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Review

Lessons learned from a lncRNA odyssey for two genes with vascular functions, *DLL4 and TIE1*

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ABSTRACT

Pervasive transcription is a feature of the human genome that requires better understanding. Over the last decade or so, RNA species longer than 200 nucleotides—dubbed long non-coding RNA (lncRNAs)—had been found in sense or anti-sense orientation within or outside of genes that encode proteins. Importantly, lncRNA-mediated gene regulation and the elements that control lncRNA expression are a source of fascination among molecular biologists. In vascular biology, a dozen or so lncRNAs had been identified, and progress occurs each day. In this review, we highlighted our laboratories' contribution to the lncRNA field by discussing lessons learned from two lncRNAs in the *tyrosine kinase containing immunoglobulin and epidermal growth factor homology1 (Tie1)* and *delta-like 4 (Dll4)* loci. These genes are responsible for basic vascular patterning and pathophysiological remodeling in angiogenesis.

1. Introduction

Pervasive transcription, the transcription of much of the mammalian genome despite its vast amount of non-protein-coding DNA, had long been observed in mammalian genomes [1], but skeptics sometimes question its existence or functional relevance [2, 3]. With the emergence of modern RNA technologies such as RNA-seq [4], however, evidence is accumulating at a breathtaking pace to support the view that largescale transcriptional activity of genomes does occur [5]. A comparative analysis of transcriptomes across the metazoan phyla of humans, worms, and flies [6] reveals co-expression modules of developmental gene expression that are shared as well as expression patterns that can align stages in worm and fly development. Interestingly, the extent of non-canonical and non-coding transcription is similar in each organism per base pair and, in the past 5 years, has become a subject of intense investigation. Non-coding transcripts are broadly classified into short non-coding transcripts, such as microRNA (miRNAs), which have fewer than 200 bp, and long non-coding RNA (lncRNAs), which have > 200 bp. This arbitrary distinction comes from biochemical protocols used to distinguish short non-coding RNAs from others.

LncRNAs are diverse: they can arise from genes, between genes, in introns, in exons, in regulatory regions, and in both sense and antisense directions. In 2009, Eric Lander's group at MIT [7] reported over a thousand highly conserved lncRNAs in mammals. Although numerous lncRNAs have been identified since then, their functionality has been challenging to ascertain [8]. Based on functional genomic approaches, their predicted diverse roles range from embryonic stem cell pluripotency to cell proliferation. In some cases, transcription of lncRNAs may influence neighboring genes, a topic that is gaining attention [9, 10]. In the field of vascular biology, our group in 2010 identified a lncRNA in the *tyrosine kinase containing immunoglobulin and epidermal growth factor homology1 (Tie1)* locus in the antisense direction, and showed that it directly interacts with *Tie1* mRNA to facilitate its degradation [11] (Fig. 1). To date, lncRNAs in a half dozen vascular genes have been identified, and functional relevance has been assigned to some of them. This topic was reviewed 2 years ago [12], and will not be the focus of this review. Here, we discuss lncRNAs in two vascular patterning genes (*TIE1 and DLL4* [13]) in the setting of current questions in the field such as conservation of function across species, the effects of context and variants on lncRNA function, technologies for functional assessment, and locations of lncRNAs in tissues. Finally, we provide a perspective on the future of the lncRNA field.

1.1. Vascular patterning genes (TIE1 and DLL4)

Angiogenesis, the development of new vasculature from existing

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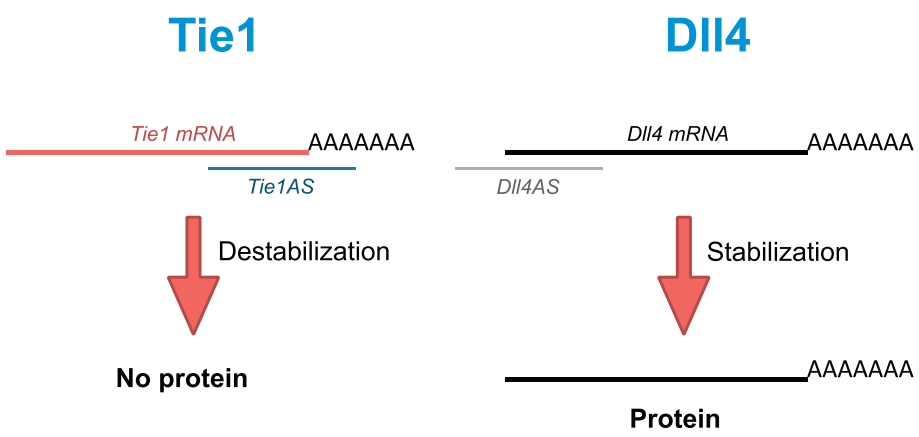

