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Review

Out of the dark: the emerging roles of lncRNAs in pain

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The dark genome, the nonprotein-coding part of the genome, is replete with long noncoding RNAs (lncRNAs). These functionally versatile transcripts, with specific temporal and spatial expression patterns, are critical gene regulators that play essential roles in health and disease. In recent years, *FAAH-OUT* was identified as the first lncRNA associated with an inherited human pain insensitivity disorder. Several other lncRNAs have also been studied for their contribution to chronic pain and genome-wide association studies are frequently identifying single nucleotide polymorphisms that map to lncRNAs. For a long time overlooked, lncRNAs are coming out of the dark and into the light as major players in human pain pathways and as potential targets for new RNA-based analgesic medicines.

Highlights

Studies of the dark genome are providing important insights into human pain insensitivity and chronic pain conditions.

Long noncoding RNAs (lncRNAs), a major portion of the dark genome, have diverse cellular functions and can dynamically regulate pain thresholds.

Often dismissed as merely fine tuners of gene expression, some lncRNAs instead play a major role in chronic pain mechanisms.

Precise spatial and temporal expression patterns and low expression levels make some lncRNAs viable analgesic drug targets.

RNA-targeted precision therapeutics may have fewer side effects than targeting broadly and highly expressed proteins.

The regulatory genome

One of the revelations from the Human Genome Project has been the discovery that less than 2% of the genome encodes proteins [1]. Remarkably, humans have similar numbers of protein-coding genes (~20 000) as less complex species such as *Caenorhabditis elegans*, the simple nematode worm [2]. However, as species have become more complex, so has the increase in the number of noncoding regulatory RNA genes that inhabit the **dark genome** (see Glossary) [3]. In fact, approximately 76% of the human genome is actively transcribed into RNA with **long noncoding RNAs (lncRNAs)** the dominant category [4,5]. These regulatory RNAs play critical roles in organismal-scale phenotypes, ranging from viability and fertility to aging [6]. Underscoring their importance, many lncRNAs are also associated with human disease [4,7,8].

Chronic pain is a major clinical problem with millions of people living with severe, debilitating and poorly treated pain [9]. It has been more than 20 years since a new class of analgesic medicine has been licensed, with many seemingly excellent validated targets yet to be successfully drugged, such as the $Na_V1.7$ voltage-gated sodium channel blockers and nerve growth factor neutralizing antibodies which are yet to be approved for clinical use [10,11]. With the added problems of current painkillers also contributing to the opioid crisis, new analgesic medicines are therefore urgently needed [12].

Recently a new potential analgesic drug target, *FAAH-OUT* was found disrupted by a deletion in a novel human pain insensitivity disorder [13]. This lncRNA gene regulates ***in cis*** the expression of the adjacent fatty acid amide hydrolase gene (*FAAH*), a critical degrader of, amongst others, the pain-relieving endocannabinoid anandamide [14]. This finding has opened the door to a new way to inhibit FAAH function, paving the way for new RNA-based analgesic medicines. Indeed, the study of long noncoding RNAs in pain is an emerging field with some notable recent findings described below. This review, which is not intended to be an exhaustive list of lncRNAs and also does not include small ncRNAs or **circular RNAs** [15–21], will give exemplars that highlight the diverse mechanistic actions of these genes recently described in the pain field. We will also

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discuss the exciting potential of lncRNA-targeted therapeutics and what challenges and opportunities remain ahead [22].

Functional versatility of lncRNAs

lncRNAs are arbitrarily defined as nonprotein-coding transcripts larger than 200 nucleotides, although it has recently been suggested to further subcategorise some with a cut-off of >500 nucleotides [3]. Most lncRNAs of this size are, like protein-coding mRNAs, transcribed by RNA polymerase II and as such can be spliced, 7-methylguanosine capped and polyadenylated. lncRNAs typically have low sequence conservation and have long been thought to be mostly unstructured. However, it is becoming clear that lncRNAs have modules of functional structure with bioinformatic tools helping to identify structural conservation and short sequence motifs (k-mers) [23–25]. The structure and function of lncRNAs may be altered by **RNA editing** and by chemical modifications which alter charge, RNA folding, RNA–DNA and RNA–protein interactions [26–28]. For example, in the cancer field, it has been shown that a single N6-methyladenosine (m^6A) modification in the lncRNA *HOTAIR* is required for it to associate with chromatin and repress tumour suppressor genes [29].

