	Exon 51 of dystrophin pre-mRNA	DMD	Phase III (completed)	51,52,58
	BMN 044 (also known as PRO044)	2′-O-methyl phosphorothioate	Exon skipping	Exon 44 of dystrophin pre-mRNA	DMD	Phase II (discontinued)	151
	BMN 045 (also known as PRO045)	2′-O-methyl phosphorothioate	Exon skipping	Exon 45 of dystrophin pre-mRNA	DMD	Phase IIb (discontinued)	151
	BMN 053 (also known as PRO053)	2′-O-methyl phosphorothioate	Exon skipping	Exon 53 of dystrophin pre-mRNA	DMD	Phase I/II (discontinued)	151
Sarepta	Eteplirsen (also known as AVI-4658)	PMO	Exon skipping	Exon 51 of dystrophin pre-mRNA	DMD	Phase III	56
	SRP-4053	PMO	Exon skipping	Exon 53 of dystrophin pre-mRNA	DMD	Phase I/II	–
	SRP-4045	PMO	Exon skipping	Exon 45 of dystrophin pre-mRNA	DMD	Phase I	–
Ionis/Biogen	Nusinersen (also known as IONIS-SMN$_{Rx}$)	2′-O-methoxyethyl and phosphorothioate chemistry	Exon inclusion	Intron 7 of *SMN2* pre-mRNA	SMA	Phase II, Phase III	57,152
	IONIS-DMPK-2.5$_{Rx}$	2′-O-methoxyethyl, cET and phosphorothioate chemistry	Gapmer	Exon 9 of *DMPK* mRNA	DM1	Phase I/IIa	–

cEt, 6′-(S)-CH$_3$ bicyclic nucleoside; DM1, myotonic dystrophy type 1; DMD, Duchenne muscular dystrophy; *DMPK*, myotonin-protein kinase; GalNAc, *N*-acetylgalactosamine; HCV, hepatitis C virus; LNA, locked nucleic acid; NA, not available; PMO, phosphorodiamidate morpholino oligomer; SMA, spinal muscular atrophy; *SMN2*, survival of motor neuron 2, centromeric. *Clinical status presented in this table is based on the information from company websites (Biogen, BioMarin Pharmaceutical, Ionis Pharmaceuticals, miRagen Therapeutics, Mirna Therapeutics, Regulus Therapeutics, Roche and Sarepta Therapeutics), ClinicalTrials.gov or the Adis Insight database (see Further information).

It is now suspected that an increased and wider distribution of the drug will be required for better efficacy[59]. As the dose of drisapersen was limited to 6 mg per kg because of its relatively high kidney toxicity, the use of modified oligonucleotide chemistries may improve the therapeutic window. Preclinical and clinical studies have suggested that another exon-skipping morpholino-based ASO, eteplirsen, may have safer biological properties for *in vivo* application, enabling the application of much higher doses (30–50 mg per kg) in the clinical trials[56].

Case study: USH1C. Usher syndrome is a hereditary hearing impairment with a prevalence as high as 1 in 6,000 (REF. 60). Some cases of type I Usher syndrome (USH1) are caused by mutations in *USH1C*, which encodes harmonin. For the Acadian population, all cases involve a mutation that creates a cryptic splice site that is used in place of the normal location[61]. Treatment of mice with an ASO capable of blocking the cryptic splice site increased expression of wild-type protein and partially restored hearing[53]. Although there have been no efforts so far to develop this approach in a clinical setting, this finding demonstrates its therapeutic potential and shows that it warrants further investigation.

Glossary

Antisense oligonucleotides
(ASOs). Synthetic single-stranded oligonucleotides that are designed to bind to complementary cellular RNA sequences by Watson–Crick base pairing.

Droplet digital PCR
(ddPCR). A PCR technology that uses nanolitre-sized oil–water emulsion droplets as PCR reaction vessels and quantifies concentrations of target DNA templates based on counting the number of PCR-positive droplets using a flow cytometer.

Long non-coding RNAs
(lncRNAs). Relatively long (>200 nucleotides) non-coding RNA transcripts.

MicroRNAs
(miRNAs). Small (~22 nucleotides) non-coding transcripts that are generally thought to silence gene translation through RNA interference.

Non-coding RNAs
(ncRNAs). RNA transcripts that do not code for protein.

Off-target effect
Phenotypic effects in a cell or animal that occur upon addition of a synthetic compound and that are not caused by interactions with the intended cellular target.

Case study: SMN2. SMA is an autosomal recessive disorder caused by a loss of function mutation within *SMN1* (REF. 62). Humans possess a paralogous gene, *SMN2*, the splicing of which differs slightly from *SMN1*, resulting in more rapid degradation that prevents it from substituting for defective *SMN1*. Nusinersen (Ionis Pharmaceuticals/Biogen), a 2′-*O*-methoxyethyl modified phosphorothioate ASO that targets intron 7 within *SMN2* pre-mRNA, restored splicing and facilitated production of full-length SMN2 in mouse models of SMA[54].

Phase I clinical trials of nusinersen have been completed in patients with type II and type III SMA. Patients ranged in age from 2 to 14 years and were treated with a single intrathecal injection of nusinersen at a dose of between 1 and 9 mg per kg[57]. The drug was well tolerated in all patients and significant improvements in motor function were observed at the highest dose 1 month after administration.

Recently released results from a phase II trial involving 15 infants with type I SMA reported no new adverse events and achievement of developmental milestones such as unsupported sitting in 8 subjects, standing with or without support in 5 subjects, and walking in 2 subjects[63]. Such milestones are rarely achieved by SMA patients; therefore, this interim data is encouraging. Further trials are ongoing and are likely to have important implications for targeting splice junctions and for neurological applications of ASOs in general.

Repetitive RNA

More than 40 neurological diseases are caused by an expansion of repetitive sequences within DNA[64,65]. These expanded repeats, and nearby sequences if they are within introns, are attractive targets for ASOs and duplex RNAs[66–68]. In contrast to single nucleotide polymorphisms, these mutations are shared by all patients; therefore, any drug would be applicable to an entire patient population.

Because the repeat mutation causes the RNA to differ from wild-type, it is possible to achieve allele-selective inhibition of mutant expression if preservation of wild-type expression is desired[68]. For some diseases, the expanded repeats are within exons that lead to production of toxic proteins (for example, Huntington disease and Machado–Joseph disease)[64,65]. Expanded repeats within introns, 3′ untranslated regions (UTRs) or 5′ UTRs can produce toxic mutant RNAs (for example, as seen in myotonic dystrophy) or affect the production of proteins (for example, as seen in Friedreich ataxia and Fragile X syndrome)[64,65].

Case study: myotonic dystrophy. Myotonic dystrophy type 1 (DM1), the most common form of muscular dystrophy, is caused by expansion of a CUG repeat within the 3′ UTR region of the myotonin-protein kinase gene (*DMPK*)[69]. This expanded repeat binds to muscleblind-like protein 1 (MBNL1), a splicing regulator, and perturbs alternative splicing. The expanded repeat also causes aberrant activation of protein kinase C (PKC), contributing to hyperphosphorylation of CUG triplet repeat RNA-binding protein 1 (CUGBP1, also known as CELF1). The hyperphosphorylation increases the half-life of CUGBP1. The resultant increase in CUGBP1 levels has pathogenic roles in DM1, disrupting alternative splicing and translation[70].

For therapy of DM1, two approaches targeting different regions of the *DMPK* transcript have been tested so far (FIG. 2a). One approach is to use a CUG repeat-targeting ASO. *In vivo*, non-RNase H-dependent 2′-*O*-methyl phosphorothioate oligonucleotides[71] and morpholino oligomers[72] complementary to the CUG repeat successfully released MBNL1 from the repeat and mitigated RNA toxicity of the repeat RNAs *in vivo*.

Another therapeutic approach is to use a gapmer ASO targeting outside of the CUG repeats to silence nuclear DMPK transcripts[73,74]. Systemic administration of a 2′,4′-constrained ethyl (cEt)-modified gapmer (named ISIS 486178) induced rapid knockdown of CUG-repeat RNAs and corrected pathogenic features of the disease[73]. A phase I/II clinical study for a similar gapmer ASO containing cEt and 2′-*O*-methoxyethyl groups (IONIS-DMPK-2.5Rx, previously known as ISIS 598769) was initiated in 2014 to evaluate its safety and tolerability[75].

Case study: Friedreich ataxia. Friedreich ataxia is caused by expansion of a GAA repeat within intronic RNA of the frataxin gene (*FXN*)[76]. Normal frataxin protein is made by cells, but transcription is reduced and the level of protein is lowered. Evidence suggests that the mechanism of action involves binding of the expanded intronic repeat RNA directly to chromosomal DNA through R-loop formation, halting transcription by inducing suppressive histone modifications[77].