Fig. 1. LncRNAs at the *Tie1* and *Dll4* loci. *Tie1AS* binds to *Tie1* mRNA and destabilizes the transcript. Conversely, *Dll4AS* stabilizes *Dll4* mRNA. The word "stabilization or destabilization," in this context implies more or less RNA respectively.

vasculature, is one of the fundamental physiological process associated with remodeling. Therefore, it is no surprise that this process is regulated at the transcriptional, post-transcriptional, and translational levels. We previously reported [11, 14] that both *tie1* and *DLL4* are regulated by natural antisense lncRNAs in the *cis* configuration (Fig. 1). TIE1 is an endothelial and hematopoietic cell-specific receptor tyrosine kinase [15] that is critical for maintaining vascular integrity during the late phase of angiogenesis and is needed for the survival of endothelial cells in mature vessels [16–19]. *Tie1* is an essential gene in development, and deleting the murine *tie1* gene leads to local hemorrhage and edema, causing embryonic lethality [17, 18]. *Tie1* is differentially expressed across vascular beds [20–22], but no ligand has yet been identified. However, TIE1 is hypothesized to function with TIE2 to trigger signaling in endothelial cells. A large body of work on TIE1 has been performed at the post-translational level, but we know very little about the context-dependent post-transcriptional mechanisms that regulate *Tie1* mRNA.

We identified three lncRNAs at the *TIE1* locus in human endothelial cells, one in mice and two in zebrafish. We further showed that one of the three lncRNAs identified in humans, *hTIE1AS2*, and one of the two lncRNAs identified in zebrafish, *tie1AS*, can regulate *tie1* mRNA levels. We have extensive data to support *tie1AS*-mediated regulation of *tie1* mRNA in zebrafish embryos. The *tie1AS* lncRNA transcript is expressed temporally and spatially *in vivo* with its native target, the *tie1* coding transcript, and in locations such as the ear and parts of the brain where only *tie1AS* is expressed [11]. *tie1AS* expression is controlled by a 3 kb genomic fragment downstream of *tie1* mRNA. We found that *tie1AS* selectively binds *tie1* mRNA in the cytoplasm and that overexpression of *tie1AS* downregulates *tie1* mRNA levels, causing defects in endothelial cell contacts that result in abnormal intersomitic vessel formation in the zebrafish trunk [11]. Our recent work elucidates the mechanism [23]. Using several novel technologies, which will be discussed later in this review, we demonstrated that zebrafish *tie1AS* lncRNA forms a complex with Embryonic lethal and abnormal vision Drosophila-like 1 (Elavl1, an RNA-binding protein) to regulate *tie1* mRNA levels in specific tissues in the head across a small window of time [23]. Thus, zebrafish *tie1AS* post-transcriptionally regulates *tie1* mRNA in a temporal and spatial manner (Fig. 2). We also showed that *hTIE1AS2* regulates *TIE1* mRNA post-transcriptionally. However, the mechanism for that regulation is yet to be identified.

In subsequent work, we hypothesized that regulation by lncRNAs of haploinsufficient gene loci such as *Delta-like 4 (DLL4)*, where 50% of the gene product is sufficient to elicit the phenotype (hypersprout) [24], regulation will be exquisite. Indeed, we identified three isoforms of natural antisense lncRNA in the murine *Dll4* locus (Fig. 3) that regulate *Dll4* mRNA levels [13]. In that locus, when lncRNAs from the *Dll4* locus were knocked down, *Dll4* mRNA levels went down; and when lncRNAs were overexpressed, *Dll4* mRNA levels went up [12]. Thus, lncRNA in the murine *Dll4* locus seems to congruently express with *Dll4* mRNA, and regulation is opposite to that of zebrafish *tie1*. These natural antisense lncRNAs from the *DLL4* locus were also identified in human endothelial cells, and investigation into its function(s) across species is ongoing.

2. Conservation of function across species

The primary sequences of lncRNAs are poorly conserved across species: only 28% for *tie1AS*. On the other hand, promoters or regulatory elements of lncRNAs and splicing patterns show a higher level of conservation [9]. We therefore suggest that conservation of secondary structures of lncRNAs and regulatory elements is more important for preserving function than conservation of primary sequences.

