Architectural RNAs for Membraneless Nuclear Body Formation

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Long noncoding RNAs (lncRNAs) are fundamental regulators of various cellular processes. A subset of lncRNAs, termed architectural RNAs (arcRNAs), function in the formation and maintenance of phase-separated membraneless organelles in multiple eukaryotic species. These membraneless organelles represent an important type of compartmentalization in the crowded cellular environment and have several distinct features. The NEAT1_2 lncRNA is a well-characterized arcRNA that functions as an essential scaffold of paraspeckle nuclear bodies. Here, we describe the biogenesis of paraspeckles on arcRNAs through phase separation, focusing on the specific functions of multiple NEAT1_2 RNA domains and their partner RNA-binding proteins. Finally, we present an updated model of paraspeckle formation and discuss future perspectives of research into arcRNA-instructed architectures of phase-separated nuclear bodies.

ARCHITECTURAL RNAs ARE ESSENTIAL SCAFFOLDS OF PHASE-SEPARATED MEMBRANELESS BODIES

Recently, membraneless organelles and biomolecular condensates, which form through phase separation, have attracted increasing interest (Banani et al. 2017; Alberti et al. 2019). These subcellular compartments play key roles in a number of processes, including the sequestration of biomolecules and the formation of organization hubs to coordinate biochemical reactions and macromolecular assembly (Shin and Brangwynne 2017). A subset of phase-separated cellular bodies are constructed by specific long noncoding RNAs (lncRNAs) (Yamazaki and Hirose 2015; Chujo et al. 2016; Chujo and Hirose 2017), which play diverse regulatory roles in various biological processes, including gene expression (Guttman and Rinn 2012; Geisler and Collier 2013; Quinn and Chang 2016; Schmitt and Chang 2017). Multiple lncRNAs can act as essential architectural scaffolds of nuclear bodies in a variety of eukaryotes, including humans, mice, *Drosophila*, and yeast; hence, we termed them architectural RNAs (arcRNAs) (Yamazaki and Hirose 2015; Chujo et al. 2016; Chujo and Hirose 2017).

A number of arcRNAs are involved in the formation of various nuclear bodies. For example, NEAT1_2, the long isoform of the nuclear paraspeckle assembly transcript 1 (NEAT1) lncRNA, is an essential scaffold of paraspeckle nuclear bodies (Chen and Carmichael 2009; Clemson et al. 2009; Sasaki et al. 2009; Sunwoo et al. 2009); highly repetitive satellite III (HSATIII) lncRNAs, which are induced by conditions such as thermal stress, promote the formation of nuclear stress bodies (nSBs) in primates (Jolly et al. 1999; Aly et al. 2019; Ninomiya et al. 2019);

HSATII RNAs are core components of cancer-associated satellite transcript bodies (Hall et al. 2017); intergenic spacer lncRNAs induce the formation of static but reversible amyloid-like solid structures termed amyloid bodies (Audas et al. 2016); histone pre-mRNAs are essential for histone locus body formation (Shevtsov and Dundr 2011); tumor-associated NBL2 transcript (TNBL) aggregates are induced by TNBL RNAs (Dumbović et al. 2018); the perinucleolar compartment is induced by the PNCTR RNA (Yap et al. 2018); Hsrø RNAs are essential cores of ω-speckles in *Drosophila* (Mallik and Lakhotia 2009); and the Mei2 dot is a nuclear body induced by meiRNA in fission yeast (Yamashita et al. 1998). The Sam68 nuclear body and DBC1 bodies are also RNA-dependent bodies, but their arcRNAs have not yet been identified (Mannen et al. 2016). In addition, disease-associated repeat RNAs can also be regarded as a type of arcRNA (Zhang and Ashizawa 2017).

