Identification and function of long non-coding RNA

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Long non-coding (lnc) RNAs are defined as non-protein coding RNAs distinct from housekeeping RNAs such as tRNAs, rRNAs, and snRNAs, and independent from small RNAs with specific molecular processing machinery such as micro- or pixi-RNAs. Recent studies of IncRNAs across different species have revealed a diverse population of RNA molecules of differing size and function. RNA sequencing studies suggest transcription throughout the genome, so there is a need to understand how sequence relates to functional and structural relationships amongst RNA molecules. Our synthesis of recent studies suggests that neither size, presence of a poly-A tail, splicing, direction of transcription, nor strand specificity are of importance to IncRNA function. Rather, relative genomic position in relation to a target is fundamentally important. In this review, we describe issues of key importance in functional assessment of IncRNA and how this might apply to IncRNAs important in neurodevelopment.

Keywords: non-coding RNA, epigenetics, gene regulation, neurodevelopment
CHARACTERIZATION OF IncRNAs

Several recent reviews have delineated ncRNA species into subcategories based on size (less or greater than 200 bases – often used as the definition of long versus short ncRNA), position (e.g., RNA species generated from the 3′UTRs or 5′UTRs), molecular interactions (e.g., Drosha- or Dicer-dependent), and molecular function, a good example of which is competitive antisense (AS) RNA that binds to microRNA and acts as a sponge to inhibit competitively microRNA from binding to a sense mRNA transcript (Cesana et al., 2011). It is unclear whether these categories are empirically determined, or whether they will prove relevant to categorization as future ncRNAs are discovered; indeed, the identification of such a wide diversity of RNA is consistent with what might be expected from an ancient, flexible molecule, capable of forming 3D structures and interacting with DNA, protein, or other RNAs.

What makes a IncRNA a IncRNA rather than some other RNA species? Are they a functionally distinct RNA product or are they a small part of the transcriptome that has been suggested to occur from large portions of the genome, mostly from recent ENCODE data (Carninci et al., 2005; Birney et al., 2007)? Certainly, a recent report (Guttman et al., 2013) suggests that intergenic IncRNAs are indeed non-coding, an issue that has been previously determined using algorithms (Liu et al., 2011) to assess whether different combinations of potential codons are similar to any other previously identified amino acid molecule. Most studies of IncRNA also attempt to determine whether an RNA species is localized to the nucleus, usually using RNA fluorescence in situ hybridization (FISH). Because translation occurs in the cytoplasm this might be evidence for the lack of coding potential. This analysis is somewhat arbitrary though, because ncRNA might be identified in the nuclear, chromatin, or cytoplasmic fraction of cells. Compartimentalization of IncRNAs in one of these fractions may be a defining feature of different IncRNA and may help to guide future classification schemes. Functional studies of IncRNA have also led to a proliferation of potential future categories for IncRNA, some of which are listed in Table 1, but this categorization creates its own problems in that many IncRNAs have overlapping features. This is a major issue at the moment and one likely to increase in complexity given the number of RNAs that can be detected from so many regions of the genome.

The current classification system will likely evolve as more RNA species are discovered, and classification of each ncRNA might follow a similar trajectory to that of protein coding gene classification. Genes that lead to an mRNA product are not divided up by length, genomic position, whether they are spliced or not for example, and numerous coding genes fit into different classification categories. Instead they are classified by function or conserved domains. Likely it is the novelty of the RNA field, facilitated by the detection of so many transcripts by massively parallel sequencing that is leading to the classification conundrum, but this may diminish as individual RNAs are functionally analyzed.

Several recent reports have carefully documented IncRNAs over a very unique range of function. To understand how IncRNAs are similar or different in both structure and function, we synthesize this information from recent papers to determine if there are any patterns or consistencies across RNA species. We focus on currently defined long RNA (>200 bp) and omit discussion of small RNAs such as microRNA, piwiRNA, or imprinting-related RNAs.