lncRNAs typically have specific developmental and tissue expression profiles and within a cell can be dynamically localised in different places such as chromatin, **nuclear condensates**, mitochondria, ribosomes, and exosomes [30,31]. lncRNAs are often expressed at low levels and techniques such as RNA CaptureSeq enable all splice isoforms of these lowly expressed genes to be identified [32]. In recent years, single-cell sequencing of mouse and human dorsal root ganglia (DRG) neurons has helped to functionally categorise somatosensory neurons based on gene expression profiles [33–35]. Single-cell profiling of long intergenic noncoding RNAs (lincRNAs); a subclass of lncRNAs transcribed from intergenic regions, has highlighted the cell-specific expression patterns of this class of gene with 200 lincRNAs shown to be highly expressed in distinct types and subtypes of mouse DRG neurons [36]. Among them, the *CLAP* lincRNA is enriched in somatostatin-positive neurons that are associated with **pruritus** and *in vivo CLAP* knockdown reduces histamine-induced scratching in mice and decreases the expression of itch-related genes [36].

Despite the low expression levels of some lncRNAs they can still have large effects due to substoichiometric actions, in particular through the formation biomolecular condensates [37]. lncRNAs are functionally versatile and depending on their localisation and specific interactions with DNA, RNA and proteins can modulate chromatin function, regulate the assembly and function of organelles and nuclear condensates, affect RNA splicing, stability and translation of mRNAs and interfere with signalling pathways [3,4,7,30].

Transcriptional regulation *in cis*

Inherited genetic pain loss disorders are rare but the identification of causative mutations can highlight new analgesic drug targets [38]. In 2019, a new pain insensitivity disorder was described in a female hypoalgesic patient with reduced anxiety symptoms and accelerated wound healing [13]. Remarkably, the patient's **hypoalgesia** only came to the attention of clinicians in her sixth decade following surgery, despite a long-standing history of injuries. Genetic analyses showed that she carried two relevant variants. The first, a **hypomorphic** SNP in the FAAH enzyme (rs324420) [39] was previously associated with significantly lower cold pain sensitivity and less need for postoperative analgesia in a female cohort of breast cancer patients [40]. Furthermore, a mouse knock-in model of the human SNP led to the finding that both the mouse and human SNP carriers display enhanced **fear-extinction learning** and decreased anxiety-linked behaviours [41]. The second variant was a microdeletion downstream of the *FAAH* gene footprint on

Glossary

Biomarker: a naturally occurring molecule, gene or characteristic by which a particular pathological or physiological process, disease, etc. can be identified.
Chronic pain: pain that lasts longer than 12 weeks, or beyond the natural healing time.
Circular RNAs: a type of single-stranded RNA which, unlike linear RNA, forms a covalently closed continuous loop.
CRISPR-Cas: a gene editing technology allowing modifications to the genome. Essentially a guide RNA is designed to match a desired target gene and directs a Cas endonuclease to cause a double-stranded DNA break.
CRISPR interference: uses a dead Cas enzyme (without endonuclease activity) and enables highly specific silencing of transcription of target genes.
CRISPR activation: also uses a dead Cas enzyme but instead induces gene expression from a gene's promoter or enhancer sequence.
Dark genome: nonprotein-coding genomic regions that are, for example, capable of regulating gene expression.
DNA methyltransferase 1 (DNMT1): an enzyme that catalyses the transfer of methyl groups to specific CpG sites in DNA, a process called DNA methylation.
Enhancer: regulatory DNA sequences that, when bound by specific proteins called transcription factors, enhance the transcription of an associated gene.
Enhancer RNAs: ncRNA molecules transcribed from the DNA sequence of enhancer regions; some actively play a role in transcriptional regulation.
Expressivity: the degree to which a phenotype is expressed by individuals having a particular genotype.
Fear-extinction learning: a lessening of conditioned fear responses following extinction training, during which subjects are exposed to repetitive presentations of conditioned stimuli alone.
Genome-wide association study (GWAS): studies that sample a large population in order to look for an association between the presence of certain genetic variants and a particular trait or condition.
H3K27me3: an epigenetic modification to the DNA packaging protein histone H3. It is a mark that indicates the tri-methylation of lysine 27 on histone H3 protein. This tri-methylation is associated with the downregulation of nearby genes.

chromosome 1 in a lncRNA, called *FAAH-OUT*, that was cloned from human brain tissue [13]. **CRISPR interference** and **activation** experiments showed that *FAAH* and *FAAH-OUT* are transcriptionally co-regulated and disruption in *FAAH-OUT* lncRNA transcription leads to **DNMT1**-dependent DNA methylation within the *FAAH* promoter that reduces its expression [14]. In addition, *FAAH-OUT* contains a conserved regulatory element in intron 1, FAAH-AMP, that acts as an **enhancer** for *FAAH* expression. FAAH is a key catabolic enzyme in the endocannabinoid system and enzymatic inhibitors have been efficacious as analgesics in rodent pain models but this success has yet to be translated to human clinical trials [42,43]. The discovery of *FAAH-OUT* and its *cis* regulation of *FAAH* raises important questions for drug development (Box 1).