2.1. Importance of studying the structure of lncRNA

Just as structural studies of proteins have greatly enhanced our understanding of protein function, structural studies of lncRNA are expected to increase our understanding of lncRNA. It has been suggested that lncRNA structures, rather than primary sequences, are conserved [25, 26], perhaps because lncRNAs are free from codon preservation constraints. Indeed, evidence for structural conservation is beginning to emerge [27, 28]. For example, *Xist* lncRNA was found to serve as a scaffold that coordinates the functions of regulatory complexes during transcription [29]. LncRNAs, like other types of RNA, can also form secondary structures such as loops, helices, and bulges. These structures can interact with other secondary structures or single-stranded primary structures to form tertiary structures. The secondary and tertiary structures can then be recognized by proteins, DNA, or RNA, leading to function. However, the structures of lncRNAs are expected to be more difficult to study than those of, say, mRNAs or ribosomal RNAs (rRNAs) as they are more complex [25] and show low abundance and high tissue specificity. Additionally, it is possible that the same lncRNA might attain unique secondary and tertiary structures under different physiological conditions. Still, attempts at studying lncRNA structures have been successful, particularly for secondary structures. The secondary structure of the entire 870 nt-long human steroid receptor activator (SRA) RNA was the first to be characterized [30]. Using chemical and enzymatic probing methods, such as selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE), in-line probing with dimethyl sulfate (DMS), and RNase VI probing, Novikova et al. showed that SRA is organized into four major subdomains [30, 31] of which domains I–III are highly conserved. These findings provide important insights into functionality. Also, next-generation sequencing coupled with chemical and enzymatic probing of lncRNA structures has

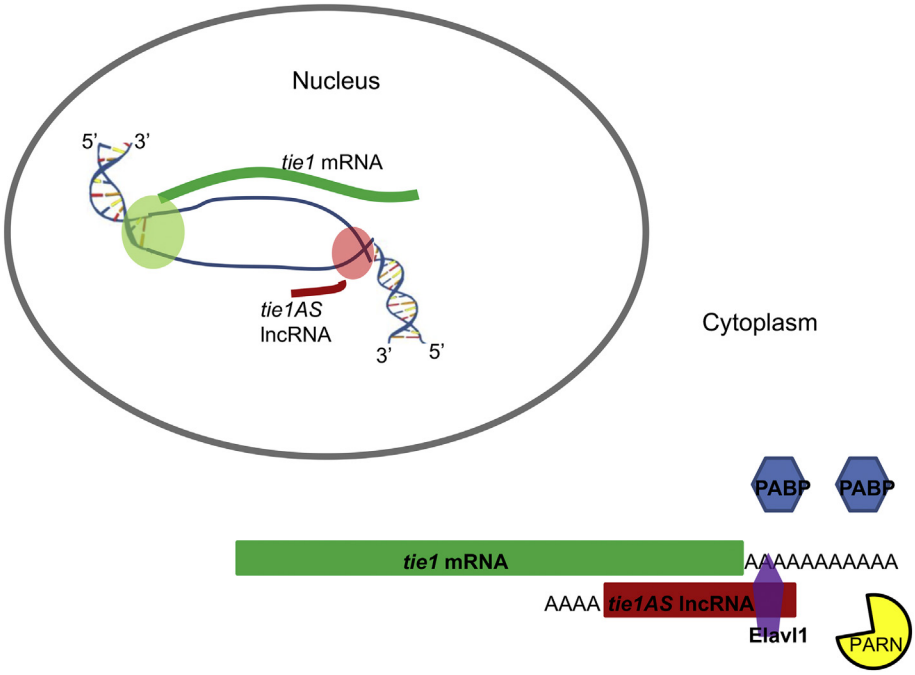

Fig. 2. Regulation of *tie1* mRNA by *tie1AS* in zebrafish embryo. *tie1* mRNA (green rectangle) and *tie1AS* lncRNA (red rectangle) are congruent during development. The *tie1:tie1AS* hybrid duplex has been isolated from cytoplasm. *tie1AS* interacts with Elavl1 (purple pentagon) at the 5′ end of *tie1AS*. We hypothesize that the interaction of *tie1AS* with Elavl1 brings Elavl1 within the proximity of the polyA tail of *tie1* mRNA, allowing Elavl1 to destabilize the interaction between poly(A)-binding protein (PABP) [blue hexagon] and *tie1* mRNA. We also hypothesize that Elavl1 recruits additional proteins involved in the degradation of *tie1* mRNA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

advanced the characterization of lncRNA structures. SHAPE-Seq and DMS-Seq methods make it feasible to study lncRNA structures with pico-molar quantities of starting material instead of nano- or micro-molar quantities. These and other high-throughput methods such as parallel analysis of RNA structures (PARS) and fragmentation sequencing (FragSeq) allow genome-wide characterization of lncRNA structures. Computational predictions also play a major role in generating structures from high-throughput datasets [32]. Our group previously used this approach to investigate stable hybrid formation between zebrafish *tie1AS* and *tie1* mRNA [11].