FUNCTIONAL STUDIES OF IncRNAs

The recognition of HOXDAR (Birn et al., 2007) as a IncRNA that regulates gene expression in cis and trans (it is transcribed on chromosome 12 from the HoxC cluster and can regulate the chromosome 2 HoxD gene cluster) opened a new chapter for RNA molecules. HOXDAR defined a class of molecules distinct from housekeeping RNAs, microRNAs, and others, and which were not involved in fundamental imprinting processes. It hinted at the existence of RNA in the genome with regulatory functions directly related to their particular sequence and

<table>
<thead>
<tr>
<th>Category</th>
<th>Description</th>
<th>Example</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intron</td>
<td>Expressed from the intron of target</td>
<td>DMD IncRNA</td>
<td>Boovska et al. (2012)</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Has a methylated H3K4 promoter</td>
<td>IncRNA-p21</td>
<td>Huarte et al. (2010)</td>
</tr>
<tr>
<td>Anti-sense</td>
<td>Expressed from the non-coding strand and acts on the complementary target</td>
<td>BACE1-AS</td>
<td>Faghri et al. (2008)</td>
</tr>
<tr>
<td>Enhancer</td>
<td>Expressed to enhance expression at a locus at some distance from target</td>
<td>p53 eRNAs</td>
<td>Melo et al. (2013)</td>
</tr>
<tr>
<td>Promoter</td>
<td>Acting on and expressed from the promoter of target</td>
<td>DBC-1</td>
<td>Cabianca et al. (2012)</td>
</tr>
<tr>
<td>Intronic</td>
<td>Expressed at some distance from coding genes</td>
<td>IncRNA00299</td>
<td>Tackeck et al. (2012)</td>
</tr>
<tr>
<td>Trans-acting</td>
<td>Acting at some distance from target</td>
<td>Ev2</td>
<td>Bond et al. (2009)</td>
</tr>
<tr>
<td>cis-acting</td>
<td>Acting on an adjacent target</td>
<td>AIRN</td>
<td>Santoro et al. (2013)</td>
</tr>
<tr>
<td>Small</td>
<td>Less than 200 bp in size</td>
<td>microRNA 137</td>
<td>Ripke et al. (2011)</td>
</tr>
<tr>
<td>Long</td>
<td>Greater than 200 bp in size</td>
<td>Fendr</td>
<td>Grotte et al. (2013)</td>
</tr>
<tr>
<td>5′UTR</td>
<td>Expressed near the 5′UTR of target</td>
<td>5′UTR ELK-1</td>
<td>Rahm et al. (2012)</td>
</tr>
</tbody>
</table>
Table 2 | Processing and positional diversity of lncRNA (in order described in text).

<table>
<thead>
<tr>
<th>LncRNA</th>
<th>Species</th>
<th>Description of all structural properties reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLDAIR (Heo and Sung, 2011)</td>
<td>A. thaliana</td>
<td>Three exons, 3.7 kb transcript predominantly cytoplasmically expressed, over 100 different targets dispersed throughout genome</td>
</tr>
<tr>
<td>IRT1 (van Werven et al., 2012)</td>
<td>S. cerevisiae</td>
<td>Transcribed from an intron of the target gene</td>
</tr>
<tr>
<td>Na;T (Gomez et al., 2013)</td>
<td>M. musculus</td>
<td>1.1 kb transcript, 13 isoforms transcribed in the antisense orientation of gene cluster, overlaps one target gene</td>
</tr>
<tr>
<td>BRAVHEART (Klattenhoff et al., 2013)</td>
<td>M. musculus</td>
<td>900 base RNA expressed from three exons, 33% confined to the nucleus</td>
</tr>
<tr>
<td>Na;ST (Gomez et al., 2013)</td>
<td>M. musculus</td>
<td>Encoded on the antisense strand, contains six exons spread over a 45 kb region, transcript is 914 bases</td>
</tr>
<tr>
<td>DBE-T (Cabianca et al., 2013)</td>
<td>H. sapiens</td>
<td>9.5 kb is one major product, transcribed from the same strand as the gene it regulates</td>
</tr>
<tr>
<td>HOTTIP (Wang et al., 2011)</td>
<td>H. sapiens</td>
<td>3,764-nucleotide, spliced and polyadenylated intergenic RNA, ~330 base product, regulates gene cluster</td>
</tr>
<tr>
<td>ANRIL (Jain et al., 2013)</td>
<td>H. sapiens</td>
<td>~126 kb transcript, spliced, 19 exons with an ~1.1 kb transcript, 13 isoforms transcribed in the antisense orientation of gene cluster, overlaps one target gene</td>
</tr>
<tr>
<td>IncMD1 (Casana et al., 2011)</td>
<td>M. musculus</td>
<td>Three exons and two introns in 14 kb of genomic space, spliced product of 521 bases, accumulates as cytoplasmic polyA+ RNA, transcribed on same strand in same orientation as microRNAs for which it acts as a decoy</td>
</tr>
<tr>
<td>TINCR (Kretz et al., 2013)</td>
<td>H. sapiens</td>
<td>Three exons, 3.7 kb transcript predominantly cytoplasmically expressed, over 100 different targets dispersed through genome</td>
</tr>
<tr>
<td>UCHL1-AS (Cadoret et al., 2012)</td>
<td>M. musculus</td>
<td>Four exons spanning 70 kb, overlaps the first 73 bases of UCHL1, including the AUS start codon, transcribed in reverse orientation in a head-to-head fashion, second intron of UCHL1 contains the TSS for UCHL1-AS, enriched in the nucleus</td>
</tr>
<tr>
<td>I2isdRNA1 (Gong and Maquat, 2011)</td>
<td>H. sapiens</td>
<td>Present in cytoplasm, polyA+; two alternative transcripts consist of 688 nucleotides, multiple targets throughout genome</td>
</tr>
</tbody>
</table>
Table 3 | Protein:RNA interacting domains.