Regulation of gene expression *in cis* can also be provided by a subclass of lncRNAs called natural antisense transcripts (NATs), which are transcribed from the opposite strand to that of the sense transcript of either protein-coding or nonprotein-coding genes (Figure 1, Key figure) [44]. The classic example of a NAT is *TSIX*, which negatively regulates the expression of *XIST*, itself a lncRNA that is essential for **X chromosome inactivation** [45]. Genome sequencing projects have shown that more than 30% of annotated transcripts in humans have antisense transcription and they can positively or negatively regulate expression using transcription-dependent and/or RNA-dependent mechanisms [5,44,46]. One example from the pain field is *SCN9A*, which encodes the voltage-gated sodium channel $Na_V1.7$ that is mutated in congenital insensitivity to pain and has a negatively regulating NAT [47,48]. Similarly, the voltage-gated potassium channel *Kcna2* gene has a negatively regulating NAT, *Kcna2-AS*, which is upregulated in dorsal root ganglia neurons following peripheral nerve injury [49]. This leads to downregulation of Kcna2, increasing excitability of DRG neurons and producing neuropathic pain symptoms. *Kcna2-AS* expression is also increased in ventricles of rats with congestive heart failure leading to silencing of Kcna2 and ventricular arrhythmias [50]. NATs for genes mutated in human Mendelian pain insensitivity disorders such as *NGF* [51], *SCN9A* [47] and *ZFHX2* [52], are listed in Table 1.

Box 1. Future directions for *FAAH/FAAH-OUT*-related research

FAAH-OUT-associated pain insensitivity, in which the index patient carries the *FAAH-OUT* deletion and the relatively common *FAAH* hypomorphic SNP (rs324420) on opposite alleles, has only been reported in one female and is accompanied by high levels of FAAH-substrates (AEA, OEA, and PEA) measured in peripheral blood. Similar-sized rare deletion variants (esv3585936 and nsv4048188) are present in GnomAD which is probably due to the deletion being flanked by genomic repeats, likely predisposing the region to rearrangement [13]. This raises some interesting questions, such as:

- What is the prevalence of *FAAH-OUT*-associated pain insensitivity in the general population?
- How does the phenotype of other individuals who also carry the *FAAH-OUT* deletion and the *FAAH* hypomorphic SNP on opposite alleles compare to the index case? Is there variable **expressivity** or differences in **penetrance** between carriers of these variants? What is the post-surgical pain phenotype of such individuals?
- Do additional genetic variants modify the phenotype and what are the effects of sex and age on disease presentation?

FAAH enzymatic inhibitors have so far failed to provide pain relief in human clinical trials yet are highly effective in rodent pain models. *FAAH-OUT*, which is proven to regulate *FAAH* gene expression *in cis*, is not present in the rodent genome. This could have important implications for drug development:

- Is the human hypoalgesic phenotype a consequence of developmental changes and would *FAAH-OUT* inhibition provide pain relief if dosed in adults (i.e. post-development)?
- Will therapies that mimic the effects of the index patient's variants (i.e. reduce the level of functional FAAH protein) fare better in clinical trials than current enzymatic small molecule inhibitors? Would **proteolysis targeting chimeras (PROTACs)** for FAAH be more efficacious?
- Which human chronic pain and mental health indications can be best treated with a *FAAH-OUT*-based therapeutic? Which peripheral and/or central tissues should be targeted?
- Does *FAAH-OUT* also function *in trans* to regulate genes at distant loci and/or have an additional cytoplasmic function?
- Is *FAAH-OUT*-associated pain insensitivity better described as a form of indifference to pain, where pain messages are received in the central nervous system but are not perceived as unpleasant?