The next step in studying lncRNA structures involves determining three-dimensional (3D) structures. Although classical methods such as nuclear magnetic resonance (NMR) and x-ray crystallography have been applied successfully to small portions of lncRNAs [33, 34], a 3D structure of an entire lncRNA has not yet been established. However, successful use of these technologies may shed light on how lncRNAs function and how they interact with other RNAs and proteins, such as RNA-binding proteins, which often associate with lncRNA for function.

2.2. Identifying interactors of lncRNA (TIE1)

All stages of RNA life—synthesis, maturation, transport, function, and degradation—are regulated by RNA-binding proteins (RBPs). Therefore, identifying interactors of lncRNA, particularly RBPs, is likely to provide insights into the mechanisms that enable lncRNAs to function. In the case of zebrafish *tie1AS*, identifying a *tie1AS* interactor allowed us to characterize the mechanism of action. We identified one RBP—Embryonic lethal and abnormal vision Drosophila-like 1 (Elavl1)—that binds zebrafish *tie1AS* lncRNA to regulate levels of *tie1* mRNA [23]. To explore the interaction between Elavl1 and *tie1AS*, we employed both *in vitro* [RNA affinity purification or RNA pulldown] and *in vivo* [RBP-immunoprecipitation (RIP)] methods. In the RNA pull-down assay, *in vitro* transcribed and biotinylated parts of the *tie1AS* lncRNA were incubated with zebrafish embryonic protein lysate. Streptavidin magnetic beads helped isolate lncRNA–protein complexes, which were then identified by mass spectrometry (MS) sequencing and subsequently validated by western blot analysis. In the RIP assay, we immunoprecipitated Elavl1 from zebrafish embryos under native conditions and then used RT-qPCR to determine whether *tie1AS* lncRNA co-precipitated with it specifically. However, these two methods do not identify protein-binding sites within RNA, which is required for functionally-relevant studies of lncRNA–protein interaction. To identify Elavl1 binding sites within *tie1AS*, we used a UV crosslinking assay in which radioactive *tie1AS* lncRNA probes were incubated with zebrafish lysate, crosslinked with UV, treated with RNase I, and resolved by SDS-PAGE. Abrogation of ribonucleoprotein complexes due to the introduction of mutations within the *tie1AS* C.

The methods used in our studies were cost-effective, relatively simple, and informative, however, additional RNA- and protein-centric methods exist that are more robust and comprehensive. Also, it is noteworthy that RNA-centric and protein-centric methods can often complement each other. Robust RNA-centric *in vivo* methods use RNA aptamers such as MS2 aptamer, PP7 stem-loop aptamer, or S1 and D8 aptamers [35–37]. In an aptamer-based approach, an lncRNA of interest is expressed in frame with a stem loop forming RNA aptamer(s) that can be specifically isolated with the help of streptavidin or stem loop binding proteins. LncRNA-RBP interactions can be fixed *in vivo* with chemical or UV crosslinking, and affinity-purified. Other RNA-

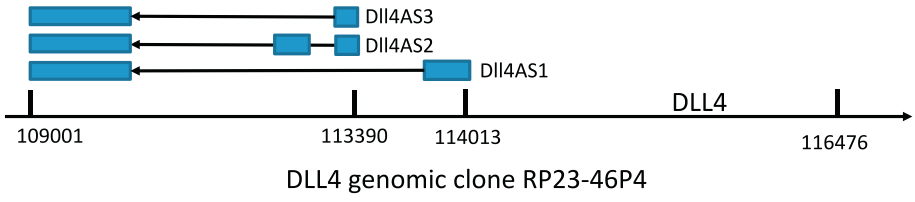

Fig. 3. Mouse *DLL4 genomic locus* and location of DLL4AS variants. The DLL4 genomic locus adapted from clone RP23-46P4 is shown. The numbers represent the genomic denotation for the clone RP23-46P4. DLL4AS variants 1, 2 and 3 are shown in the antisense direction to the DLL4 genomic locus. The blue boxes depict putative exon sequences and the black lines with arrows at the end depict genomic sequences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

centric approaches use hybridization-based purification methods, which include capture hybridization analysis of RNA targets (CHART), chromatin isolation by RNA purification (ChIRP), the peptide nucleic acid (PNA) analog-based PAIR approach, and locked nucleic acid (LNA)-based hybridization [38–40]. In each method, short (20 − 120) oligonucleotide probes tiling the lncRNA of interest are hybridized *in vivo*, crosslinked, washed, and purified. In both affinity tag and hybridization-based methods, RBPs interacting with lncRNAs are commonly identified through MS sequencing. In protein-centric approaches, antibodies are used to immunoprecipitate the RBP of interest and are probed for the presence of lncRNA similar to RIP. One major limitation of RIP is the loss or gain of interactors during purification. Incorporation of crosslinking and nuclease digestion into a RIP protocol, as seen in the case of the crosslinking and immunoprecipitation (CLIP) assay, is a significant improvement on the RIP assay [41]. Several modifications of the CLIP assay exist: high-throughput sequencing of RNA isolated by CLIP (HITS-CLIP) [42], photoactivatable-ribonucleoside-enhanced CLIP (PAR-CLIP) [43], individual-nucleotide resolution CLIP (iCLIP) [44], enhanced CLIP (eCLIP) [45], and infrared-CLIP (irCLIP) [46]. Each of these second- and third-generation CLIP assays allows identification of protein binding sites on lncRNA.