**Cysteine domains**

CXC (redox-like), CXXC (redox), or C-X-B-X (zinc finger or ring finger) motifs refer to the cysteine residue (C) with any amino acid (X) in between. These Cys residues may be active, meaning they can use their highly active sulfhydryl (SH) group to form a covalent bond with the OH group on the RNA sugar ring. These motifs can also interact with Ser, Thr, or Tyr amino acid residues to form S-S or S-O bonds on other proteins. An example of the cysteine RNA interacting domain are the Enhancer of zeste-related proteins with conserved X-B-I-X-D-X-C-C motifs.

**WD domains**

WD domains refer to peptide domains with rich repeats of tryptophan (W), hydrophilic and aspartic acid (D), negatively charged that are present in a large range of proteins. WD domains are non-catalytic and are thought to form a platform for the interaction of different cellular partners.

and deacetylases suggests that IRT1 can physically hinder TFs but also guide repressive chromatin complexes. Here the repressive effects are different than COLDAIR in that repression is due to the deposition of H3K4me2 and H3K36me by factors traveling with the RNA polymerase transcribing IRT1. Little is known about the regulation of IRT1, but it must be under tight control to hinder or allow expression of the target gene within such a timeframe of only hours.

NeST is an lncRNA that functions to increase transcription of the target gene and appears to act in trans although it is physically proximal to its target gene, Ifng. Evidence for trans action comes from NeST being genetically unlinked to its target gene and from experimental injection of NeST into cells. NeST action on the target gene is similar to IRT1 and COLDAIR in that it acts through a histone complex, but in this case it physically interacts with WDR5, which has a WD repeat domain of ~40 amino acids (see Table 3). WDR5 is a core subunit of complexes that catalyze the methylation of histone H3 at lysine 4, a mark of active gene expression, so NeST interacts directly with the histone modifying complex, unlike COLDAIR. It is likely that NeST functions to physically bring the histone modifying complex in close proximity to the target gene; it is 59 kb downstream from its target in mouse and 166 kb in human.

Bvht is a cis-acting lncRNA expressed only in mouse, meaning it may be an lncRNA that has recently gained a function. In a continuing theme for lncRNAs, it interacts directly with Suz22 (a component of the PRC2 complex) and functions upstream of a key gene in lineage commitment. This differs from the action of COLDAIR that requires a binding partner for interaction with PRC2, whereas Bvht directly interacts with one of the subunits. Notably, Suz22 has a zinc finger motif, which may explain the protein/RNA binding (see Table 3).