The existence of arcRNAs in a large number of organisms suggests that they are suitable for nucleating subnuclear bodies through phase separation. RNA-binding proteins (RBPs) are enriched with prion-like domains (PLDs), low-complexity domains (LCDs), and intrinsically disordered regions (IDRs), which form weak multivalent molecular interactions that promote biological phase separation (Hennig et al. 2015; Yamazaki and Hirose 2015). Thus, arcRNAs can induce phase separation by increasing the local concentration of these RBPs. More than 1000 human RBPs have been reported to date; these proteins possess a wide variety of biological functions, such as transcriptional regulation and epigenetic chromatin regulation (Baltz et al. 2012; Castello et al. 2012, 2016). RNAs can assemble a specific set of RBPs to combine and integrate their functions, even when the RBPs

would not directly interact (Chujo et al. 2016; Engreitz et al. 2016). This property allows arcRNA-induced bodies to perform multiple tasks in the cell, including acting as molecular sponges to sequester proteins and/or RNAs, forming reaction crucible to enhance biochemical reactions, and organizational hubs of chromatin (Chujo et al. 2016; Chujo and Hirose 2017; Hirose et al. 2019). In addition, noncoding RNAs can act as functional molecules themselves, with roles that depend on their specific sequences and do not require protein coding capacity. Architectural noncoding RNAs are able to alter their specific sequences to adapt to circumstantial changes. Furthermore, multiple copies of lncRNAs are synthesized from their gene loci; this process is important for phase separation as it promotes multivalent interactions between high local concentrations of associated IDR proteins. Therefore, lncRNAs act as markers of specific genomic loci. This property of lncRNAs is beneficial to the amplification of signals from specific genomic loci, such as super-enhancers, and phase separation would enhance this effect (Engreitz et al. 2016). Transcription of arcRNAs is essential for the formation of phase-separated nuclear bodies, and inhibition of their transcription leads to rapid disinte-

gration of the bodies. As most arcRNAs are stress-inducible or specifically expressed in certain developmental and pathological conditions, phase-separated nuclear bodies are transiently formed upon the transcription of arcRNAs. Therefore, it is conceivable that arcRNAs spatiotemporally control cellular signals and act as integral hubs for gene regulation.

In the sections below, we focus on NEAT1_2 as a model arcRNA. We describe how NEAT1_2 arcRNAs construct paraspeckle nuclear bodies and determine their biophysical properties, with a special focus on the functional RNA domains of NEAT1 and their partner proteins.

THE CHARACTERISTICS OF PHASE-SEPARATED PARASPECKLE NUCLEAR BODIES CONSTRUCTED BY THE NEAT1_2 arcRNA

Paraspeckles were originally identified as distinct nuclear bodies called interchromatin granule-associated zones (IGAZs) (Visa et al. 1993), which were located adjacent to interchromatin granule clusters (also known as nuclear speckles) (Fig. 1A). In 2002, paraspeckles were identified

Figure 1. The NEAT1_2 architectural RNA (arcRNA) is essential for maintaining paraspeckle integrity. (A) An electron micrograph of a paraspeckle (electron dense structures were detected using gold particles [probes against NEAT1 5' regions]) and nuclear speckles (black dashed circle) in human HAP1 cells treated with MG132 (5 μ M) for 17 h. Scale bar, 500 nm. The image was kindly provided by Dr. Gerard Pierron (Institut Gustave Roussy, CNRS). (B) Visualization of paraspeckles in wild-type (WT) and NEAT1 knockout human cells by NONO immunofluorescence (green foci). The diffuse green signals seen in the knockout cells indicate disruption of proper paraspeckle formation. DNA was stained with DAPI (blue). Scale bar, 5 μ m. (C) Schematic illustration of the NEAT1_1 and NEAT1_2 isoforms of the NEAT1 gene. (D) A super-resolution microscopy image (structured illumination microscopy) of paraspeckles stained with a NEAT1 FISH probe (green, the 5' and 3' regions; magenta, the middle region). Scale bar, 500 nm. (E) A schematic illustration of NEAT1_2 configuration within a paraspeckle and the core-shell-patch localizations of paraspeckle-localizing proteins (PSPs).