Our laboratory has targeted steric-block locked nucleic acid (LNA) ASOs and duplex RNAs to the expanded GAA repeat in cells derived from patients with Friedreich ataxia[78] (FIG. 2b). These compounds reduce R-loop formation and increase frataxin mRNA and protein levels to those found in wild-type cells. The *FXN* locus is a good example of the unexpected mechanisms used by repeat RNA and the ability of designed duplex RNAs and ASOs to manipulate mechanisms and alter gene expression towards a therapeutically valuable end point.

Repeat-targeting double-stranded RNAs (dsRNAs) or ASOs may have some advantages when allele-specific gene silencing or activation is required to avoid adverse effects that might be caused by knocking down gene expression from normal alleles. However, targeting simple repeat-sequence motifs increases the potential risk of off-target effects. Using gapmer ASOs that target sequences within the mutant gene that are outside of the expanded repeat may be more effective for diseases such as DM1 that are caused by nuclear RNA gain-of-function toxicity[74].

lncRNA

lncRNAs are a diverse group of transcripts whose natural functions and potential as drug targets remain largely undefined. These RNA species are greater than 200 nucleotides in length and do not encode protein. Transcriptome analysis over the past decade has identified many lncRNAs[79–82] that fall into various subclasses depending on length, association with protein-encoding genes, sense or antisense orientation relative to protein-encoding genes, association with repeats, and other criteria[3,83,84]. Some lncRNAs are encoded between genes and are known as long intergenic ncRNAs (lincRNAs)[85]. Intergenic ncRNAs that are greater than 50 kb in length are known as very long intergenic ncRNAs (vlincRNAs)[86].

Although transcriptome studies have revealed that approximately 80% of the genome is transcribed into RNA[79–82], it remains unclear how many lncRNAs are functional, how many are expressed at substantial levels inside cells, or even how to define how much RNA is necessary to achieve a biological effect[87–89]. It is likely that many lncRNAs have no function, complicating predictions and analysis. For lncRNAs that might play a part in controlling gene expression, mechanisms will often be unknown and require a substantial effort to establish.

The first step towards understanding the existence or potential function of a lncRNA is to examine databases such as FANTOM5, ENCODE, and NONCODE. However, the information in these databases does not provide quantitative measures of lncRNA expression and is derived from selected cell lines or tissues.

Confirming the existence of an RNA transcript and determining the number of RNA transcripts per cell (using a combination of quantitative reverse transcription PCR (RT-qPCR), 5′ and/or 3′ rapid amplification of complementary DNA ends (RACE) analysis and droplet digital PCR (ddPCR)) is crucial to understanding potential mechanisms and functions[90,91]. A transcript present at a just a few copies per cell would be sufficient to act 'in cis' relative to the locus that encodes it (that is, at the chromosomal locus encoding the lncRNA). A transcript present at dozens, hundreds, or thousands of copies per cell might act 'in trans' (at a distant locus or even in the cytoplasm) because sufficient numbers are available to diffuse to distant sites and exert an effect. A transcript present at much less than one per cell would be less likely to have an important function, although it should not be dismissed.

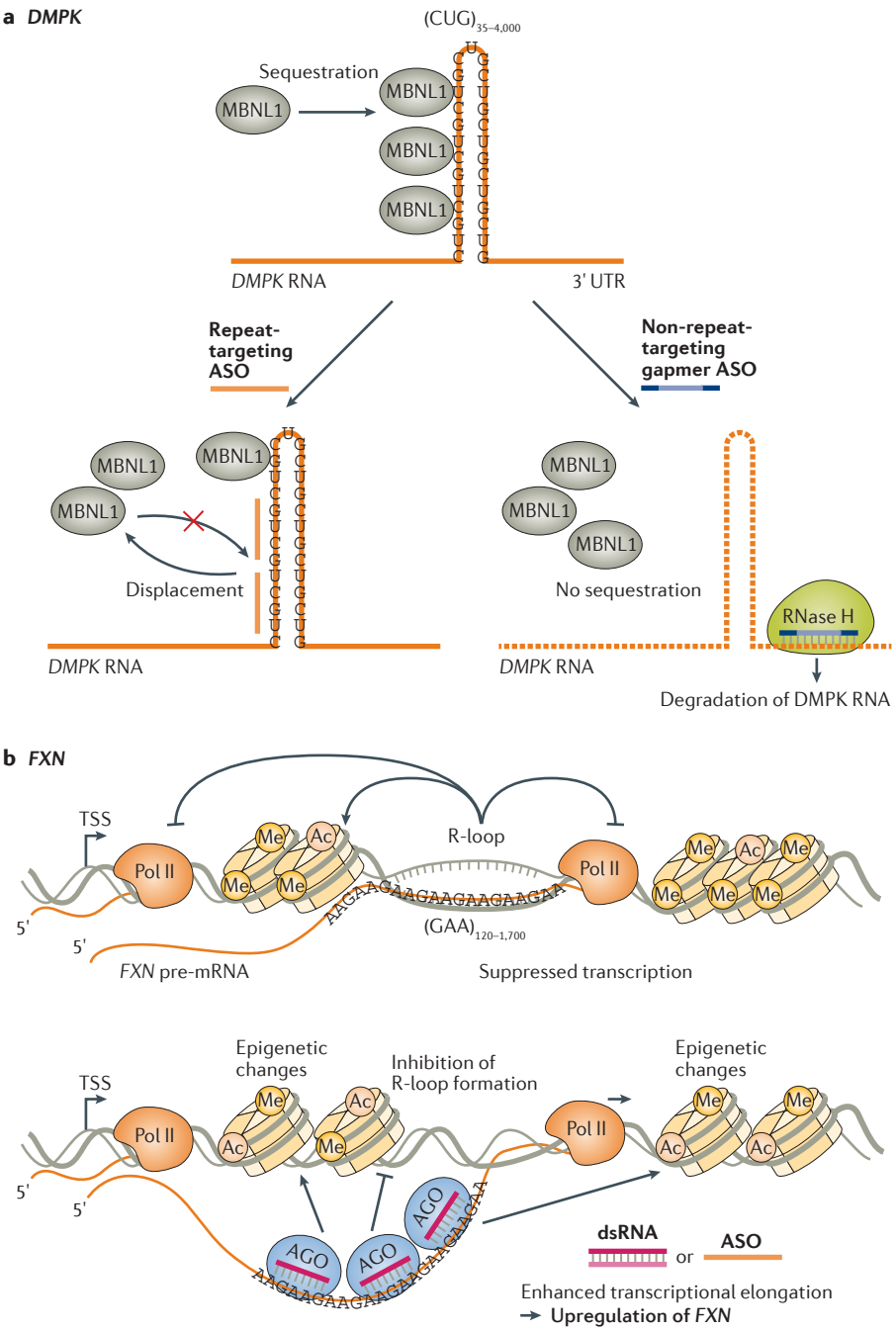

Figure 2 | **ASOs and duplex RNAs targeting repetitive RNAs. a** | Antisense oligonucleotides (ASOs) used for therapy of myotonic dystrophy type 1 (DM1). DM1 is caused by gain-of-function toxicity from CUG-repeat containing myotonin-protein kinase (DMPK) transcripts. ASOs targeting the CUG repeats can be used to prevent binding of muscleblind-like protein 1 (MBNL1) to the repeats. Gapmer ASOs targeting outside the repeat are also used for depleting toxic nuclear DMPK transcripts. **b** | Activating frataxin (FXN) expression using GAA repeat-targeting oligonucleotides. *FXN* is a causative gene for Friedreich ataxia and its expression is suppressed at least partly because of the R-loop formation between the expanded GAA repeats within the intron 1 of FXN pre-mRNA and the genomic DNA in patients (upper panel). Double-stranded RNAs (dsRNAs) or single-stranded locked nucleic acid (LNA) ASOs targeting the GAA repeats can inhibit the R-loop formation in the repeat region and change histone modifications surrounding the repeats, leading to activation of FXN expression (lower panel)[78]. Ac, acyl group; Me, methyl group; Pol II, RNA polymerase II; TSS, transcription start site; UTR, untranslated region. Part **b** was modified from Groh, M., Lufino, M. M. P., Wade-Martins, R. & Gromak, N. R-loops associated with triplet repeat expansions promote gene silencing in Friedreich ataxia and fragile X syndrome. *PLoS Genet.* **10**, e1004318 (2014), with permission from PLOS (http://dx.doi.org/10.1371/journal.pgen.1004318).

lncRNAs can act in either the nucleus or cytoplasm. Therefore, determining the subcellular localization is also important for understanding mechanism and designing strategies for manipulating lncRNA expression and function. This can be done by stringent isolation of nuclei followed by demonstration of purity[92,93]. Fluorescence *in situ* hybridization (FISH) assay is also useful for semi-quantitative evaluation of the subcellular localization of lncRNAs. Localization in the nucleus would be consistent with an effect on transcription or splicing, whereas a predominant localization in the cytoplasm would suggest the possibility of an effect on translation and imply action in trans. Another important step in identifying potential functionality of an ncRNA and its relevance to disease, is to search for potential correlations between lncRNA expression and disease outcomes or symptoms.