Hypoalgesia: decreased sensitivity to painful stimuli.
Hypomorphic: a mutation that causes a partial loss of function.
***In cis*:** the Latin prefix '*cis*' means 'on this side' – that is, on the same DNA molecule.
***In trans*:** the Latin prefix '*trans*' means 'across from' – that is, on a different DNA molecule.
Intrathecal: a route of administration for drugs via an injection into the spinal canal, or into the subarachnoid space, so that it reaches the cerebrospinal fluid.
Induced pluripotent stem cells (iPSCs): a type of pluripotent stem cell that can be generated directly from a somatic cell.
Long noncoding RNAs (lncRNAs): nonprotein-coding transcripts larger than 200 nucleotides in size.
Nuclear condensates: specific functional compartments within the nucleoplasm which lack a lipid membrane and contain different macromolecules such as DNA, RNA, and proteins.
Penetrance: proportion of individuals carrying a particular variant of a gene that also expresses an associated phenotype.
Proteolysis targeting chimeras (PROTACs): heterobifunctional molecules that degrade proteins by hijacking the ubiquitin–proteasome system.
Pruritus: severe itching of the skin.
RNA editing: a molecular process through which some cells can make discrete changes to specific nucleotide sequences within an RNA molecule after it has been generated by RNA polymerase.
RNA therapeutics: a new class of medications based on ribonucleic acid.
Translatome: the collection of proteins actively being created in a cell.
X chromosome inactivation: the process by which one of the copies of the X chromosome is inactivated and involves epigenetic silencing of genes.

Key figure

Schematic overview of the long noncoding RNA (lncRNA)-provided regulatory network for gene expression *in cis* and *in trans*

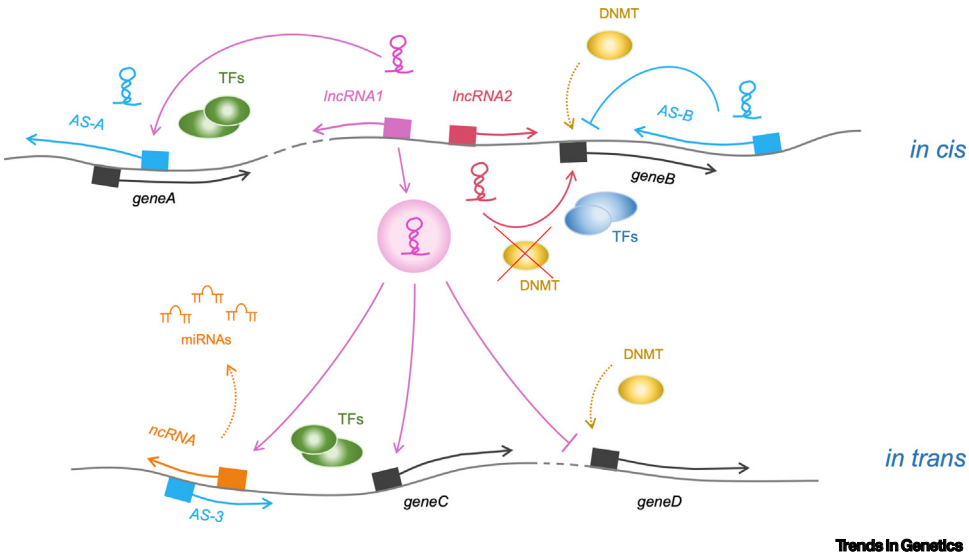

Figure 1. Protein-coding genes *A* and *B* (in black) each have a natural antisense transcript (*AS-A* and *AS-B*, in light blue) transcribed in directions opposite to those of respective mRNAs *A* and *B*. *AS-A* can regulate expression of geneA mRNA by simple transcriptional competition between opposing promoters of two strands. This, *in cis*, is facilitated by an enhancer (*lncRNA1*, in magenta) by recruiting transcriptions factors (TFs, in green) to the promoter of *AS-A*. *AS-B* lncRNA can compete with the opposing strand of *geneB*, but also can regulate methylation levels of *geneB* promoter, via recruiting a DNA methyltransferase (DNMT, in yellow) thus leading to its silencing. This can be counterplayed by the *lncRNA2* gene product (in red) which blocks DNMT binding and instead recruits transcription factors (TFs, in blue) to the *geneB* promoter, thus controlling *in cis* the regulatory loop between *geneB* and *AS-B* transcription. *lncRNA1* (in magenta) regulates other distant genes *in trans*. It facilitates transcription of *geneC* by recruiting transcription machinery (TFs, in green) to the *geneC* promoter. It negates *geneD* transcription by recruiting DNA methyltransferase (DNMT, in yellow). *lncRNA1*-facilitated recruitment of transcription factors to the *ncRNA* promoter (in orange) results in transcription of miRNA precursors of one or more miRNAs that subsequently regulate levels of mRNAs of other genes. Short *ncRNA* gene transcription (in orange) can be counter-acted by transcription of *AS-3* (in light blue).