DBE-T is a human lncRNA expressed only in a diseased condition only that acts in cis and affects genes in a large chromosomal region, in contrast to COLDAIR, IRT1, NeST, or Bvht, which appear to regulate a single target. Although these targets often trigger expression of many other genes. DBE-T is transcribed from the first repeat of the DAZ4 repeat domain that is important for recruitment of PRC2. Repression of the region, controlled by PRC2 binding and spreading, commences at the repeat region, thus the basal state in adult cells is the repression of genes at this chromosomal locus. Loss of PRC2 at the repeat region corresponds with the binding of ASH1L, a histone lysine N-methyltransferase that is part of the TriuG group, which recruits DBE-T to chromatin. Thus, this lncRNA is at the crossroads of crosstalk between conflicting histone modifying complexes. While little is known about the regulation of IncRNAs, DBE-T may be an example of a positive feedback loop which may be a common theme for other IncRNAs – in other words, lncRNA expression may be regulated by targets of the target that the lncRNA itself regulates.

Similar to the positive feedback observed between DBE-T and ASH1L, HOTTIP lncRNA and WDR5 operate analogously. Similar to NeST, HOTTIP physically interacts with WDR5, and WDR5 forms a complex with MLL1, which is a H3K4 methyltransferase, triggering gene expression. HOTTIP maintains an appropriate level of the WDR5/MLL1 at a gene cluster, and its influence over the gene cluster dissipates as a function of distance from its site of transcription. Thus, this lncRNA interacts indirectly with a histone modifying complex, is involved in a feedback loop with its interacting partner, and activates expression of a cluster of genes as a function of distance from its site of expression.

ANRIL is a lncRNA transcribed immediately upstream of a cluster of genes important in human cell proliferation and is probably the most studied lncRNA to date because of its important role in cancer. ANRIL is transcribed on the AS strand of three intimately linked genes. It can bind to the transcript of the nearest gene at the locus, INK4, through complementary base pairing and can act at the promoter to recruit both PRC1 and PRC2 to repress transcription. ANRIL, while seemingly with a wider diversity of function than other polycomb recruitment IncRNAs, may actually foreshadow the function of other PRC-recruiting IncRNAs. Specifically, that they may have a wide variety of functions at a particular locus, and the only reason this has not yet been identified is because of experimental design strategies. We suspect many PRC-interacting IncRNAs will have many other functions that complement their effects. The multi-mechanistic function of ANRIL also showcases the idea that not all lncRNAs operate by recruiting large histone modifying complexes. Instead, recently identified IncRNA often operate by binding to the primary target or acting as a decoy of repressive effectors of the target.

lincMD1 and TINCR are two examples of non-PRC-recruiting IncRNAs with novel function to refine expression of a target. In contrast to lncRNAs, COLDAIR, IRT1, NeST, Bvht, DBE-T, HOTTIP, and ANRIL, lincMD1 is an lncRNA that appears to be a by-product or remnant of microRNA processing (Ali et al., 2013). Specifically, this lncRNA can act as a decoy for the targets of microRNA produced from the same locus as lincMD1 (Cesana et al., 2011). TINCR also differs from all reported IncRNAs to date as it appears to bind to a 25 bp “TINCR-box” present in the RNA of different coding transcripts and influence levels of those transcripts in a STAU-dependent manner. STAU is an RNA guidance protein initially identified for its
FIGURE 1 | Different mechanistic action of lncRNAs that overlap an mRNA target. (A) lncRNA expressed from the antisense strand may have an overlapping domain, providing specificity, and a non-overlapping activation domain. This activation domain could be a sequence that allows single-strand binding for different molecules, leading to stabilization or degradation of the RNA:RNA complex. (B) Complementary binding of overlapping mRNA and lncRNA could create a double-stranded binding site for protein binding, leading to selective degradation of stabilization of the RNA:RNA complex.

The idea of repeat elements in the genome, acting through IncRNAs, has also been described with respect to Alu repeats, one of the most common repeats in the human genome. The description of overlapping Alu repeats, one in an AS strand and one in the 3′UTR of the sense strand, can lead to formation of a STAU1 binding site, which allows for STAU1 to stabilize base pairing and target the RNA duplex for degradation. Similar to all IncRNAs described here, these Alu-containing IncRNAs can regulate the levels of a transcript through an mRNA decay pathway. This was specifically demonstrated for SERPINE1 and FLJ21870 mRNAs between their 3′UTR Alu element and the Alu element in a single IncRNA (see Figure 1B).