Lack of conservation between lncRNA sequences in humans and experimental animal models may complicate development of related therapeutics. The divergence of mRNA sequences is constrained by the need to maintain critical protein functions and structures, making it more likely that some sequences will be shared and that a single ASO or duplex RNA will be effective in more than one species. lncRNAs, by contrast, are expressed from intronic or intergenic regions that are often less conserved between species (for example, human versus mouse or rat). Drug candidates obtained from screening using human cells may not be applicable to *in vivo* evaluation using rodent or other disease models, because the functions of lncRNAs may differ in humans relative to other animals. As a result, it may be necessary to engineer animal models to express the human lncRNA. Engineering the complex interplay of lncRNA and target gene is likely to be challenging and will require a detailed understanding of mechanism.

Gene silencing can be used to investigate the function of a lncRNA, using either ASOs or duplex RNAs. However, the use of gene silencing for lncRNAs is less straightforward than for mRNA because, as indicated above, cellular localization differs among lncRNAs, as does the efficacy of different gene silencing strategies[94]. Duplex RNAs are a reliable approach for targeting mRNAs or lncRNAs in the cytoplasm and inhibiting gene expression. In the nucleus, RNAi factors are present and can promote cleavage of target sequences[92], suggesting that RNAi can also be used to silence nuclear lncRNAs. Recently, it has been shown that ASOs are more reliable gene-silencing agents than duplex RNAs for RNAs that are localized to cell nuclei[94]. Therefore, ASOs may be the silencing method of choice when a target RNA is thought to function in the nucleus, whereas duplex RNAs may be a better choice when a target is thought to function in the cytoplasm.

Finally, as lncRNAs are novel targets with often undefined mechanisms, it is crucial that proper controls be used during gene-silencing studies to ensure that observed phenotypes are due to on-target interactions.

Regulation of gene expression. A central question for drug discovery is whether a lncRNA modulates expression at a specific locus. Linkage of a specific lncRNA to a specific gene suggests that the lncRNA might provide an alternative to small molecule or antibody approaches for controlling the activity of the gene product[95].

Polycomb repressive complex 2 (PRC2) presents a model for how lncRNAs might contribute to the regulation of gene expression[96,97]. PRC2 is a multi-protein complex that includes EZH2, RBBP4, AEBP2, SUZ12 and EED subunits and is involved in modifying chromatin with trimethylation of Lys 27 on histone H3 (H3K27me3) and epigenetic silencing[98]. The non-coding X-inactive specific transcript (*XIST*) has been implicated in PRC2 recruitment during X-chromosome inactivation[99,100], and thousands of other lncRNAs have been reported to bind PRC2 by studies using RNA sequencing (RNA-seq)[101].

The hypothesis that selective binding of PRC2 affects gene silencing has obvious implications for drug development, as the use of ASOs to block the binding of PRC2 to specific lncRNAs by ASOs would be expected to activate gene expression. Supporting this hypothesis, ASOs were reported to be able to displace PRC2 and disrupt interactions between lncRNA and chromatin[102]. The ASOs used in this study were not designed to induce cleavage of the lncRNA and were thought to act by competing with PRC2 for association with the target lncRNA.

To successfully cooperate during gene silencing, PRC2 must be selective for target RNA sequences. However, PRC2 is a promiscuous RNA binding protein that, presumably, would have little preference on its own for one sequence versus another[103]. Similar promiscuous binding has also been shown for FUS protein, another protein factor that had been thought to associate with specific RNAs[104]. A subsequent study reported that different sequences embedded within RNAs of similar lengths could confer differences in FUS binding (three- to eightfold), but also that results could vary depending on experimental conditions and the nature of the RNAs used for comparisons[105]. Although a three- to eightfold difference cannot explain selective recognition by PRC2 acting alone, it remains possible that specificity is achieved through combinatorial binding of several protein factors. These studies of PRC2 are significant because they reveal that the mechanism for lncRNA action, even for a relatively well-studied model target, remains incompletely understood.

Recognition of lncRNAs by small RNAs and the RNAi machinery is another potential mechanism for selective control of gene expression[106,107]. Both miRNAs and RNAi protein factors exist in the nuclei of human cells, providing the components necessary for recognition[92]. RNAi-mediated recognition by synthetic duplex RNAs and miRNAs has been demonstrated to alter transcription through recognition of RNAs complementary to transcripts that overlap gene promoters and mRNA transcription start sites (described below)[108] and splicing through recognition of sequences at intron/exon junctions[109].

As lncRNAs often overlap genes, they might be able to affect expression *in cis* without relying on sequence-specific binding by proteins. In that case, inactivation of a lncRNA by an ASO would be sufficient to affect expression of the nearby gene. LNA gapmers targeting an antisense transcript at the brain-derived neurotrophic factor (*BDNF*), glial cell line-derived neurotrophic factor (*GDNF*), and ephrin type B receptor 2 (*EPHB2*) loci were also reported to lead to increased expression of the parent gene[110].

Examples of lncRNA function and mechanism. The discovery and development of agents that target lncRNAs will benefit from insights connecting expression, mechanism and function. Below, we discuss selected lncRNAs that have been the focus of studies that provide insights into their mechanisms.

Case study: MALAT1. Metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) is a nuclear localized RNA that is upregulated in cancer cells[111]. It is an unusually abundant lncRNA, present at concentrations of a few thousand per cell, which is well above that of essential

house-keeping genes[91,111]. The abundance of *MALAT1* makes it a representative for candidate lncRNAs that might possess activity in trans.

MALAT1-mediated regulation of alternative splicing has been implicated in human HeLa cells[112]. RNA immunoprecipitation revealed an interaction of *MALAT1* with the serine/arginine-rich family of nuclear phosphoproteins (so-called SR proteins) that have been well characterized as alternative-splicing regulators. Depletion of *MALAT1* using ASOs increased cellular levels of SR proteins and changed the ratio and the cellular distribution of phosphorylated and dephosphorylated SR proteins, leading to a change in alternative splicing of pre-mRNAs.

However, another study investigating the involvement of *MALAT1* in splicing regulation, using *MALAT1* knockout mice or cultured cells, found that elimination of *MALAT1* does not significantly change global pre-mRNA splicing and phosphorylation status of SR proteins[113]. Other groups have also reported that knockout of *MALAT1* in mice does not lead to obvious defects in development, fertility or normal adult functions[114,115]. These inconsistent observations between different groups emphasize the unsettled state of insight into the physiological functions of *MALAT1* for splicing regulation.

In contrast to the lack of effects on normal development, depletion of *MALAT1* by genetic knockout or treatment with ASOs can reduce metastasis in mouse mammary or lung carcinoma models[116,117]. Reduced cancer metastasis by siRNA-mediated knockdown of *MALAT1* has also been shown in human bladder cancer cells[118]. The molecular mechanism proposed in this study is that *MALAT1* functions as a scaffold for recruiting SUZ12 (one of the PRC2 components) and mediates downregulation of the E-cadherin gene (*CDH1*). Depletion of *MALAT1* decreases H3K27me3 levels at the E-cadherin promoter, leading to upregulation of E-cadherin and concurrent downregulation of N-cadherin and fibronectin as epithelial–mesenchymal transition (EMT) markers. The mechanism by which *MALAT1* can affect the expression of other genes remains uncertain. Studies have suggested that *MALAT1* may bind to active chromatin directly or to nascent RNAs[119,120], although the molecular interactions that might mediate such binding remain unknown.

Although *MALAT1* is highly abundant and well studied, fundamental gaps remain in our knowledge of its mechanism and function[121]. ASOs targeting *MALAT1* have been shown to reduce tumour growth and metastasis, but converting these observations into a viable drug development programme will require reliable methods for delivering ASOs to tumour cells and be facilitated by a better understanding of the role of *MALAT1* in normal human cells and tumours.

Case study: Angelman syndrome antisense transcript. Angelman syndrome is a severe neurodevelopmental disorder with a prevalence of 1 in 10,000–20,000 individuals and no known curative treatments[122]. Angelman syndrome is caused by a defect affecting expression of the maternal gene *UBE3A*, which encodes an E3 ubiquitin protein ligase. An antisense transcript is expressed at the paternal *UBE3A* locus and represses gene expression of that allele[123–125] (FIG. 3a), raising the possibility that inactivating the non-coding transcript might increase transcription of the paternal allele to compensate for the lack of maternal expression.

Reduction of murine *Ube3a* antisense transcript (*Ube3a-ATS*) levels was achieved in mice using phosphorothioate 2′-methoxyethyl gapmer ASOs[126]. Reducing transcript levels using two different ASOs led to increased expression of the paternal allele of *Ube3a*.