Transcriptional regulation *in trans*

lncRNAs can also activate gene expression at distant loci instead of modulating local gene expression, with a well-known example being the *Firre* locus which produces a *trans*-acting lncRNA that has physiological roles in haematopoiesis (Figure 1) [53]. FIRRE lncRNA (and its variants) functions ***in trans*** as a chromosome scaffold lncRNA via its interaction with the nuclear matrix factor hnRNPU, thus allowing to maintain a nuclear domain and bringing different chromosomes into proximity [53–55].

In addition, some lncRNAs can promote chromatin looping by interacting with scaffold proteins such as the transcriptional coactivator Mediator and/or the Cohesin multiprotein complex allowing for DNA looping. These interactions allow physical and functional connection of promoters and enhancers of distant genes [56,57]. lncRNAs can exercise their gene-regulatory

Table 1. NATs for genes mutated in human Mendelian pain insensitivity disorders [38]

Gene	Disease	GenBank accession number of an example of EST support for putative NAT
ATL3	Hereditary sensory and autonomic neuropathy type 1	AA757229
DST	Hereditary sensory and autonomic neuropathy type 6	BM273293
NGF	Hereditary sensory and autonomic neuropathy type 5	AK130715
RETREG1	Hereditary sensory and autonomic neuropathy type 2	AK097784
SCN9A	Congenital insensitivity to pain	KM096550
ZFHX2	Marsili syndrome	DA054698

roles via scaffolding with multiple RNA binding proteins and chromatin-remodelling protein complexes [37,58–67].

Recently, a novel RNA transcript called sensory-neuron specific lncRNA (*Ss-lncRNA*) was shown to alleviate nerve injury-induced mechanical hypersensitivity by rescuing expression of Kcnn1 (calcium-activated potassium channel subfamily N member 1) in DRG [68]. *Ss-lncRNA* is exclusively expressed in mouse dorsal root ganglia and trigeminal ganglia from chromosome 1 and binds to heterogenous nuclear ribonucleoprotein M (hnRNPM). In injured DRG after nerve injury, *Ss-lncRNA* is significantly downregulated which results in recruitment of less hnRNPM to the *Kcnn1* promoter on chromosome 8, silencing *Kcnn1* gene transcription and leading to neuropathic pain behaviour [68]. In addition, *Ss-lncRNA* downregulation reduces its binding to lysine-specific demethylase 6B (KDM6B) with consequent less recruitment of KDM6B to the *Kcnn1* promoter and an increase of **H3K27me3** enrichment [69]. Upregulation of *Ss-lncRNA* in small nonpeptidergic sensory neurons is therefore a potential therapeutic strategy for alleviating neuropathic pain.

Another novel lncRNA-based analgesic strategy focuses on the conserved nerve-injury specific lncRNA (*Nis-lncRNA)*, which is upregulated in injured DRGs exclusively in response to nerve injury [70]. *Nis-lncRNA* acts *in trans* to recruit more binding of the RNA-interacting protein FUS to the promoter of the *Ccl2* gene, which encodes C-C chemokine ligand 2, an endogenous initiator of neuropathic pain [71]. In a potential clinical translation of these findings, **intrathecal** injection of *Nis-lncRNA* antisense oligonucleotides attenuate neuropathic pain caused by nerve injury, chemotherapy or diabetes mellitus [72].

RNA stabilisation and miRNA sponging

lncRNAs can also exert their effects post-transcriptionally. *LINC01119* was identified as a lncRNA that is significantly upregulated in the spinal cord in the rat spare nerve injury neuropathic pain model [73]. The RNA binding protein, ELAVL1, was shown using RNA immunoprecipitation to directly interact with *LINC01119*. This complex binds to brain-derived neurotrophic factor (BDNF) mRNA, strengthening its RNA stability and increasing the expression level of BDNF at both transcript and protein levels, which in turn promotes neuropathic pain. Silencing of *LINC01119* using short hairpin RNAs targeting *LINC01119* injected intrathecally significantly attenuates neuropathic pain [73]. Importantly, serum levels of *LINC01119* were shown to be significantly upregulated in human Shingles-induced neuropathic pain patients, suggesting that levels of this lncRNA transcript could also be used as a **biomarker** of neuropathic pain [73].

lncRNAs may also act as sponges of miRNAs if they bear miRNA-complementary sites [74]. This competing endogenous RNA (ceRNA) activity reduces miRNA availability to target mRNAs. This is a suggested mechanism of action for *lncRNA-NONRATT021203.2* which is upregulated in a

cancer-induced pain model and leads to an increase in C-X-C motif chemokine ligand 9 (CXCL9) [75]. Downregulation of *lncRNA-NONRATT021203.2* by siRNA intrathecal injection alleviates cancer-induced bone hyperalgesia in rats [75]. Likewise, *LncRNA71132* was also found to be upregulated in cancer-induced bone pain and acts as a ceRNA which sponges miR-143 [76]. This modulates the expression of the G-protein-coupled receptor, GPR85, in the spinal cord, which regulates pain hypersensitivity.