Irrespective of the method, the evidence for functional conservation across species is best demonstrated by determining whether the human ortholog of the identified protein binds to human lncRNA of the gene of interest. Of course, lncRNAs of a given gene are not necessarily found in all species. However, without comprehensive analysis of a given human gene locus, it is difficult to dismiss the lack of human orthologues for a lncRNA.

3. Context- and isoform-specific function of lncRNAs

3.1. LncRNA variants

Determining the functional relevance of lncRNAs is often confounded by the presence of multiple variants, many of which are nonfunctional. (We prefer the term “variant” over “isoform” for lncRNAs). As mentioned earlier, we identified three *TIE1AS* variants in humans, but only one proved functional [11]. Similarly, lncRNAs identified in the murine *Dll4* locus have multiple variants (Fig. 3), the functions of which are currently under investigation. In an extreme case that is listed in the Ensembl database, *PCBP1-AS1* has 40 variants, many of which are presumably nonfunctional. Furthermore, some lncRNAs, such as *SRA*, function as both protein-coding and non-coding variants [47]. Thus, identifying the correct lncRNA variant is necessary for assessing biological function.

LncRNA variants arise through various mechanisms, namely alternative promoters, alternative transcription start sites (TSS) [48], alternative splicing [49], alternative cleavage, and polyadenylation [50]. One sensitive method for identifying lncRNAs is RNA-seq, which can be robust and informative in combination with bioinformatics [8]. Traditional assays such as northern blotting is also useful, especially for determining numbers and lengths of variants. However, northern blotting cannot provide complete sequences of lncRNAs. In addition to RNA-seq, rapid amplification of cDNA ends (RACE) and cap analysis gene expression (CAGE) can effectively annotate the sequences of full-length lncRNAs.

3.2. Independent regulatory elements for variants and associated genes (the DLL4 locus)

An understudied area of lncRNA biology is the identification of regulatory elements that control expression. Furthermore, if there are multiple variants, it is important to determine whether each variant has its own regulatory element or shares regulatory sequences. As we are noticing with lncRNAs from the murine *Dll4* locus, the origin and regulation of lncRNA variants can be dynamic. The first long noncoding RNA (Accession #AA111531) at the mouse *Dll4* locus was cloned from 13.5 days post coitum (dpc) mouse embryos by the WashU-HHMI Mouse EST Project: an RNA sequence spliced at the 5′ end and consisting of 4 exons that mapped upstream of *Dll4* mRNA. Subsequently, a full-length lncRNA (Accession # AK039958) at the mouse *Dll4* locus was cloned from day 0 neonate thymus by the Mouse Genome Encyclopedia Project of the Genome Exploration Research Group in Riken. It shares most of its sequence with AA111531, but has a different TSS: AK039958 transcribes from the first exon of *Dll4*, and overlaps with *Dll4*. Subsequently, many expressed sequence tags (ESTs) from cDNA libraries have been mapped onto the *Dll4* locus. These ESTs have various TSS and exons, reflecting their different tissue origins. Based on the conserved regions of the ESTs, we performed rapid amplification of cDNA ends (RACE) to acquire full-length lncRNAs in mouse endothelial cells. Three lncRNA variants were amplified and named *Dll4-AS1*, *Dll4-AS2*, and *Dll4-AS3*, which are oriented in the opposite direction to *Dll4* mRNA. *Dll4-AS1* overlaps with *Dll4* mRNA, but *Dll4-AS2* and *Dll4-AS3* do not. *Dll4-AS2* and *Dll4-AS3* have the same TSS, 210 bp away from the TSS of *Dll4* mRNA.

To identify the genomic elements that regulate the expression of the lncRNA variants, we used Promega's pGL4.14 vector luciferase-based reporter assay system in cells [13]. We identified a common genomic fragment (844 bp) that drives the expression of *Dll4AS* variants and *Dll4* mRNA. It spans the 5′ region of all three DLL4AS variants and part of the first exon of *Dll4*. Because activity was observed in both orientations of this fragment, we referred to it initially as a “dual promoter” element. However, we now prefer genomic regulator terminology over the previously used promoter terminologies as the classical definitions may need to be revisited in the context of lncRNA biology. Promoters by classical definition are regions of DNA that initiate gene transcription, and they are often located proximal to the TSS. Our 844 bp fragment showed activity in both directions, and thus could also fit the classical definition of an enhancer. Transcription factors bind to enhancers to promote transcription, and enhancers act in a position-independent fashion, with activity observed irrespective of orientation. However, enhancers are often located several kilobases upstream or downstream of a gene. The original term “promoter” is fine because of the proximity of 844 bp to *Dll4* TSS [13], but the ever-changing understanding of lncRNA regulation prompts us to use the more conservative term “genomic regulator element” as we move forward.