Although the antisense transcript that overlaps the *Ube3a* locus also encodes the genes *Snrpn*, *Snord115* and *Snord116*, the expression of these genes was not affected[126]. This was thought to be due to the fact that, because the genes are far upstream of the target site for the ASOs, they are fully spliced before transcription reaches the ASO target site. This complex interplay between coding RNA and ncRNA is a good example of why understanding mechanisms in detail is necessary for informed interpretation of results. Administration of the ASOs to animals partially reversed some cognitive defects in a mouse model[126].

Case study: targeting transcripts that overlap gene promoters. Cyclooxygenase 2 (COX2, encoded by *PTGS2*) catalyses the conversion of arachidonic acid into prostaglandins and is a critical regulator of gene expression. *PTGS2* is controlled by a TATA box, and almost all transcription of this gene begins at a single +1 transcription start site.

The *PTGS2* locus encodes more than mRNA. There are additional transcripts that overlap the promoter in both the sense and antisense directions[108] (FIG. 3b). miR-589 has two adjacent binding sites in the *PTGS2* promoter transcripts and acts to upregulate COX2 expression by eightfold[108]. Designed duplex RNAs that are fully complementary to the promoter transcript enhanced COX2 expression by up to twenty-fold. A gapmer targeting the promoter transcript reduced levels of the transcript and COX2 expression in A549 lung cancer cells, which is consistent with the hypothesis that the promoter RNA contains a binding site for an activating miRNA.

As little as one mismatch within the seed sequence of the transcript was sufficient to disrupt activation by the promoter-targeted dsRNAs[108]. Activation of COX2 expression was dependent on expression of the RNAi factors argonaute 2 (AGO2) and trinucleotide repeat-containing gene 6A protein (TNRC6A) and also required WD repeat-containing protein 5 (WDR5), a protein that stimulates histone methyltransferase activity. While AGO2 expression was required, cleavage of the promoter transcript by AGO2 was not necessary. Activity in the absence of cleavage suggests that the promoter transcripts act as a scaffold for assembly of factors near the promoter, rather than a trigger whose cleavage provokes a change in gene expression.

The gene adjacent to *PTGS2* is phospholipase A2 group IVA (*PLA2G4A*, which encodes cytosolic phospholipase A2 (cPLA2)). cPLA2 produces the substrate for COX2 enzyme, so the genes are both spatially and functionally linked. Analysis of *PLA2G4A* expression revealed that duplex RNAs targeting the *PTGS2* promoter also increased *PLA2G4A* expression[108]. As with *PTGS2*, activation of *PLA2G4A* was dependent of expression of AGO2, TNRC6A and WDR5. Analysis by chromosome conformation capture (known as '3C') showed that the promoters for *PTGS2* and *PLA2G4A* are physically linked, providing a means for RNA recognition to control two genes that are separated by almost 150,000 bases. For drug development, promoter-targeted duplex RNAs might be an effective strategy for upregulating gene expression when additional amounts of a therapeutic protein are desired.

Case study: NORAD, an ncRNA that sequesters PUMILIO proteins. The lncRNA *NORAD* was identified as being upregulated in response to DNA damage in human cells[127]. In contrast to *PTGS2* promoter RNA, but similar to *MALAT1*, *NORAD* is well expressed and present at up to 1,000 copies per cell. Genetic inactivation

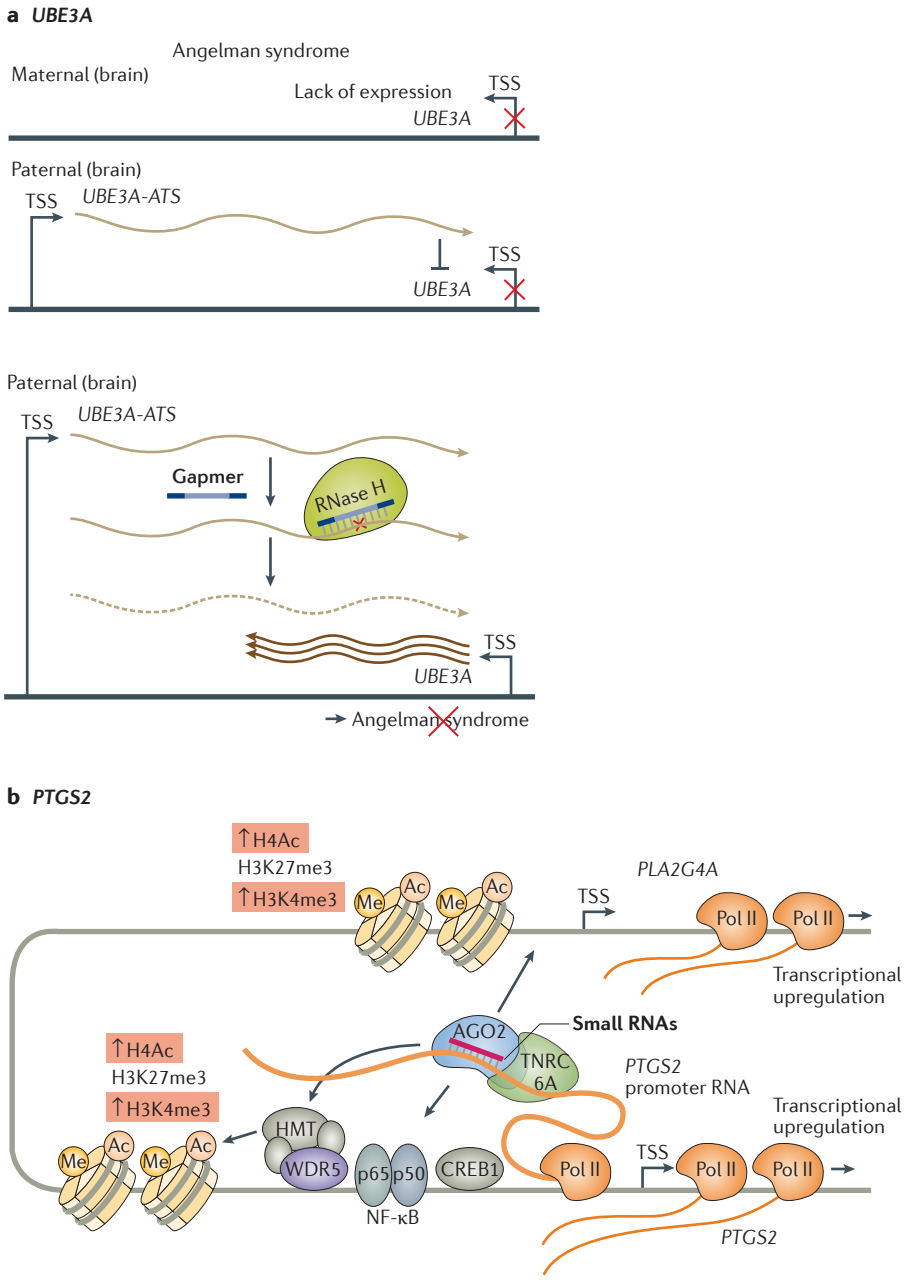

Figure 3 | **Modulating gene expression by targeting cis-acting non-coding RNAs or promoter RNAs. a** | Targeting the *UBE3A* antisense transcript (*UBE3A-ATS*) non-coding RNAs (ncRNAs) using antisense oligonucleotides (ASOs). *UBE3A* encodes an E3 ubiquitin ligase and is the imprinted gene. In the brain, paternal *UBE3A* is silenced by *UBE3A-ATS*, and maternal deficiency of *UBE3A* causes Angelman syndrome. Depletion of nuclear *UBE3A-ATS* using specific gapmers activates paternal *UBE3A* expression[126]. **b** | Regulation of *PTGS2* (the gene encoding cyclooxygenase 2 (COX2)) expression is mediated by promoter RNAs that are produced upstream of the *PTGS2* promoter. The transcript could function as a scaffold for interaction of complementary small RNAs in complex with argonaute (AGO) and trinucleotide repeat-containing gene 6A protein (TNRC6A). Binding of the protein–small RNA complex to the promoter RNAs could further trigger recruitment of some histone modifiers (for example, WD repeat-containing protein 5 (WDR5)) and transcription factors (for example, nuclear factor-κB (NF-κB) and cyclic AMP-responsive element-binding protein 1 (CREB1)) to the *PTGS2* promoter, causing transcriptional upregulation of *PTGS2*. These molecular recognitions also affect expression of the adjacent phospholipase A2 group IVA (*PLA2G4A*) gene[108]. H3K4me3, trimethylation of Lys 4 on histone 3; H4Ac, acylation of histone 4; HMT, histone methyltransferase; Pol II, RNA polymerase II; TSS, transcription start site. Figure part **b** was modified from Matsui, M. *et al.*, Promoter RNA links transcriptional regulation of inflammatory pathway genes. *Nucleic Acids Res.* (2012) **41**(22): 10086–10109, by permission of Oxford University Press.

of *NORAD* triggered chromosomal instability[127]. *NORAD* possesses a repetitive 400-nucleotide domain and binds to PUMILIO protein 1 (PUM1) and PUM2, which mediate chromosomal instability when overexpressed. These data implicate *NORAD* as a regulatory factor maintaining genomic stability.