In addition, to already mentioned regulatory properties of lncRNAs, there are multiple examples where they can affect mRNAs turnover [77–79] and translational rate [31,80], modulating pre-mRNAs splicing [81] and mRNAs decay [82–84], protein–protein interactions [85], protein activity [86,87], and signalling pathways [88,89]. We will most certainly see cases of lncRNAs engaged in such regulations in nociception to be discovered and described in the future.

Gene dysregulation in chronic pain – a domino effect

lncRNAs, as key players in the regulatory dark genome, effectively modulate gene expression profiles. As chronic neuropathic pain is so difficult to treat, numerous transcriptional studies have been carried out to pinpoint specific genes dysregulated in neuropathic pain and other chronic pain states with one aim being to identify new analgesic targets. Such studies inevitably lead to the finding of massive gene dysregulation in both protein-coding and noncoding genes (see Table 2 for examples of key lncRNAs identified) [90–96]. This is perhaps unsurprising, as even a change in expression levels of one lncRNA could, for example, modulate the activity of a transcription factor which in turn could lead to multiple dysregulated downstream genes. Over time, this domino effect multiplies making the identification of potential analgesic targets all the more challenging. This complexity is further complicated by the heterogeneity of cells which contribute to pain signalling, the multiple underlying chronic pain mechanisms [97], sex-specific [98] and rodent strain effects [91], compensatory mechanisms, activation of nerve regeneration pathways [96] and the sheer diversity of ncRNAs that exist that can impact on gene expression, such as **enhancer RNAs** [99]. That's without mentioning the effect of lncRNAs on the pain **translatome** [100].

A key question to ask as chronic pain develops is what are the primary, secondary and tertiary effects of lncRNA regulation? As eloquently demonstrated by the Rinn lab using the *Firre* lncRNA,

Table 2. Examples of lncRNAs dysregulated in pain models with downstream targets identified

lncRNA	Pain model (species)	Downstream target affected	Refs
Ds-lncRNA	Spinal nerve ligation (mouse)	Ehmt2/G9a (euchromatic histone lysine methyltransferase 2)	[155]
Kcna2-As	Spinal nerve ligation (rat)	Kcna2 (potassium voltage-gated channel subfamily A member 2)	[49]
Linc01119	Spare nerve injury (rat)	BDNF (brain-derived neurotrophic factor)	[73]
LncRNA71132	Cancer-induced bone pain (rat)	GPR85 (G protein-coupled receptor 85)	[76]
lncRNA-Nonratt021203.2	Cancer-induced bone pain (rat)	CXCL9 (C-X-C motif chemokine ligand 9)	[75]
Nis-lncRNA	Spinal nerve ligation (mouse)	CCL2 (C-C chemokine ligand 2)	[70]
Snhg5	Chronic constriction injury (rat)	Scn9a ($Na_V1.7$ voltage-gated sodium channel)	[156]
Uc.153	Chronic constriction injury (mouse)	pre-miR-182-5p (miRNA)	[157]

the timescales of gene activation by lncRNAs can be in the order of minutes [101]. This means that the earliest effects of lncRNA regulation are obscured by layers of gene regulatory cascades and compensation mechanisms within neurons [101]. If the power of single-cell sequencing can be applied over multiple time points as chronic pain develops, the domino effect of lncRNA regulation may be unpicked. Studies of temporal expression patterns in neuropathic pain states are beginning to be published, offering new insights into gene dysregulation networks that may underpin the development of chronic pain states [102,103].

Dark genome: an untapped resource of analgesic targets for lncRNA medicine

The rising numbers of lncRNA genes characterised for their key roles in either regulation of pain sensory genes or developmental processes for pain-related neurons is giving hope that these lncRNAs can be used either as targets or as drugs in the rapidly advancing **RNA therapeutics** field.