While analyzing genomic regulatory elements of lncRNAs, especially in cases such as *Dll4*, where independent regulatory elements are within proximity, one should be mindful that genetic manipulations can have unintended consequences. Recent findings demonstrate that genetic manipulations in 12 lncRNAs enable genomic loci to influence neighboring genes in *cis* [10]. Furthermore, none of these effects require actions by lncRNAs themselves but involve transcriptional processes associated with their production.

LncRNA regulatory elements are more conserved than their exons, and are almost as conserved as protein-coding gene promoters. Strong conservation in regulatory sequences of lncRNAs [51] and weaker conservation in the sequences of their transcripts [52] are consistent with the act of transcription having greater biological consequences than transcript sequence [9]. Given the importance of lncRNA regulatory elements, identifying them is a prerequisite for functional investigations. Thus, emerging results argue that the act of transcribing lncRNAs can perhaps mediate both *cis* and *trans* effects on gene expression [9], a subject that will come under scrutiny in the future.

3.3. Subcellular localization of variants

LncRNAs are often present in low copy number per cell, and heterogeneity exists across population of cells. However, most lncRNAs have been found to be highly specific to tissue type or developmental stage. Because each lncRNA may have variants, it is plausible that the location of each lncRNA and its variants in space and time and of the

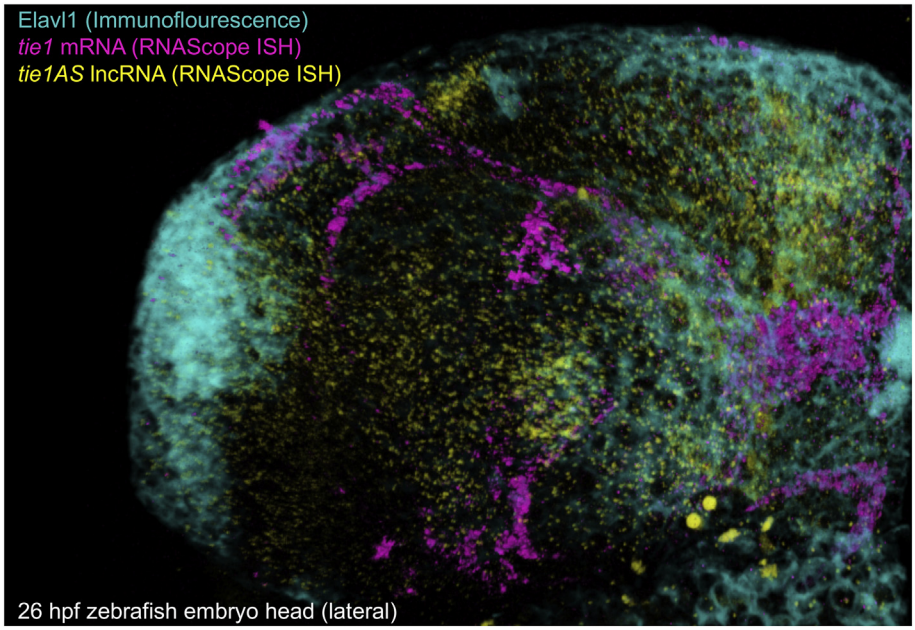

Fig. 4. *tie1AS* lncRNA, *tie1* mRNA, and Elavl1 co-localize in zebrafish embryo. Confocal photomicrograph of a lateral zebrafish embryo head 26 h post fertilization (hpf). Embryos were fixed at 26 hpf, and RNAScope™-based fluorescence *in situ* hybridization (ISH) was performed to detect the expression of *tie1* mRNA and *tie1AS* lncRNA. Following ISH, an anti-Elavl1 antibody was used to perform immunofluorescence to detect the expression of Elavl1. *tie1* mRNA, *tie1AS,* and Elavl1 can be seen to co-localize in select cells. This image was reproduced from publication [23] with permission.

proteins they interact with in a specific cell type in a tissue is critical. Traditional *in situ* hybridization approaches are unable to detect low levels of lncRNA in space and time. Thus, techniques that improve target and signal amplification (tyramide-based signal amplification and QuantiGene View RNA assays) to increase the sensitivity of *in situ* hybridization have taken precedence. In this case, the length of the probe is a determining factor. For example, the QuantiGene assay requires lncRNA probes to be at least 1 kb long, and therefore may be a limiting factor for some 200–800 bp lncRNAs such as *tie1AS* lncRNA.