With regard to drug discovery, these findings suggest that compounds that mimic *NORAD* might be useful for maintaining chromosome stability. Alternatively, reducing the level of *NORAD* might increase instability and make cancerous cells more susceptible to some anticancer agents.

The experimental strategy used in the study emphasizes the importance of quantitating RNA. Understanding of the mechanisms involved depends on accurate evaluation of the relative amounts of PUMILIO protein and *NORAD* RNA present, owing to the possibility that PUMILIO function depends on *NORAD*–PUMILIO interactions. A two-fold increase in *NORAD* expression is able to affect cellular processes profoundly, because increased *NORAD* provides ~7,000 additional binding sites per cell[127]. It is worth considering that the pool of highly expressed and conserved lncRNAs might form a group of outstanding targets for studies exploring biological functions, with the *NORAD* study providing a useful roadmap.

Case study: SAMMSON. The lncRNA *SAMMSON* (survival-associated mitochondrial melanoma-specific oncogenic ncRNA) has recently been linked to melanomagenesis and may represent a potential therapeutic target[128]. Based on comprehensive qPCR and RNA-Seq analysis data, for 60 different cancer cell lines and more than 8,000 tumour specimens, *SAMMSON* appears to be expressed exclusively in melanoma cells. Subcellular fractionation and FISH assays revealed that *SAMMSON* is mainly localized in the cytoplasm, especially in the mitochondria. *SAMMSON* was found to exhibit in trans pro-oncogenic functions in melanomas by interacting with p32, which is a known critical regulator of mitochondrial homeostasis and metabolism[129]. Intravenous injection of ASO gapmers targeting *SAMMSON* in patient-derived xenograft (PDX) melanoma models significantly suppressed tumour growth, decreased cell proliferation and increased apoptosis, suggesting substantial therapeutic potential[128].

Although these findings are promising, additional confirmatory studies would be useful before further development of this therapeutic approach. The studies described above that used gapmer ASOs used only one control oligonucleotide that was not based on the sequence of either active ASO. In addition, the animal studies did not demonstrate reduction in *SAMMSON* expression or provide evidence of ASO uptake in the target tissue. Furthermore, the number of *SAMMSON* molecules are not calculated, nor related to the number of p32 proteins per cell. Finally, the mass spectrometry data identifying p32 as an outstanding interacting partner are difficult to interpret because data are not provided for individual proteins. We anticipate that future research will reveal the potential of targeting *SAMMSON* and its mechanism of action.

Conclusions

Cellular RNAs have a crucial role during disease progression and comprise a diverse class of targets for drug discovery. Three decades of research have demonstrated that synthetic nucleic acids are a promising starting point for drug development, but that they have the potential to mislead the unwary. This valuable experience can be applied to ncRNAs. If used wisely, prior experience will speed up the development process by helping investigators to avoid past mistakes.

ncRNAs act through mechanisms that are novel and often poorly understood. Although it is tempting to believe that simply observing a physiological effect is sufficient as the basis for a drug discovery platform, knowledge of the mechanism is also necessary. Projects that build a strong case that a physiological effect is due to interactions at ncRNAs will be much more likely to succeed over the long term than those that do not.

Which class of ncRNAs has the most potential to be developed as therapeutic targets? In the near term, compounds that bind to miRNAs or that affect splicing function act through the best understood mechanisms and will be the focus of most clinical development. In the longer term, it is likely that our understanding of the mechanism of lncRNAs and other ncRNAs will grow. In addition, new classes of potential non-coding targets may emerge. As our understanding of ncRNAs and their mechanisms improve, the design of effective development programmes will gain a firmer foundation and the likelihood of clinical success will increase.

Drug discovery involving ncRNA will need to walk a fine line. On the one hand, RNA has repeatedly been proven capable of surprising biological activities, and daring hypotheses and remarkable results should be taken seriously. On the other hand, RNA has been prone to irreproducible research. Drug discovery and development should combine an eagerness to test the limits of the possible with a scepticism that demands a deep focus on the link between molecular interactions and biological outcomes.

Masayuki Matsui and David R. Corey are at the Departments of Pharmacology and Biochemistry, UT Southwestern, Dallas, Texas 75390–9041, USA.

Correspondence to D.R.C.
david.corey@utsouthwestern.edu

doi:10.1038/nrd.2016.117
Published online 22 Jul 2016

1. Morris, K. V. & Mattick, J. S. The rise of regulatory RNA. *Nat. Rev. Genet.* **15**, 423–437 (2014).
2. Ling, H., Fabbri, M. & Calin, G. A. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. *Nat. Rev. Drug. Discov.* **12**, 847–865 (2013).
3. St. Laurent, G., Wahlestedt, C. & Kapranov, P. The landscape of long noncoding RNA classification. *Trends Genet.* **31**, 239–251 (2015).
4. Ebbersen, K. K., Kjems, J. & Hansen, T. B. Circular RNAs: identification, biogenesis and function. *Biochim. Biophys. Acta* **1859**, 163–168 (2016).
5. Sato-Kuwabara, Y., Melo, S. A., Soares, F. A. & Calin, G. A. The fusion of two worlds: noncoding RNAs and extracellular RNAs — diagnostic and therapeutic implications. *Int. J. Oncol.* **46**, 17–27 (2015).
6. van Rooij, E. & Kauppinen, S. Development of microRNA therapeutics is coming of age. *EMBO Mol. Med.* **6**, 851–864 (2014).
7. Zamencnik, P. C. & Stephenson, M. L. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligonucleotide. *Proc. Natl Acad. Sci. USA* **75**, 280–284 (1978).
8. Sharma, V. K. & Watts, J. K. Oligonucleotide therapeutics: chemistry, delivery and clinical progress. *Future Med. Chem.* **7**, 2221–2242 (2015).
9. Schmidt, M. F. Drug target miRNAs: chances and challenges. *Trends Biotechnol.* **32**, 578–585 (2014).
10. Deleavey, G. F. & Damha, M. J. Designing chemically modified oligonucleotides for targeted gene silencing. *Chem. Biol.* **19**, 937–954 (2012).
11. Ozcan, G., Ozpolat, B., Coleman, R. L., Sood, A. K. & Lopez-Berestein, G. Preclinical and clinical development of siRNA-based therapeutics. *Adv. Drug Deliv. Rev.* **87**, 108–119 (2015).
12. Sah, D. W. Y. & Aronin, N. Oligonucleotide therapeutic approaches for Huntington disease *J. Clin. Invest.* **121**, 500–507 (2011).
13. Doak, B. C., Over, B., Giordanetto, F. & Kihlberg, J. Oral druggable space beyond the rule of 5: insights from drugs and clinical candidates. *Chem. Biol.* **21**, 1115–1142 (2014).
14. Bennett, C. F. & Swayze, E. E. RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. *Annu. Rev. Pharmacol. Toxicol.* **50**, 259–293 (2010).
15. Abramova, T. Frontiers and approaches to chemical synthesis of oligodeoxyribonucleotides. *Molecules* **18**, 1063–1075 (2013).
16. Juliano, R. L. The delivery of therapeutic oligonucleotides. *Nucleic Acids Res.* http://dx.doi.org/10.1093/nar/gkw236 (2016).
17. Geary, R. S., Henry, S. P. & Grillone, L. R. Fomivirsen: clinical pharmacology and potential drug interactions. *Clin. Pharmacokinet.* **41**, 255–260 (2002).
18. Rader, D. J. & Kastelein, J. J. Lomitapide and mipomersen: two first-in-class drugs for reducing low-density lipoprotein cholesterol in patients with homozygous familial hypercholesterolemia. *Circulation* **129**, 1022–1032 (2014).
A demonstration that ASOs can have profound effects on gene expression in humans.
19. McClorey, G. & Wood, M. J. An overview of clinical application of antisense oligonucleotides for RNA-targeting therapies. *Curr. Opin. Pharmacol.* **24**, 52–58 (2015).
20. Childs-Disney, J. L. & Disney, M. D. Approaches to validate and manipulate RNA targets with small molecules in cells. *Annu. Rev. Pharmacol. Toxicol.* **56**, 123–140 (2016).
21. Watts, J. K. & Corey, D. R. Silencing disease genes in the laboratory and the clinic. *J. Pathol.* **226**, 365–379 (2012).
22. Wilson, R. C. & Doudna, J. A. Molecular mechanisms of RNA interference. *Annu. Rev. Biophys.* **42**, 217–239 (2013).
23. Crooke, S. T. Proof of mechanism of antisense drugs. *Antisense Nucleic Acid. Drug Dev.* **6**, 145–147 (1996).
24. Stein, C. A. Keeping the biotechnology of antisense in context. *Nat. Biotechnol.* **17**, 209 (1999).
25. Crooke, S. T. Evaluating the mechanism of action of antiproliferative antisense drugs. *Antisense Nucleic Acid. Drug Dev.* **10**, 123–126 (2000).
26. [No authors listed.] Whither RNAi? *Nat. Cell. Biol.* **5**, 489–490 (2003).
Along with references 23–25, this work provides common sense guidelines for interpreting experiments and designing controls.
27. Sledz, C. A. & Williams, B. R. RNA interference and double-stranded-RNA-activated pathways. *Biochem. Soc. Trans.* **32**, 952–956 (2004).
28. Svoboda, P. Off-targeting and other non-specific effects of RNAi experiments in mammalian cells. *Curr. Opin. Mol. Ther.* **9**, 248–257 (2007).
29. Juliano, R. L. & Carver, K. Cellular uptake and intracellular trafficking of oligonucleotides. *Adv. Drug. Deliv. Rev.* **87**, 35–45 (2015).
30. Myers, K. J. & Dean, N. M. Sensible use of antisense: how to use oligonucleotides as research tools. *Trends Pharmcol. Sci.* **21**, 19–23 (2000).
31. Stein, C. A. The experimental use of antisense oligonucleotides: a guide for the perplexed. *J. Clin. Invest.* **108**, 641–644 (2001).
32. Krieg, A. M. CpG still rocks. Update on an accidental drug. *Nucleic Acid. Ther.* **22**, 77–89 (2012).
33. Chen, Z., Monia, B. P. & Corey, D. R. Telomerase inhibition, telomere shortening, and decreased cell proliferation by cell permeable 2′-*O*-methoxyethyl oligonucleotides. *J. Med. Chem.* **45**, 5423–5425 (2002).
34. Stein, C. A. *et al.* Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. *Nucleic Acids Res.* **38**, e3 (2010).
Reported application of 'gymnotic' oligonucleotide delivery, providing an important tool for experimenters using ASOs.
35. Jonas, S. & Izaurralde, E. Towards a molecular understanding of microRNA-mediated gene silencing. *Nat. Rev. Genet.* **16**, 421–433 (2015).
36. Hammond, S. M. An overview of microRNAs. *Adv. Drug Deliv. Rev.* **87**, 3–14 (2015).
37. Li, Z. & Rana, T. M. Therapeutic targeting of microRNAs: current status and future challenges. *Nat. Rev. Drug Discov.* **13**, 622–638 (2014).
38. Kaboli, P. J., Rahmat, A., Ismail, P. & Ling, K.-H. MicroRNA-based therapy and breast cancer: a comprehensive review of novel therapeutic strategies from diagnosis to treatment. *Pharm. Res.* **97**, 104–121 (2015).
39. Agostini, M. & Knight, R. A. miR-34: from bench to bedside. *Oncotarget* **5**, 872–881 (2014).
40. Thakral, S. & Ghoshal, K. miR-122 is a unique molecule with great potential in the diagnosis, prognosis of liver disease and therapy both as miRNA mimic and antimir. *Curr. Gene Ther.* **15**, 142–150 (2015).
41. Gomez, I. G. *et al.* Anti-microRNA-21 oligonucleotides prevent Alport nephropathy progression by stimulating metabolic pathways. *J. Clin. Invest.* **125**, 141–156 (2015).
42. Venter, J. C. *et al.* The sequence of the human genome. *Science* **291**, 1304–1351 (2001).
43. Aartsma-Rus, A. & van Ommen, G.-J. B. Antisense-mediated exon skipping: a versatile tool with therapeutic and research applications. *RNA* **13**, 1609–1624 (2007).
44. Järver, P., O'Donovan, L. & Gait, M. J. A chemical view of oligonucleotides for exon skipping and related drug applications. *Nucleic Acid. Ther.* **24**, 37–47 (2014).
45. Rigo, F., Seth, P. P. & Bennett, C. F. Antisense oligonucleotide-based therapies for diseases caused by pre-mRNA processing defects. *Adv. Exp. Med. Biol.* **825**, 303–352 (2014).