There is a broad range of available approaches already used for mRNA targeting that can also be used to target lncRNAs [104–108]. These include antisense oligonucleotides (ASOs), siRNAs and miRNAs to promote lncRNA degradation, or using aptamers to modulate lncRNA interaction with proteins [109–111]. Defective lncRNA genes can potentially be corrected, repressed or activated using CRISPR-Cas systems [112].

RNAi approaches use siRNAs – short double-stranded RNAs (20–24 nucleotides) to sequence-specifically target RNAs via argounate2 (AGO2)-mediated RNA cleavage of the target RNA [113]. miRNAs are short intrinsic endogenous single-stranded ncRNAs that modulate RNA levels via RNAi, which also can be used to degrade key lncRNA targets [114,115]. In a similar way, shRNAs that exploit the miRNA pathway can be used to degrade target lncRNAs [116,117].

ASOs are short, single-stranded oligonucleotides (12–24 nucleotides) complimentary to the specific RNA [114,118]. ASOs can be designed to target lncRNAs for degradation by RNAse H1 that selectively cleaves RNA-DNA heteroduplexes [119]. RNAse H1 can target lncRNA transcripts complexed with ASOs equally efficiently in cytoplasm and nuclei [120]. Alternatively, ASOs can be used to target miRNAs, thus protect key lncRNAs from degradation [121,122]. Finally, ASOs can be used to modulate splicing by targeting intron-exon junctions of lncRNAs [114]. Most of the anti-RNA drugs that are currently in clinical trials are based on ASOs [105,107].

Aptamers are single-stranded oligonucleotides with a defined 3D structure that bind and inhibit proteins. Aptamers can be designed to disrupt interaction between protein-protein and/or protein/ligand interactions and to deliver other therapeutic agents in a cell-specific way [123,124]. In the case of lncRNAs, specific aptamers can be designed to interact with lncRNA-binding proteins such as transcription factors and chromatin remodellers, thus efficiently modulating downstream pathway(s) [125,126]. Similarly, lncRNA structures can be targeted by small molecule compounds to disrupt their spatial organisation and/or lncRNA-protein interactions [127–129].

With more advances in our understanding of the role of lncRNAs in pain control, more cases will be identified in which the sequence and therefore the function of key lncRNA genes will be affected by mutations; for example, point mutations and/or deletions and insertions. It will be then reasonable to use **CRISPR-Cas**-based therapies [112,130] where the affected lncRNA gene can be edited back to the form that negates pain (if this is initially the main function of the wild type) or edit the target lncRNA sequence by introducing a mutation that will inactivate or diminish the pain-promoting role of the newly discovered lncRNA molecule. A similar approach has been used to unsilence the *Ube3a-ATS* lncRNA to treat Angelman syndrome [131,132]. In addition, the CRISPR-Cas system can be used to either suppress (with CRISPR-KRAB) or activate

(with CRISPR-VP64) transcription of target lncRNA genes depending on their roles [133,134]. Finally, CRISPR-Cas tools can be used to target miRNAs to protect their targets [135].

Whenever a key lncRNA has been lost or mutated an RNA replacement strategy can also be used. To that end a viral delivery vector expressing the desired lncRNA, or liposomes loaded with modified lncRNA can be used for delivery in a tissue-specific manner, similar to routes used for mRNA vaccines [104–107]. Specifically engineered RNA molecules can also be used to act as sponges for miRNAs, thus protecting key lncRNAs which those miRNAs target for degradation [136,137].

These approaches will transcend the use of pain-related lncRNA targeting from altering their levels for research purposes into the domain of RNA medicine, in particular the development of novel analgesic treatments based on either delivering those lncRNAs that negate pain or targeting those lncRNA molecules that promote and/or sustain pain. Targeting lncRNAs and/or their genes would benefit from their low and specific expression thus reducing potential side effects [104,106–108].

The novel anti-lncRNA drugs can be cost-effective, easy to make and personalise, and may allow efficient and specific targeting of previously undruggable pathways. That could prove to be a game-changer, especially for patients with intractable pain when current painkillers and even opioids do not work, thus treating these patients' conditions and avoiding subsequent side effects, such as addiction, which is linked to the current use of opioids.