For *in vivo* detection in tissues, the detection challenges are exponentially higher, so our lab has utilized the novel RNAscope method [53, 54]. The simultaneous signal amplification and background suppression produced by RNAscope preserves tissue morphology, permitting single molecules to be visualized *in vivo* and *in vitro*. We adopted this method for detecting *tie1AS* lncRNA in zebrafish embryos [54], which allowed us to obtain an exquisite understanding of lncRNA function during embryonic development. Our results with *tie1AS* lncRNA and RNAscope-based ISH showed that the lncRNA is widely expressed in the brain but not in trunk regions; moreover, cognate *tie1* mRNA is excluded from some of these locations at a given time in development. We used double fluorescence *in-situ* hybridization to detect *tie1* mRNA and *tie1AS* lncRNA followed by immunofluorescence staining for Elav-like proteins in 26–28 hpf embryos. Both *tie1AS* and Elav-like proteins had broad regions of expression in the head (Fig. 2, [22]) but not in the tail or trunk (Supplemental Fig. III, [23]). In the 26–28 hpf head, we observed close apposition of *tie1* mRNA expression (Fig. 2A, [22]) with head vessels, and the region of expression showed sharp demarcation. In contrast, *tie1AS* lncRNA (Fig. 2B, [22]) was expressed almost exclusively in regions of head where *tie1* mRNA was absent. In the merged image (Fig. 2D, [22]), the regions of exclusivity for *tie1* mRNA and *tie1AS* lncRNA along carotid arteries (CrDI) and primordial mid-brain channels (PMBCs) is clearly noticeable. Expression of Elav-like proteins (Fig. 2C, [22]) was abundant in the forebrain (f), hindbrain (hb), and developing eyes (e). These observations and other data [23] suggest that *tie1* lncRNA, acting *via* Elavl1 protein, facilitates the degradation of *tie1* mRNA at critical times and locations in embryonic zebrafish development.

We are thus beginning to capture the unappreciated temporal and spatial aspects of lncRNA regulation of its cognate targets. Numerous online tools, such as RNALocate [55] and LncATLAS [56], that have just become available, are valuable resources for RNA subcellular localization studies. Just as the subcellular localization of mRNA [57] is known to participate in nuclear export, transport, anchoring, translation, and degradation, we anticipate associations of lncRNAs with each of these functions. We also predict that determining the distribution of lncRNAs in organelles, especially those in the cytoplasm, with their cognate mRNA targets and protein interactors will be the next milestone in this field. Indeed, localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution has already begun [58]. Such analyses show that lncRNAs, like mRNAs, show cell-to-cell variability as pockets of cells expressing high levels of lncRNAs. Finally, exclusive RNA motifs that mediate strict nuclear localization have been reported in lncRNAs [59]. For example, adding the pentamer sequence motif AGCCC (with sequence restrictions at positions 8 (T or A) and 3 (G or C) relative to the first nucleotide of the pentamer) to a cytoplasmic lncRNA shifts it to the nucleus. These novel findings further underscore the importance of the subcellular localization of lncRNAs in biology, a topic that will no doubt gain increased prominence.

3.4. Functional analysis and challenges

Functional analysis of lncRNAs may require multiple approaches, some of which depend on the context of lncRNA study. We and others have used a variety of gene manipulation tools such as CRISPR-based interference (CRISPRi) in fish, CRISPR-mediated genome editing in cell lines, anti-sense oligonucleotides (morpholinos) in fish, and traditional siRNA-mediated strategies. It is worth noting that it is not possible to use all those methods with all lncRNAs and that some methods are more appropriate for certain model systems over others. We focus this section on our experience with targeting zebrafish *tie1AS*.

As mentioned earlier, we determined that *tie1AS* lncRNA interacts with Elavl1 in zebrafish embryos [23]. Using fluorescence *in situ* hybridization (FISH) and immunofluorescence (IF) on 26–28 hpf zebrafish embryos, we observed that *tie1* mRNA is expressed in endothelial cells in the vasculature, whereas *tie1AS* and Elavl1 are broadly expressed in cells surrounding the vasculature (Fig. 4). Detailed co-localization analysis showed that *tie1AS* and Elavl1 expression overlap in the forebrain, hindbrain, and eye; however, overlap between *tie1* mRNA, *tie1AS,* and Elavl1 could be identified only in a small number of eye cells [23]. The mutual exclusivity of *tie1* mRNA and the *tie1AS*-Elavl1 complex led us to hypothesize that the *tie1AS*-Elavl1 complex restricts