as nuclear bodies enriched in PSPC1, a member of the *Drosophila* behavior human splicing (DBHS) family of proteins that includes SFPQ and NONO (Fig. 1B, left; Fox et al. 2002). Subsequently, paraspeckles were shown to be identical to IGZs and were found to be RNase-sensitive structures, suggesting a requirement of RNAs for their maintenance (Fox et al. 2005). In 2009, four groups independently reported that the NEAT1 lncRNA, also called Men ϵ / β , is an essential architectural scaffold of paraspeckles, as shown by a lack of discrete paraspeckles in NEAT1 knockdown cells (Fig. 1B, right; Chen and Carmichael 2009; Clemson et al. 2009; Sasaki et al. 2009; Sunwoo et al. 2009). NEAT1 has two isoforms: the longer NEAT1_2/Men β isoform (22.7 kb in human) is essential for paraspeckle formation, whereas the short NEAT1_1/Men ϵ isoform (3.7 kb in human) is dispensable (Fig. 1C; Naganuma et al. 2012; Yamazaki et al. 2018a,b).

Several subsequent studies identified more than 60 paraspeckle-localizing proteins (PSPs) in humans (Naganuma et al. 2012; Fong et al. 2013; Kawaguchi et al. 2015; Yamazaki and Hirose 2015; Mannen et al. 2016). Most PSPs are RBPs that contain PLDs, LCDs, or IDRs that show significant overlaps with a number of genes associated with amyotrophic lateral sclerosis (Yamazaki and Hirose 2015). Electron microscopy studies revealed that paraspeckles are roundish or oblong structures with constant short axes (~360 nm in human HeLa cells) (Fig. 1A) and become elongated when NEAT1_2 is up-regulated (Souquere et al. 2010; Hirose et al. 2014b). Notably, the NEAT1_2 lncRNA is spatially organized within paraspeckles; the 5' and 3' regions of NEAT1_2 are located in the shells of paraspeckles, whereas the middle region is located in the core region, suggesting that NEAT1_2 forms a looped structure within paraspeckles (Fig. 1A, D; Souquere et al. 2010; West et al. 2016). In addition, PSPs are also spatially organized within paraspeckles, with distinct localizations at the core, shell, and patch regions (Fig. 1E; Kawaguchi et al. 2015; West et al. 2016).

Multiple lines of evidence support the concept that paraspeckles are phase-separated nuclear bodies. First, fluorescence recovery after photobleaching (FRAP) experiments revealed that PSPs are dynamically exchanged between paraspeckles and the surrounding nucleoplasm (Mao et al. 2011; Audas et al. 2016; Wang et al. 2018). Second, paraspeckles are sensitive to 1,6-hexanediol, which mainly disrupts hydrophobic interactions and contributes to the formation of phase-separated subcellular bodies (Yamazaki et al. 2018b). Third, the abilities of the PSPs FUS and RBM14, which are essential for paraspeckle formation, to form hydrogels in vitro are essential for paraspeckle assembly (Hennig et al. 2015; Fox et al. 2018). Fourth, paraspeckles undergo a fusion and fission process termed “kiss-and-run fusion” (Mao et al. 2011; Yang et al. 2019). A recent review article reported that paraspeckles are induced by polymer–polymer phase separation rather than the typical liquid–liquid phase separation (Peng and Weber 2019). Taken together, these findings suggest that paraspeckles are massive phase-separated subnuclear structures with a distinct shape and ordered interior configuration.