SYNTHESIS OF lncRNA FEATURES FROM DIFFERENT SPECIES

These examples support an important role for IncRNAs in the genome, and highlight the diverse function of IncRNAs, but also some similarities. First, there appears to be no relationship between the particular function of a IncRNA, its size, or how it is processed. This suggests that IncRNAs will represent a diverse range of characteristics. Second, neither transcriptional direction nor strand specificity appears to have an effect on function. The key element is that IncRNAs are produced either within their target gene or in the vicinity of target genes. Those IncRNAs produced from overlapping regions of their target gene are more likely to bind to the target, however, due to direct complementarity with the target. Whether these IncRNAs come from the same or the AS strand as a target appears not to have functional impact. Future experiments should document this for all newly described
IncRNAs to determine whether this remains the case. These ideas may help guide issues of categorization of IncRNAs, and we propose a system that anchors IncRNAs in the target molecule. This may not prove useful for those IncRNAs, like the intergenic IncRNAs, that do not appear to have nearby targets. Their function may prove to be completely different and independent from those IncRNAs expressed in relationship to mRNAs.

Most IncRNAs are modulators of a primary transcript suggesting that, evolutionarily, they arose after the primary transcript. For example, HOTTIP, either evolved with or after the HOX gene cluster that it regulates. There is little evidence for IncRNAs that operate in isolation (although the lincRNAs may be an exception, reflected by their distinct locations and conservation across species (Managadze et al., 2013), but rather form part of a transcriptional regulation complex of a specific target or a cluster of targets. This suggests that characterizing IncRNA might best be done grounded in the primary target rather than through effector status.

Many IncRNAs act through histone modifying complexes and appear to affect either a single target gene or a cluster of genes in a local region. They may require an intermediate binding partner for recruitment of the histone complex or interact directly with one of the proteins in these complexes. Determining whether IncRNAs bind directly to the target, interact directly with a histone modifying complex, or require a partner to bind histone modifying complex, will be important information as new IncRNAs are uncovered. Most IncRNAs do not share any sequence similarity (i.e., no indication yet of any conserved domain within IncRNAs) and it seems the position of IncRNAs in relation to the target(s) are of fundamental importance to their function. While there are many remarkable functions attributed to IncRNAs, we strongly suspect that the function of even these IncRNA will prove more diverse as they undergo further investigation.

**IncRNAs IN CNS DEVELOPMENT**

Functional and mechanistic data generated by studying IncRNAs in different molecular systems and species suggests IncRNAs likely play an important role in all cellular systems. As evidenced in the previous sections, IncRNAs most likely act as modifiers of a complementary RNA, interact with large histone complexes, interact with complementary DNA sequences, or act completely independently in the nucleus with no obvious partners required. Given this diverse potential, the complexity of the nervous system in any species might be partially due to the additional level of control over the cellular machinery by IncRNAs. IncRNAs may provide a means to tweak a cellular system at many levels and to operate rapidly in response to external signals whether axon guidance cues or environmental exposure. In line with these ideas, we synthesize recent reports of IncRNAs in the developing nervous system.