the expression of *tie1* mRNA within the developing vasculature. To test this hypothesis, we disrupted the interaction between *tie1AS* and Elavl1 with the help of morpholino antisense oligonucleotides (MOs). LncRNA-protein interaction-blocking MOs (ib-MO) were designed to target Elavl1-binding sites within *tie1AS*, thus blocking the interaction of *tie1AS* with Elavl1. Injecting ib-MOs into 1-cell-stage zebrafish embryos disrupted the formation of the *tie1AS*-Elavl1 complex, increased *tie1* mRNA levels, particularly in the head, between 28 and 31 hpf. The spatial distribution of *tie1* mRNA also changed. The increase in *tie1* mRNA levels and the change in spatial expression of *tie1* mRNA in the head coincided with smaller eyes, reduced ventricular space, and dilated primordial midbrain channel (PMBC) phenotypes [23].

To further analyze the temporal and spatial aspects of the regulation of *tie1* mRNA by *tie1AS*-Elavl1, we used a photoactivatable MO (p-MO) approach (Supplemental Fig. VIII, [23]). In the p-MO approach, a photo-cleavable MO complementary to the ib-MO was premixed with ib-MO at a ratio of 1.2:1 (p-MO: ib-MO). The p-MO: ib-MO complex was injected into 1-cell-stage zebrafish embryos and allowed to develop normally. Between 14 - 16 hpf, the embryos were exposed to UV light. UV light cleaved the photo-cleavable MO in the p-MO:ib-MO complex, facilitating release of the ib-MO, which then bound to its target. Photo-cleavable MOs have been shown to be non-toxic to embryonic zebrafish [60], as has exposure to UV for > 10 min post gastrulation (5 hpf) [60]. Our photo-cleavable approach recapitulated the results we observed with constitutive morpholino: increased *tie1* mRNA levels in the head, smaller eyes, reduced ventricular space, and dilated PMBCs [23].

In addition, we used CRISPR inteference (CRISPRi) to reduce the expression of *tie1AS* because the proximity of Elavl1 binding sites within *tie1AS* to *tie1 mRNA* coding sequences and the paucity of canonical protospacer adjacent motif (PAM) sites in our region of interest made it difficult to use conventional CRISPR-Cas9 editing. For CRISPRi method, we designed guide RNAs to TSS of *tie1AS*, and injected them into zebrafish embryos along with a catalytically inactive Cas9 (dCas9) fused to the repressive Krüppel-associated box domain (dCas9-KRAB). The binding and recruitment of dCas9-KRAB into the TSS site down-regulated the expression of *tie1AS*, resulting in concomitant upregulation of *tie1* mRNA, a functional readout. Multiple gRNAs (3) were injected and, as controls, scrambled guide RNAs were co-injected with dCas9-KRAB. CRISPRi-mediated knockdown of *tie1AS* resulted in concomitant upregulation of *tie1* mRNA, which, as with our MO-approaches, coincided with smaller eyes, reduced ventricular space, and dilated PMBCs [23]. Thus, CRISPRi confirmed that *tie1AS* functionally regulates *tie1* mRNA expression. While performing CRISPR-mediated regulation or editing of lncRNA, we recommend investigating the expression of adjacent genes in the targeted locus and the expression of other genes in the pathway. This idea was reinforced by a recent study that showed that manipulation at the lncRNA (*Lockd*) locus that was located downstream of a gene (*Cdkn1b*), influenced *Cdkn1b* expression [61]. In general, multiple orthogonal approaches help establish the functions of lncRNAs *in vivo*.

4. The next frontier

The lncRNA field is clearly moving at a breathtaking pace—the number of publications each day is a clear indicator of this trajectory. We consider structural determination, context-dependent space and time function, and the role of transcription *vs.* the transcript as questions that will drive the field. Of course, structural determinations of RNAs and their associated protein complexes and comparisons across species will perhaps unravel novel mechanisms that are yet undetermined.

Although the pathogenic roles of a handful of lncRNAs have been considered, systematic analysis of lncRNAs' contribution to disease is still in its infancy. We predict that the vast amount of data generated by genome wide association studies (GWAS) in the last decade, especially data about associations in non-coding variant regions, is likely to open new insights into connections between lncRNAs and disease. With the availability of CRISPR and 3D organoid models for studying tissue architecture, we anticipate that patient-specific mutated cell lines, produced by CRISPR, and generation of organoids from those lines *ex vivo* will provide novel tools for investigation. We also predict that extracellular vesicles from cells (exosomes) and the presence of lncRNA in those organelles will only increase the value of lncRNAs as tools for biomedical research into physiological process, and as potential biomarkers for disease progression or ontogeny, bringing us to the cusp of a new RNA frontier in science.

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