Concluding remarks and future perspectives

Studies of the dark genome are leading to an exciting new era for pain research and for other complex disorders. Advanced sequencing technologies now enable whole genomes to be surveyed in large sample cohorts, with **genome-wide association study (GWAS)** variants mapping more frequently to the dark genome than to protein-coding genes and disease gene deleterious mutations also mapping to lncRNAs [8,138]. One of the main challenges now is to understand how these variants affect the function of regulatory ncRNA genes. Fortunately, methods to understand RNA localisation and function are also developing at a pace. For example, RNA molecules can be imaged for subcellular localisation using single-cell spatial transcriptomic techniques such as RNAscope and Molecular Cartography [139]; interactions with chromatin may be assessed using RNA affinity purification followed by DNA sequencing (RAP–DNA-seq) [140] or RNA and DNA interacting complexes ligated and sequenced (RADICL-seq) [141]; RNA–RNA interactions may be mapped using RAP–RNA [142] or RNA *in situ* conformational sequencing (RIC-seq) [143]; and interactions with proteins identified with RNA immunoprecipitation followed by mass spectrometry [144] or incPRINT (in cell protein–RNA interaction) [145]. CRISPR-Display, whereby lncRNAs are localised to target genomic sites, can be used to study *in cis* functions to determine if an RNA molecule alone is functional when decoupled from the act of its transcription [146]. Inducible lncRNA systems in combination with RNA-seq, Pro-seq [147] and ATAC-seq [148] enable gene regulatory events to be resolved temporally [101].

Rodent model systems are extremely useful for better understanding lncRNA functions and their effect on pain behaviour. However, it is important to note that some lncRNAs are primate specific – these may be missed by focussing on rodent pain models. Conversely, some rodent lncRNAs are not present in the human genome. In this respect, the use of mature human **induced pluripotent stem cell** nociceptors is advantageous as lncRNAs in these cells can be manipulated using the CRISPR toolbox. These cells respond to noxious stimuli and exhibit mature electrophysiological characteristics making them relevant for studies of nociception [149]. Four-part human ascending

Outstanding questions

How do the spatial and temporal expression profiles of lncRNAs change as different chronic pain conditions develop?

How do chemical modifications and RNA editing of lncRNAs impact on their structure and function? What is the contribution of these modifications to chronic pain states?

Are specific lncRNA functions conserved across species?

Will the financial cost of gene therapies be reduced to a level that will make them feasible treatments for a wide array of human chronic pain conditions?

How do functional SNPs in lncRNAs identified from GWASs impact on lncRNA structure and cellular functions?

What is the impact of sex and age on lncRNA-related pain mechanisms?

Will putative novel regulatory ncRNAs such as natural antisense transcripts to validated pain genes (e.g. to *NGF*) prove to be useful therapeutic targets?

Which lncRNAs will be suitable as biomarkers from liquid biopsies for different chronic pain conditions?

somatosensory assembloid model systems are now also being developed [150]. These are generated from human pluripotent stem cells and integrate somatosensory, spinal, diencephalic, and cortical organoids to model the human ascending spinothalamic pathway.

However, high-throughput functional screening to identify lncRNAs that are important in pain pathways remains a major challenge. The effects of inducing point mutations in the open reading frames of protein-coding genes are predictable (e.g., frameshifts that lead to protein truncations); however, the consequences of point mutations in lncRNAs are less obvious. Saturation genome editing is one potential tool that could enable high-throughput analysis of different lncRNA mutations, although an appropriate functional readout is needed [151]. CRISPR editing in human dorsal root ganglia is possible which means that relevant readouts of lncRNA mutations in damage-sensing neurons can be designed, although developing high throughput screens is difficult [152]. Instead, insights into lncRNA mutations may be provided by deep analyses of genetic Biobank data (e.g., UK BioBank, which currently contains deep genetic and phenotype data on ~500 000 individuals) [153]. Individuals with recessive loss-of-function deletions of single lncRNAs can be searched for and pain phenotyping data analysed. The study of consanguineous populations, such as using the Qatar Biobank, may lead to a greater yield of homozygous human knockouts [154].

Future research (see Outstanding questions) will elucidate the myriad of functions of lncRNAs, how they ensure the correct expression of genes at the right place and at the right time, and how they contribute to chronic pain mechanisms. It is remarkable that despite the massive unmet clinical problem of pain, the impact of regulatory ncRNAs on chronic pain states is still largely overlooked in favour of drugging protein-coding genes. Nevertheless, the tide is turning and parallel developments in the new frontiers of RNA medicine give hope that advances in lncRNA research will be translated to new effective and safe analgesic medicines to help treat the millions of people living in chronic pain.

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Declaration of interests

PCT applications relate to this work that protects gene therapy IP for *FAAH-OUT* (PCT/GB2019/050368) and a DRG promoter sequence (PCT/GB2020/053296). The authors declare no additional competing interests.

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