46. Dominski, Z. & Kole, R. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc. Natl Acad. Sci. USA* **90**, 8673–8677 (1993).
Classic work introducing the concept of ASO-mediated regulation of splicing.
47. Rigo, F. *et al.* Synthetic oligonucleotides recruit ILF2/3 to RNA transcripts to modulate splicing. *Nat. Chem. Biol.* **8**, 555–561 (2012).
48. Sierakowska, H., Sambade, M. J., Agrawal, S. & Kole, R. Repair of thalassemic human β-globin mRNA in mammalian cells by antisense oligonucleotides. *Proc. Natl Acad. Sci. USA* **93**, 12840–12844 (1996).
49. Mann, C. J. *et al.* Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc. Natl Acad. Sci. USA* **98**, 42–47 (2001).
50. van Deutekom, J. C. *et al.* Local dystrophin restoration with antisense oligonucleotide PRO051. *N. Engl. J. Med.* **357**, 2677–2686 (2007).
51. Goemans, N. M. *et al.* Systemic administration of PRO051 in Duchenne's muscular dystrophy. *N. Engl. J. Med.* **364**, 1513–1522 (2011).
52. Voit, T. *et al.* Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory randomised, placebo-controlled Phase 2 study. *Lancet Neurol.* **13**, 987–996 (2014).
53. Lentz, J. J. *et al.* Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. *Nat. Med.* **19**, 345–350 (2013).
54. Passini, M. A. *et al.* Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci. Transl. Med.* **3**, 72ra18 (2011).
55. Kole, R. & Krieg, A. M. Exon skipping therapy for Duchenne muscular dystrophy. *Adv. Drug. Deliv. Rev.* **87**, 104–107 (2015).
56. Mendell, J. R. *et al.* Longitudinal effect of eteplirsen versus historical control on ambulation in Duchenne muscular dystrophy. *Ann. Neurol.* **79**, 257–271 (2016).
57. Chiriboga, C. A. *et al.* Results from a phase 1 study of nusinersen (ISIS-SMN_{Rx}) in children with spinal muscular atrophy. *Neurology* **86**, 890–897 (2016).
Compelling early stage clinical results of an ASO designed to change splicing and upregulate functional SMN protein.
58. [No authors listed.] FDA briefing document: Peripheral and Central Nervous System Drugs Advisory Committee Meeting, November 24, 2015, NDA 206031. *US Food and Drug Administration* http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm473737.pdf (2014)
59. Lu, Q. L., Cirak, S. & Partridge, T. What can we learn from clinical trials of exon skipping for DMD? *Mol. Ther. Nucleic Acids* **3**, e152 (2014).
60. Kimberling, W. J. *et al.* Frequency of Usher syndrome in two pediatric populations: implications for genetic screening of deaf and hard of hearing children. *Genet. Med.* **12**, 512–526 (2010).
61. Ebermann, I. *et al.* Deafblindness in French Canadians from Quebec: a predominant founder population in USH1C gene provides the first genetic link with the Acadian population. *Genome. Biol.* **8**, R47 (2007).
62. Faravelli, I., Nizzardo, M., Comi, G. P. & Corti, S. Spinal muscular atrophy — recent therapeutic advances for an old challenge. *Nat. Rev. Neurol.* **11**, 351–359 (2015).
63. Ionis Pharmaceuticals, Inc. Ionis Pharmaceuticals Reports Data Update from Nusinersen Phase 2 Study in Infants with Spinal Muscular Atrophy and Reviews Neurological Disease Franchise. *Ionis Pharmaceuticals Press Release* http://ir.ionispharma.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=2158750 (2016).
64. Schmidt, M. H. M. & Pearson, C. E. Disease-associate repeat instability and mismatch repair. *DNA Repair* **38**, 117–126 (2016).
65. Nelson, D. L., Orr, H. T. & Warren, S. T. The unstable repeats — three evolving faces of neurological disease. *Neuron* **77**, 825–843 (2013).
66. La Spada, A. R. & Taylor, J. P. Repeat expansion disease:progress and puzzles in disease pathogenesis. *Nat. Rev. Genet.* **11**, 247–258 (2010).
67. Kay, C., Skotte, N. H., Southwell, A. L. & Hayden, M. R. Personalized gene silencing therapeutics for Huntington disease. *Clin. Genet.* **86**, 29–36 (2014).
68. Fiszer, A. & Krzyzosiak, W. J. Oligonucleotide-based strategies to combat polyglutamine diseases. *Nucleic Acids Res.* **42**, 6787–6810 (2014).
69. Mankodi, A. *et al.* Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. *Science* **289**, 1769–1772 (2000).
70. Kuyumcu-Martinez, N. M., Wang, G.-S. & Cooper, T. A. Increased steady-state levels of CUGBP1 in myotonic dystrophy 1 are due to PKC-mediated hyperphosphorylation. *Mol. Cell* **28**, 68–78 (2007).
71. Mulders, S. A. *et al.* Triplet-repeat oligonucleotide-mediated reversal of RNA toxicity in myotonic dystrophy. *Proc. Natl Acad. Sci. USA* **106**, 13915–13920 (2009).
72. Wheeler, T. M. *et al.* Reversal of RNA dominance by displacement of protein sequestered on triplet repeat RNA. *Science* **325**, 336–339 (2009).
This study demonstrated that ASOs can target trinucleotide repeats and detailed the investigation of the mechanism.
73. Pandey, S. K. *et al.* Identification and characterization of modified antisense oligonucleotides targeting DMPK in mice and nonhuman primates for the treatment of myotonic dystrophy type 1. *J. Pharmacol. Exp. Ther.* **355**, 329–340 (2015).
74. Wheeler, T. M. *et al.* Targeting nuclear RNA for *in vivo* correction of myotonic dystrophy. *Nature* **488**, 111–115 (2012).
75. US National Library of Medicine. ClinicalTrials.gov https://www.clinicaltrials.gov/ct2/show/NCT02312011 (2014).
76. Sandi, C., Sandi, M., Anjomani Virmouni, S., Al-Mahdawi, S. & Pook, M. A. Epigenetic-based therapies for Friedreich ataxia. *Front. Genet.* **5**, 165 (2014).
77. Groh, M., Lufino, M. M. P., Wade-Martins, R. & Gromak, N. R-loops associated with triplet repeat expansions promote gene silencing in Friedreich ataxia and fragile X syndrome. *PLoS Genet.* **10**, e1004318 (2014).
This paper outlines the mechanism for inhibition of transcription in triplet-repeat disease genes, *FXN* and Fragile X mental retardation 1 (*FMR1*), by R-loop formation.
78. Li, L., Matsui, M. & Corey, D. R. Activating frataxin expression by repeat-targeted nucleic acids. *Nat. Commun.* **7**, 10606 (2016).
An example of how targeting an intron with ASOs or dsRNAs can derepress a gene by blocking R-loop formation.