BIOGENESIS OF THE NEAT1_2 arcRNA AND PARASPECKLES

Synthesis of the NEAT1 isoforms is regulated by alternative 3'-end processing governed by HNRNPK and TDP-43 (Naganuma et al. 2012; Modic et al. 2019). The 3' end of NEAT1_2 contains an unusual triple helix structure (3'TH) that is essential for stabilization of the arcRNA (Fig. 1C; Brown et al. 2012; Wilusz et al. 2012; Yamazaki et al. 2018b). Seven PSPs and the SWI/SNF complex are essential for paraspeckle formation (Fig. 2; Naganuma et al. 2012; Kawaguchi et al. 2015; West et al. 2016). Specifically, SFPQ, NONO, and RBM14 are essential for NEAT1_2 stability (Naganuma et al. 2012), and HNRNPK controls isoform switching to the NEAT1_2 isoform by inhibiting polyadenylation of the NEAT1_1

Figure 2. Two distinct processes of paraspeckle formation. NEAT1_2 ribonucleoproteins (RNPs) are transcribed by RNA polymerase II (RNAPII) and then approximately 50 NEAT1_2 RNPs assemble into paraspeckles through phase separation. PSPs that are essential for paraspeckle integrity can be divided into two categories: category 1A includes PSPs required for NEAT1_2 expression and category 1B includes PSPs required for paraspeckle assembly. Putative RNA elements are shown on the NEAT1_2 structure (left).

isoform (Fig. 2; Naganuma et al. 2012). NONO, FUS, DAZAP1, HNRNP3, and the SWI/SNF complex are essential for paraspeckle assembly (Naganuma et al. 2012; Kawaguchi et al. 2015; Yamazaki et al. 2018b), and RNA polymerase II transcription is also essential for paraspeckle formation (Fig. 2). Upon transcriptional inhibition, paraspeckles are rapidly disassembled alongside NEAT1_2 degradation and relocation of PSPs to nucleolar cap structures (Fox et al. 2005; Chujo et al. 2017). Paraspeckles are formed in close proximity to the *NEAT1* gene loci, likely because NEAT1_2 lncRNAs are highly concentrated at these regions during transcription, and thus the environment favors phase separation by facilitating molecular interactions among RNAs and proteins. This evidence suggests cotranscriptional formation of paraspeckles via phase separation (Mao et al. 2011; Yamazaki et al. 2018b). During this process, approximately 50 NEAT1_2 lncRNAs are incorporated into a single spherical paraspeckle (Fig. 2; Chujo et al. 2017). The paraspeckle is a highly dynamic structure; FRAP studies revealed that 60%–70% of PSPs within paraspeckles are recovered within several minutes (Mao et al. 2011; Audas et al. 2016; Wang et al. 2018). In addition, a recent study showed that elongated, oblong paraspeckles show slower mobility than spherical paraspeckles (Wang et al. 2018). These data indicate the existence of slow or immobile fractions of paraspeckles and that paraspeckle dynamics are related to their shape.

FUNCTIONAL RNA DOMAINS OF THE NEAT1_2 arcRNA

Although the biological importance of various lncRNAs has been shown, the specific sequences and structural elements required for their biogenesis and functions remain poorly understood (Hirose et al. 2014a). Nonetheless, some lncRNAs have been studied intensively. A previous study examining the XIST lncRNA revealed the existence of functional modular repeat domains involved in X-chromosome inactivation (Wutz et al. 2002). Subsequent studies identified the subdomains of the XIST lncRNA and their partner proteins required for specific functions (Chu et al. 2015; Monfort and Wutz 2017; Colognori et al. 2019). We recently identified multiple modular domains of NEAT1 involved in its biogenesis and the formation of paraspeckles (Yamazaki et al. 2018b). Similar to that of many other lncRNAs, the cross-species homology of mammalian NEAT1_2 sequences is markedly lower than those of protein-coding mRNAs. In general, mammalian NEAT1_2 sequences only display homology at the 5' region (~1.6 kb), short stretches in the internal regions, and the 3' TH (Fig. 3A, B). Nevertheless, mouse *Neat1_2* can form paraspeckles in human cells, suggesting that the functions of NEAT1_2, at least in paraspeckle formation, are conserved between mouse and human (Fig. 3C; Naganuma et al. 2012).