**IncRNA IN NEURAL STEM CELLS**

Some of the first experiments to underscore the importance of IncRNAs were done in mouse or human stem cells from. Stem cells used for research are either derived from the inner cell mass of a fertilized embryo (Thomson et al., 1998) or induced to pluripotency by the experimental increase of transcription factors normally present in early embryonic stages (Takahashi and Yamanaka, 2006) in terminally differentiated cells. These stem cells can be differentiated to a neural stem cell (NSC) fate and these NSCs can then give rise to glia and neurons (Hu et al., 2010). In a wide ranging, exploratory analysis, Ng and colleagues (Ng et al., 2012) examined neuronal differentiation from human embryonic stem cells (hESCs). They used a two-step differentiation protocol from radial glial-like cells to largely dopaminergic cells, and then assessed global gene expression levels of pre-selected IncRNAs in radial-glia cells compared to dopaminergic-like cells. They identified 35 IncRNAs that were differentially expressed between progenitor and mature states, and then tested some of these for functionality. Following similar designs of non-neuronal studies of IncRNA, we assessed the association of differentially expressed IncRNA with SUZ12 and the neurogenesis repressor complex REST/nRbF (neural restrictive silencer factor; Naruse et al., 1999). In a study using just three IncRNAs, their data supported interaction of one IncRNA with REST and another IncRNA with SUZ12. While the SUZ12 interaction is consistent with previous IncRNA studies, the interaction with REST/nRbF is novel for IncRNAs in neurons, although it does associate with HOTAIR in non-neuronal cell types to repress expression of neuronal genes. This suggests that the IncRNA in the Ng et al. (2012) study may interact with REST to regulate neuronal gene expression.

While ES (embryonic stem cells)- and iPSC (induced pluripotent stem cell)-derived NSCs may not perfectly capture the developmental progression of the human brain, they provide an excellent model with which to screen for important factors as the cells develop from stem cells to electrically active neurons. A study monitoring iPSC-NSC differentiation accompanied by RNA sampling at different timepoints, contrasted with brain temporal lobe brain tissue RNA levels from the same donor has revealed a gradual increase in the expression of different IncRNAs as NSCs differentiate (Hjelm et al., 2013). This is supported by our own study, where we observed an increase in the neurodevelopmentally important intergenic IncRNA00199 as iPSC-NSCs differentiated (Takahashi et al., 2012). A recent report using adult NSCs in mice has further confirmed that IncRNAs increase as cells differentiate; Ramos et al. (2013) sorted stem cells of the sub-ventricular zone of mice and screened these cells for expression levels of different IncRNA creating publically accessible expression maps for IncRNAs that may be relevant to glial-neuron specification in adult brain.

**IncRNA IN DEVELOPING BRAIN**

In the mammalian brain, IncRNAs have long been recognized as important in neurodevelopment, although they were traditionally referred to as AS transcripts. An example of this is the AS transcripts near the Sox4 and Sox11 loci produced during development of the mouse cerebral cortex (Ling et al., 2009). Sox proteins contain a high mobility group, and this refers to the ability of these proteins to bind and bend DNA. Using global gene expression analysis tools, Ling et al. (2011) showed that AS Sox4 and Sox11 transcripts are produced during proliferating and differentiating states, suggesting that the regulation of these important genes is by complementary IncRNAs. Recently, this same group documented a similar effect with respect to Ngn2 and Camk2a gene product in mouse cerebral corticogenesis. A recent study in adult brain also suggests that electrical activity in neurons stimulates IncRNA


H3K4. This model conforms nicely to what is known of lncRNA transcription and splicing in this region are complex. Recently, Powell et al. (2013) reported the first experiments to determine the function of lncRNA 116HG. They found that 116HG forms RNA “clouds” specific to nuclei in mouse brain, and that these 116HG RNA clouds change size and shape through predictable ways as the brain develops. Using RNA and DNA FISH mapping, they show that 116HG likely interacts with the paternal UBE3A locus, a gene found immediately upstream of the 116HG locus and known to be important in neurodevelopment. Their data further suggest that 116HG interacts with RBBP5, a subunit of the MLL complex, which acts as a transcriptional activator by methylation of H3K4. This model conforms nicely to what is known of lncRNA functions in other species; 116HG might associate with MLL complex and interact with histones at the UBE3A locus. How 116HG itself is regulated is unknown, but this will be clearly important to understand better the neurobiology of PWS.

lncRNAs likely have a role in many aspects of the cell, and brain development might be an area where their structure and function is particularly suited. This may suggest that many more lncRNAs await discovery in novel systems as well as in added layers of control for well known processes of neurodevelopment.

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Ernst and Morton. Identification and function of long non-coding RNA.


Willi locus lncRNA cloud modulates diurnal genes and energy expenditure.

Willi phenotype caused by paternal deficiency for the HBII-85 C/D box small nucleolar RNA.

Trends Genet.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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