79. Yelin, R. *et al.* Widespread occurrence of antisense transcription in the human genome. *Nat. Biotechnol.* **21**, 379–386 (2003).
80. Katayama, S. *et al.* Antisense transcription in the mammalian transcriptome. *Science* **309**, 1564–1566 (2005).
81. Carninci, P. *et al.* The transcriptional landscape of the mammalian genome. *Science* **309**, 1559–1563 (2005).
82. Kapranov, P. *et al.* RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* **316**, 1484–1488 (2007).
83. Djebali, S. *et al.* Landscape of transcription in human cells. *Nature* **489**, 101–108 (2012).
84. Bergmann, J. H. & Spector, D. L. Long non-coding RNAs: modulators of nuclear structure and function. *Curr. Opin. Cell Biol.* **26**, 10–18 (2014).
85. Guttman, M. *et al.* Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. *Nature* **458**, 223–227 (2009).
86. St. Laurent, G. *et al.* Functional annotation of vlinc class of non-coding RNAs using systems biology approach. *Nucleic Acids Res.* **44**, 3233–3252 (2016).
87. van Bakel, H., Nislow, C., Blencowe, B. J. & Hughes, T. R. Most "dark matter" transcripts are associated with known genes. *PLoS Biol.* **8**, e1000371 (2010).
88. Clark, M. B. *et al.* The reality of pervasive transcription. *PLoS Biol.* **9**, e1000625 (2011).
89. van Bakel, H., Nislow, C., Blencowe, B. J. & Hughes, T. R. Response to "The Reality of Pervasive Transcription". *PLoS Biol.* **9**, e1001102 (2011).
Together with references 87 and 88, this work illustrates the spirited debate over the broader importance of lncRNAs.
90. Bustin, S. A. Why the need for qPCR publication guidelines? — The case for MIQE. *Methods* **50**, 217–226 (2010).
91. Dodd, D. W., Gagnon, K. T. & Corey, D. R. Digital quantitation of potential therapeutic target RNAs. *Nucleic Acids Ther.* **23**, 188–194 (2013).
An example of how RNA copy numbers can be quantified.
92. Gagnon, K. T., Li, L., Chu, Y. Janowski, B. A. & Corey, D. R. RNAi factors are present and active in human cell nuclei. *Cell Rep.* **6**, 211–221 (2014).
93. Gagnon, K. T., Li, L., Janowski, B. A. & Corey, D. R. Analysis of nuclear RNA interference in human cells by subcellular fractionation and Argonaute loading. *Nat. Protoc.* **9**, 2045–2060 (2014).
94. Lennox, K. A. & Behlke, M. A. Cellular localization of long non-coding RNAs affects silencing by RNAi more than by antisense oligonucleotides. *Nucleic Acids Res.* **44**, 863–877 (2016).
This study makes the important observation that ASOs function best in nuclei, whereas duplex RNAs function best in the cytoplasm.
95. Wahlestedt, C. Targeting long non-coding RNA to therapeutically upregulate gene expression. *Nat. Rev. Drug Discov.* **12**, 433–446 (2013).
96. Froberg, J. E., Yang, L. & Lee, J. T. Guided by RNAs: X-inactivation as a model for lncRNA function. *J. Mol. Biol.* **425**, 3698–3706 (2013).
97. Davidovich, C. & Cech, T. R. The recruitment of chromatin modifiers by long noncoding RNAs: lessons from PRC2. *RNA* **21**, 2007–2022 (2015).
98. Simon, J. A. & Kingston, R. E. Occupying chromatin: polycomb mechanisms for getting to genomic targets, stopping transcriptional traffic, and staying put. *Mol. Cell* **49**, 808–824 (2013).
99. Plath, K. *et al.* Role of histone H3 lysine 27 methylation in X inactivation. *Science* **300**, 131–135 (2003).
100. Zhao, J., Sun, B. K., Erwin, J. A., Song, J.-J. & Lee, J. T. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science* **322**, 750–756 (2008).
101. Khalil, A. M. *et al.* Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. *Proc. Natl Acad. Sci. USA* **106**, 11667–11672 (2009).
102. Sarma, K., Levasseur, P., Aristarkhov, A. & Lee, J. T. Locked nucleic acids (LNAs) reveal sequence requirements and kinetics of Xist RNA localization to the X chromosome. *Proc. Natl Acad. Sci. USA* **107**, 22196–22201 (2010).
103. Davidovich, C., Zheng, L., Goodrich, K. J. & Cech, T. R. Promiscuous RNA binding by Polycomb repressive complex 2. *Nat. Struct. Mol. Biol.* **20**, 1250–1257 (2013).
104. Wang, X., Schwartz, J. C. & Cech, T. R. Nucleic acid-binding specificity of human FUS protein. *Nucleic Acids Res.* **43**, 7535–7543 (2015).
105. Davidovich, C. *et al.* Towards a consensus on the binding specificity and promiscuity of PRC2 for RNA. *Mol. Cell* **57**, 552–558 (2015).
A balanced and nuanced investigation from two groups with different viewpoints of the role of PRC2 and its interactions with RNA.
106. Kalantari, R., Chiang, C.-M. & Corey, D. R. Regulation of mammalian transcription and splicing by nuclear RNAi. *Nucleic Acids Res.* **44**, 524–537 (2015).
107. Roberts, T. C. The microRNA biology of the mammalian nucleus. *Mol. Ther. Nucleic Acids* **3**, e188 (2014).
108. Matsui, M. *et al.* Promoter RNA links transcriptional regulation of inflammatory pathway genes. *Nucleic Acids Res.* **41**, 10086–10109 (2013).
An in-depth study of one mechanism for controlling transcription with miRNAs and synthetic duplex RNAs.
109. Liu, J., Hu, J. & Corey, D. R. Expanding the action of duplex RNAs into the nucleus: redirecting alternative splicing. *Nucleic Acids Res.* **40**, 1240–1250 (2012).
110. Modarresi, F. *et al.* Inhibition of natural antisense transcripts *in vivo* results in gene-specific transcriptional upregulation. *Nat. Biotechnol.* **30**, 453–459 (2012).
111. Ji, P. *et al.* MALAT-1, a novel noncoding RNA, and thymosin β4 predict metastasis and survival in early-stage non-small cell lung cancer. *Oncogene* **22**, 8031–8041 (2003).
112. Tripathi, V. *et al.* The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol. Cell* **39**, 925–938 (2010).
113. Zhang, B. *et al.* The lncRNA Malat1 is dispensable for mouse development but its transcription plays a *cis* regulatory role in the adult. *Cell Rep.* **2**, 111–123 (2012).
114. Eissmann, M. *et al.* Loss of the abundant nuclear non-coding RNA MALAT1 is compatible with life and development. *RNA Biol.* **9**, 1076–1087 (2012).
115. Nakagawa, S. *et al.* MALAT1 is not an essential component of nuclear speckles in mice. *RNA* **18**, 1487–1499 (2012).