Figure 3. Conservation of the sequences and functions of NEAT1_2 between human and mouse. (A) A dot plot showing the homologous regions of the human and mouse NEAT1_2 sequences. The BLAST search parameter was “somewhat similar.” (B) A screenshot of the UCSC genome browser record for the human *NEAT1* gene locus with modifications. Sequence conservation (PhastCons) and positions of repetitive elements identified by a RepeatMasker algorithm are shown. (C) Transient expression of mouse *Neat1_2* (mNeat1_2) induces paraspeckles in human HeLa cells (magenta signals). Scale bar, 5 μ m.

To dissect the roles of specific sequences within the human NEAT1_2 lncRNA, we performed extensive deletion analyses using CRISPR–Cas9 and the human haploid HAP1 cell line (Yamazaki et al. 2018b). These analyses revealed that multiple functional RNA domains of NEAT1_2 dictate its own biogenesis and the coupled formation of paraspeckles. The 5' end (first 1 kb) and 3' TH of NEAT1_2 (termed the A domains) are necessary for stability of the lncRNA (Yamazaki et al. 2018b). The regions upstream (2.1–2.8 kb) and downstream (4–5 kb) of the polyadenylation site of NEAT1_1 (termed the B domains) promote the expression of NEAT1_2 by repressing NEAT1_1 polyadenylation, indicating a role in isoform switching from NEAT1_1 to NEAT1_2 (Yamazaki et al. 2018b). The middle domain of NEAT1_2 (termed the C domain) is necessary and sufficient for paraspeckle assembly. Notably, our CRISPR dissection analysis also revealed that the C domain contains several subdomains (C1: 9.8–12 kb; C2: 12–13 kb; C3: 15.4–16.6 kb) that have redundant functions in paraspeckle assembly (Yamazaki et al. 2018b). Such redundancy is also found among the C domain and the unidentified outside domain, suggesting that the presence of multiple redundant domains confers a robust competency of NEAT1_2 to build paraspeckles (Fig. 4).

In our recent study, artificial tethering of NONO, SFPQ, or FUS, but not RBM14, rescued paraspeckle assembly in cells expressing a NEAT1 mutant lacking the C1 and C2 subdomains (Yamazaki et al. 2018b). Moreover, the rescue activity of NONO required dimerization with DBHS family proteins, including SFPQ, PSPC1, and NONO itself, which is also required for the oligomerization of these proteins along the RNA molecule. CLIP-seq data showed that the C domain of NEAT1_2 contains multiple binding sites for NONO/SFPQ, which are likely to be essential for paraspeckle formation (Naganuma et al. 2012; Passon et al. 2012; Lee et al. 2015; Huang et al. 2018; Yamazaki et al. 2018b). The in vitro–transcribed RNA derived from the C2 domain preferentially binds to NONO/SFPQ and induces the formation of higher-order structures that are sensitive to 1,6-hexanediol and depend on NONO/SFPQ (Yamazaki et al. 2018b). In addition, the NONO/SFPQ oligomer seems to recruit additional essential PSPs such as FUS and RBM14, which in turn likely induce phase separation (Hennig et al. 2015; Yamazaki et al. 2018b). We recently found that the NEAT1_2 arcRNA is poorly extracted by conventional RNA purification methods using acid guanidinium thiocyanate–phenol–chloroform reagents such as TRIzol (Thermo Fisher Scientific). This unusual feature,

which we termed “semiextractability,” depends on NONO, FUS, or the NEAT1_2 C domain, suggesting that the tenacious interactions between these proteins and the C domain, which can probably tolerate guanidinium thiocyanate and phenol-containing harsh conditions, contribute to arcRNA-induced cellular body formation (Chujo et al. 2017; T Yamazaki, unpubl. observ.). SFPQ forms higher-order structures by cooperative spreading and coating the nucleic acids, and a similar spreading mechanism is also proposed to play a role in splicing regulation by HNRNPA1 (Zhu et al. 2001; Lee et al. 2015). Hence, this spreading mechanism might play an important role in the initial process of paraspeckle formation.

In addition to the A, B, and C domains, NEAT1_2 contains UG repeat stretches that are evolutionally conserved among multiple species. These repeat sequences are thought to be the major binding sites of TDP-43 (Tollervey et al. 2011). When we deleted the UG repeats from NEAT1_2 using CRISPR–Cas9, recruitment of TDP-43 to the paraspeckles was reduced dramatically (Modic et al. 2019). Furthermore, knock-in of a long UG repeat stretches into the UG-deleted NEAT1_2 mutant recovered the recruitment of TDP-43, suggesting that the UG repeat stretches are necessary and sufficient for TDP-43 recruitment to paraspeckles (Fig. 4; Modic et al. 2019).

As described above, NEAT1_2 is looped and spatially organized within paraspeckles (Fig. 4). Our super-resolution microscopy analyses revealed that deletion of the 3' region of NEAT1_2 (Δ 16.6–22.6-kb mutant) alters its configuration (Yamazaki et al. 2018b). In wild-type cells, the 3' end of NEAT1_2 is located in the shell of the paraspeckle, but the 3' end of the Δ 16.6–22.6-kb mutant localizes to the paraspeckle core. This finding suggests that the configuration of NEAT1_2 is determined by one or more of its domains. By extension, we speculate that other NEAT1_2 domains will also contribute to the organization of the lncRNA within paraspeckles.

NEAT1_1, which can be regarded as one of the functional domains of the *NEAT1* gene, forms “microspeckles,” suggesting a role outside of paraspeckles (Li et al. 2017a). A recent study showed that NEAT1_1-specific knockout mice do not show an aberrant phenotype, although a function of NEAT1_1 under specific conditions cannot be excluded (Adriaens et al. 2019; Isobe et al. 2019). Further investigations may reveal specialized roles of NEAT1_1 under certain cellular conditions.

Several recent studies have suggested the presence of additional functional NEAT1 domains. Genome-wide

Figure 4. The functional modular domain structure of the human NEAT1_2 arcRNA. The functional domains of NEAT1_2 and their positions (kb, kilobases from the 5' end of NEAT1_2) are shown. (PAS) Polyadenylation signal.

mapping of R-loop-forming regions revealed that the 5'-terminal region of NEAT1 (~1400 nt) forms a long R-loop (DNA–RNA hybrid), although its biological importance remains unknown (Fig. 4; Dumelie and Jaffrey 2017). Another recent study showed that the 5'-terminal regions of NEAT1, including the GA- and CU-rich regions, form DNA:RNA triplex structures with many human genomic regions (Fig. 4; Senturk Cetin et al. 2019). Consistent with these two reports, the 5'-terminal sequence of NEAT1 is evolutionarily conserved (Fig. 3A), suggesting a sequence-based mechanism for the biological functions of NEAT1, including target gene recognition. In addition, the 3'-terminal region of human NEAT1_2 harbors a pseudo-microRNA (miR-612) that is not processed into mature microRNA (miRNA) but attracts microprocessors to process pri-miRNAs to pre-miRNAs in the nucleus (Jiang et al. 2017). Overall, as described in this section, the multiple modular domains of NEAT1 determine its biogenesis and the composition, biophysical properties, and functions of paraspeckles.

CURRENT MODEL OF PARASPECKLE FORMATION

Based on the studies described above, we propose a model of paraspeckle formation (Fig. 5). In this model, NEAT1_2 lncRNAs are transcribed by RNA polymerase II and PSPs associate with nascent NEAT1_2 transcripts at the *NEAT1* gene loci (Fig. 5, step (1); Mao et al. 2011). PSPs such as NONO and SFPQ, which are essential for paraspeckle assembly, are loaded onto the multiple functionally redundant high-affinity binding sites of NEAT1_2 and then spread and coat NEAT1_2 domains to form base ribonucleoprotein (RNP) complexes through the NOPS and coiled coil domains of NONO and SFPQ (Fig. 5, steps (2) and (3); Lee et al. 2015; Yamazaki et al. 2018b). Subsequently, the NEAT1_2 RNPs further assemble into paraspeckles (50 NEAT1_2 RNPs per paraspeckle sphere), likely through phase separation. Concurrently, FUS and RBM14 are recruited to the RNPs, likely by NONO and SFPQ, and promote paraspeckle assembly via multivalent interactions between their PLDs (Fig. 5, steps (4) and (5); Hennig et al. 2015; West et al. 2016; Chujo et al. 2017; Yamazaki et al. 2018b). During this process, NEAT1_2 RNPs are bundled and folded (Fig. 5, step (5); West et al. 2016).