116. Arun, G. *et al.* Differentiation of mammary tumors and reduction in metastasis upon Malat1 lncRNA loss. *Genes Dev.* **30**, 34–51 (2016).
117. Gutschner, T. *et al.* The noncoding RNA *MALAT1* is a critical regulator of the metastasis phenotype of lung cancer cells. *Cancer Res.* **73**, 1180–1189 (2013).
118. Fan, Y. *et al.* TGF-β-induced upregulation of malat1 promotes bladder cancer metastasis by associating with suz12. *Clin. Cancer Res.* **20**, 1531–1541 (2014).
119. Engreitz, J. M. *et al.* RNA–RNA interactions enable specific targeting of noncoding RNAs to nascent pre-mRNAs and chromatin sites. *Cell* **159**, 188–199 (2014).
120. West, J. A. *et al.* The noncoding RNAs NEAT1 and MALAT1 bind active chromatin sites. *Mol. Cell* **55**, 791–802 (2014).
121. Yoshimoto, R., Mayeda, A., Yoshida, M. & Nakagawa, S. MALAT1 and noncoding RNA in cancer. *Biochim. Biophys. Acta* **1859**, 192–199 (2016).
122. Bird, L. M. Angelman syndrome: review of clinical and molecular aspects. *Appl. Clin. Genet.* **7**, 93–104 (2014).
123. Rougeulle, C., Cardoso, C., Fontés, M., Colleaux, L. & Lalande, M. An imprinted antisense RNA overlaps UBE3A and a scond maternally expressed transcript. *Nat. Genet.* **19**, 15–16 (1998).
124. Chamberlain, S. J. & Brannan, C. I. The Prader-Willi syndrome imprinting center activates the paternally expressed murine Ube3a antisense transcript but represses paternal Ube3a. *Genomics* **73**, 316–322 (2001).
125. Meng, L., Person, R. E. & Beaudet, A. L. Ube3a-ATS is an atypical RNA polymerase II transcript that represses the paternal expression of Ube3a. *Hum. Mol. Genet.* **21**, 3001–3012 (2012).
126. Meng, L. *et al.* Towards a therapy for Angelman syndrome by targeting a long noncoding RNA. *Nature* **518**, 409–412 (2015).
127. Lee, S. *et al.* Noncoding RNA *NORAD* regulates genomic stability by sequestering PUMILIO proteins. *Cell* **164**, 69–80 (2016).
This study provides an unusually detailed mechanism of action for a well-expressed lncRNA.
128. Leucci, E. *et al.* Melanoma addiction to the long non-coding RNA SAMMSON. *Nature* **531**, 518–522 (2016).
129. Fogal, V. *et al.* Mitochondrial p32 protein is a critical regulator of tumor metabolism via maintenance of oxidative phosphorylation. *Mol. Cell. Biol.* **30**, 1303–1318 (2010).
130. Begley, C. G. & Ellis, L. M. Drug development: raise standards for preclinical cancer research. *Nature* **483**, 531–533 (2012).
This paper points out the low rate of reproducibility for many published results.
131. Freedman, L. P. & Inglese, J. The increasing urgency for standards in basic biologic research. *Cancer Res.* **74**, 4024–4029 (2014).
132. [No authors listed.] Oblimersen: Augmersosen, BCL-2 antisense oligonucleotide — Genta, G 3139, GC 3139, oblimersen sodium. *Drugs R. D.* **8**, 321–334 (2007).
133. Kim, R., Emi, M., Tababe, K. & Toge, T. Therapeutic potential of antisense Bcl-2 as a chemosensitizer for cancer therapy. *Cancer* **101**, 2491–2501 (2004).
134. Bedikian, A. Y. *et al.* Bcl-2 antisense (oblimersen sodium) plus dacarbazine in patients with advanced melanoma: the Oblimersen Melanoma Study Group. *J. Clin. Oncol.* **24**, 4738–4745 (2006).
135. Bedikian, A. Y. *et al.* Dacarbazine with or without oblimersen (a Bcl-2 antisense oligonucleotide) in chemotherapy-naive patients with advanced melanoma and low-normal serum lactate dehydrogenase: 'the AGENDA trial'. *Melanoma Res.* **24**, 237–243 (2014).
136. Lai, J. C. *et al.* G3139 (oblimersen) may inhibit prostate cancer cell growth in a partially bis-CpG-dependent non-antisense manner. *Mol. Cancer Ther.* **2**, 1031–1043 (2003).
137. Benimetskaya, L. *et al.* Relative Bcl-2 independence of drug-induced cytotoxicity and resistance in 518A2 melanoma cells. *Clin. Cancer Res.* **10**, 8371–8379 (2004).
138. Anderson, E. M. *et al.* Gene profiling study of G3139- and Bcl-2 targeting siRNAs identifies a unique G3139 molecular signature. *Cancer Gene Ther.* **13**, 406–414 (2006).
139. Winkler, J., Stessl, M., Amartey, J. & Noe, C. R. Off-target effects related to the phosphorothioate modifications of nucleic acids. *ChemMedChem.* **5**, 1344–1352 (2010).
140. Gjertsen, B. T., Bredholt, T., Anensen, N. & Vintemyr, O. K. Bcl-2 antisense in the treatment of human malignancies: a delusion in targeted therapy. *Curr. Pharm. Biotechnol.* **8**, 373–381 (2007).
141. Westphal, S. P. Behind the mask. *New Scientist* **179**, 32–35 (2003).
142. Beg, M. S. *et al.* Safety, tolerability, and clinical activity of MRX34, the first-in-class liposomal miR-34 mimic, in patients with advanced solid tumors. *Mol. Cancer. Ther.* **14** (12 Suppl. 2) C43 (2015).
143. Regulus Therapeutics Inc. All HCV patients treated with a single SC administration of 4 mg/kg of RG-101 responded with mean viral load reduction of 4.8 log10 at day 29 and 9/14 patients are below the limit of quantification at day 57. *Regulus Therapeutics press release*, http://ir.regulusrx.com/releasedetail.cfm?ReleaseID=895314 (2015).
144. Regulus Therapeutics Inc. RG-101 interim analysis shows 97% response at 8 week follow-up. *Regulus Therapeutics press release*, http://ir.regulusrx.com/releasedetail.cfm?ReleaseID=955249 (2016).
145. Regulus Therapeutics Inc. Regulus to present additional preclinical data supporting RG-012 as a novel microRNA therapeutic in development for Alport Syndrome at ASN's Kidney Week 2015. *Regulus Therapeutics press release*, http://ir.regulusrx.com/releasedetail.cfm?ReleaseID=935181 (2015).
146. Regulus Therapeutics Inc. RG-125(AZD4076), a microRNA therapeutic targeting microRNA-103/107 being developed for the treatment of NASH in patients with type 2 diabetes/pre-diabetes, enters Phase I clinical development. *Regulus Therapeutics press release*, http://ir.regulusrx.com/releasedetail.cfm?ReleaseID=947579 (2015).
147. Janssen, H. L. A. *et al.* Treatment of HCV infection by targeting microRNA. *N. Engl. J. Med.* **368**, 1685–1694 (2013).
148. Gebert, L. F. *et al.* Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. *Nucleic Acids Res.* **42**, 609–621 (2014).
149. Ottosen, S. *et al. In vitro* antiviral activity and preclinical and clinical resistance profile of miravirsen, a novel anti-hepatitis C virus therapeutic targeting the human factor miR-122. *Antimicrob. Agents Chemother.* **59**, 599–608 (2015).
150. van der Ree, M. H. *et al.* Miravirsen dosing in chronic hepatitis C patients results in decreased microRNA-122 levels without affecting other microRNAs in plasma. *Aliment. Pharmacol. Ther.* **43**, 102–113 (2016).
151. BioMarin Pharmaceutical Inc. BioMarin announces withdrawal of market authorization application for Kyndrisa™ (drisapersen) in Europe. *BioMarin Pharmaceutical press release*, http://investors.bmrn.com/releasedetail.cfm?ReleaseID=973536 (2016).
152. Haché, M. *et al.* Intrathecal injections in children with spinal muscular atrophy: Nusinersen clinical trial experience. *J. Child Neurol.* **31**, 899–906 (2016).

Acknowledgements
This work was supported by the National Institutes of Health (GM106151, GM73042 and GM118103) and the Robert Welch Foundation (I-1244). D.R.C. holds the Rusty Kelley Professorship in Medical Sciences.

Competing interests statement
The authors declare no competing interests.

FURTHER INFORMATION
Adis Insight: http://adisinsight.springer.com
Biogen: https://www.biogen.com/en_us/home.html
BioMarin Pharmaceutical: http://www.biomarin.com
ClinicalTrials.gov: https://clinicaltrials.gov/ct2/home
ENCODE: https://www.endcodeproject.org
FANTOM5: http://fantom.gsc.riken.jp/5/
Ionis Pharmaceuticals: http://www.ionispharma.com
miRagen Therapeutics: http://miragentherapeutics.com
Mirna Therapeutics: http://www.mirnatherapeutics.com
NONCODE: http://www.noncode.org
Regulus Therapeutics: http://www.regulusrx.com
Roche: http://www.roche.com/index.htm
Sarepta Therapeutics: https://www.sarepta.com

ALL LINKS ARE ACTIVE IN THE ONLINE PDF