It is possible that NEAT1_2 lncRNAs interact with each other via direct intermolecular RNA–RNA interactions to contribute to the phase separation process (Fig. 5, step (2); Van Treeck and Parker 2018). This possibility is supported by the fact that weak NEAT1_2 focal signals remain detectable when PSPs are almost completely obliterated by 1,6-hexanediol treatment (Yamazaki et al. 2018b). Several studies have highlighted the importance of RNA–RNA interactions in the formation of cellular bodies, including stress granules, and disease-associated RNA foci caused by aberrant repeat expansions. It is proposed that the sum of the RNA–RNA, RNA–protein, and protein–protein interactions determines whether arcRNA–

protein complexes undergo phase separation that eventually leads to cellular body formation (Van Treeck and Parker 2018).

FUTURE PERSPECTIVES

Despite recent advances in our understanding of how the NEAT1_2 domains and their partner proteins dictate paraspeckle formation and functions, many questions still remain. First, although NONO and SFPQ have been identified as partner proteins of the NEAT1_2 C domain, it is important to identify the partner RBPs and specific RNA motifs (e.g., sequences and/or secondary structures) required for proper functioning of the other NEAT1_2 functional domains, especially those involved in inducing phase separation. Second, as NEAT1_2 is involved in various physiological and pathological conditions, it is important to investigate how the molecular functions driven by specific RNA domains/motifs link to physiological functions.

We identified NONO and SFPQ as partner proteins of the NEAT1_2 C domain based on prior knowledge of the protein composition of paraspeckles and the results of experiments using immunofluorescence analyses of PSPs on NEAT1 mutants, MS2 tethering, CLIP-seq, and in vitro RNA pulldown (Yamazaki et al. 2018b). These approaches should also enable the identification of partner proteins of the other functional NEAT1_2 domains. To narrow down the functional domains of NEAT1_2 to precise RNA motifs, a large CLIP-seq data set, including eCLIP data, would be a great resource to differentiate between direct and indirect binding proteins and determine their binding sites (Van Nostrand et al. 2016). Analyses of the secondary structure of NEAT1_2 would also be important (Lu et al. 2016; Lin et al. 2018). For example, it would be useful to identify the specific RNA motifs in the NEAT1_2 C domain required for paraspeckle assembly via phase separation. It would also be interesting to understand how specific NEAT1_2 motifs are conserved between species. Furthermore, it would be helpful to understand how the specific domains and motifs within NEAT1_2 can initiate cellular body formation and determine their properties and functions.

Various molecular and physiological or pathological functions of NEAT1_2 have been reported. One of the molecular functions of paraspeckles is to regulate gene expression by acting as molecular sponges that incorporate specific RBPs and RNAs such as IRAlu-containing RNAs, CTN RNA, AG-rich RNAs, and miRNAs (Chen and Carmichael 2009; Hirose et al. 2014b; Imamura et al. 2014; West et al. 2016; Jiang et al. 2017). In addition, the global association of NEAT1/paraspeckles with chromatin has been shown by multiple methods, including CHART-seq, ChIA-PET, GRID-seq, and RADICL-seq (West et al. 2014; Cai et al. 2016; Li et al. 2017b; Bonetti et al. 2019). Under physiological conditions, NEAT1_2 is required for the establishment of pregnancy, mammary gland development, mitochondrial functions, and efficient regulation of gene expression for pluripotency and

Figure 5. An updated model of paraspeckle formation showing the processes involved in *NEAT1_2* transcription through paraspeckle formation via phase separation. See the main text for more details.

cellular differentiation (Nakagawa et al. 2014; Standaert et al. 2014; Wang et al. 2018; Modic et al. 2019). A number of studies have highlighted the importance of *NEAT1* as a direct target of the p53 central tumor suppressor protein in cancer (Dong et al. 2018; Nakagawa et al. 2018). *NEAT1* is also involved in various other pathological conditions, including neurodegenerative diseases and viral/bacterial infections (Nishimoto et al.

2013; Imamura et al. 2018). Additional research into the relationship between the RNA motifs of *NEAT1* and its physiological functions is required to clarify the mechanisms of actions of arcRNAs and the biological significance of nuclear body formation on these molecules.

Several recent studies have shown that arcRNAs are still hidden within genomes. Using our recently developed

method that relies on the semiextractable feature of arcRNAs, we performed a genome-wide screen of HeLa cells and identified 45 candidate arcRNAs (Chujo et al. 2017). These candidates include lncRNAs, pre-mRNAs, and repeat-derived RNAs and form nuclear foci that are distinct from known nuclear bodies (Chujo et al. 2017). In addition, another recent study showed that short tandem repeat-containing RNAs (strRNAs) might be a rich source of arcRNAs (Yap et al. 2018). One of the strRNAs is a core of the perinucleolar compartment. As most arcRNAs are induced by various stimuli and/or specific physiological or pathological conditions, searches performed under various conditions will likely expand the repertoire of known arcRNAs.

Because known arcRNAs have partner proteins, several approaches can be used to identify the components of arcRNA-induced cellular bodies. Most PSPs identified to date were found by colocalization screening using a cDNA library and fluorescent fusion proteins (Naganuma et al. 2012; Fong et al. 2013; Mannen et al. 2016). Our recent comprehensive ChIRP-MS (chromatin isolation by RNA purification followed by mass spectrometry) analysis revealed the compositions of nSBs, among which most of the proteins (141) were newly identified (Ninomiya et al. 2019). Proximity labeling methods such as APEX and BioID have also been used to determine the compositions and dynamic compositional changes of phase-separated cellular bodies (Markmiller et al. 2018; Youn et al. 2018; Padron et al. 2019). These approaches could be used to obtain a comprehensive list of the components and their dynamic exchanges within cellular bodies under various cellular conditions. Future studies should also reveal the mechanisms by which cellular body compositions are determined by arcRNAs and their motifs.

It is well known that arcRNAs can induce phase transitions between several material states (e.g., liquid, hydrogel, and solid) of cellular bodies. Exactly how these material properties are determined remains unanswered. In particular, it is unclear which protein components contribute to each state. The binding sites of the proteins on arcRNAs, including their affinities and densities, are still unknown, and the motifs and properties (e.g., amino acid compositions in PLDs, LCDs, or IDRs) of the proteins that determine the material states also require clarification. In vitro reconstitution of arcRNA-induced bodies using purified components or cellular/nuclear extracts should elucidate how they are constructed and how their biophysical properties are determined (Maharana et al. 2018). In addition to paraspeckles, other arcRNA-induced bodies also show various distinct morphologies. For example, nSBs display island structures that are composed of dense cores and shells (Kawaguchi et al. 2015), and amyloid bodies have filamentous structures (Audas et al. 2016). Therefore, it would be interesting to delineate how these morphologies are determined by arcRNAs and their partner proteins.

Identification of both the partner proteins and the precise binding sites as RNP modules should enable artificial control and reprogramming of arcRNAs. Motif-specific functional inhibition (or activation) might be

achievable using antisense oligos, small molecules, or CRISPR-based strategies. In future analyses of arcRNAs, theoretical models and simulations, in addition to new methodologies and quantitative analyses, would offer novel frameworks to explain the mechanisms involved in complex biological phase separation.

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