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Substoichiometric action of long noncoding RNAs

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Low expression levels and stoichiometric imbalances of long noncoding RNAs (lncRNAs) are often used as evidence for their probable lack of function or for limiting the scope of their potential influence. Recent advances in our understanding of the substoichiometric functions of lncRNAs challenge these notions and suggest routes through which unabundant lncRNAs can affect cellular functions and gene regulatory networks.

High expression levels are considered a hallmark of functional lncRNAs. This makes sense as low expression imposes several restrictions on our ability to study lncRNAs. These limitations include difficulties in detecting and quantifying molecules present in low abundance and further challenges in functional interrogations in vitro and in vivo (reviewed in ref. [1]). Conceptually, as lncRNAs often interface with substantially more abundant factors, low expression challenges several biochemical principles of competition, cooperativity, stoichiometry and the law of mass action[2], which govern key aspects of genome regulation and, more broadly, cellular function[3,4]. However, many lncRNAs associated with specific and robust phenotypes appear to exert regulatory influences despite stoichiometric disadvantages compared with their targets or cofactors[1,5]. This is especially true for proteins, which are typically expressed at ratios tens of thousands of times higher than their RNA interactors[1]. Nonetheless, lncRNAs can evidently utilize mechanisms to overcome such imbalances, prominently the formation of biomolecular condensates[6,7]. Such mechanisms not only diversify the potential ways in which RNAs engage with their targets but, together with the higher cell, tissue and context specificity of these molecules, they also potentially endow organisms with increased complexity[7,8].

Until recently, these mechanisms have remained elusive and difficult to probe, but new techniques and re-emerging biological concepts now offer additional evidence to support the connection between lncRNA biology and substoichiometric modes of action[9].

Substoichiometric action of lncRNAs through biomolecular condensates

Biomolecular condensates are emerging as relevant cell organizing units. Regardless of whether they form through phase separation or other mechanisms, the formation of membraneless compartments within the cell provides a dynamic layer of regulation that could be behind the function of a growing number of stoichiometrically challenged lncRNAs.

Formation of phase-separated compartments. In the past decade, phase separation has experienced a resurgence owing in part to a pioneering study that described membraneless compartments or P granules in *Caenorhabditis elegans*. These granules showed liquid-like properties, which meant that they could deform, drip, fuse and dynamically exchange content with their surroundings, traits now recognized as hallmarks of phase-separated condensates[10,11].

Like P granules, many of the so far characterized phase-separated compartments depend on RNA for their biogenesis, size, liquidity, composition, positioning and/or functionality[7,12]. Although several RNA biotypes are capable of phase separation, including ribosomal RNAs in nucleoli[13], pre-mRNAs in histone locus bodies[14] or mRNAs in stress granules[15,16], lncRNAs appear to be among the most versatile facilitators of phase separation across cell compartments and species[1,17]. For example, the abundant lncRNA NEAT1 is essential in the formation of paraspeckles, while also being responsible for many of their biophysical properties, functional traits and localization within nuclei[18,19]. More recently, the formation of phase-separated condensates has been suggested as a mechanism to explain the substoichiometric action of lncRNAs in several contexts, including DNA damage repair[20], signal transduction[21], development[22] and chromatin architecture[23], among others. Notably, in many studies, condensate formation is assigned as a functional principle through descriptive or phenomenological evidence; however, a thorough quantitative assessment of the abundance of lncRNAs and their main partners within the relevant compartments and under physiological conditions remains scarce[24]. Perhaps two of the best-described examples to date are those of the lncRNAs NORAD and Xist.

The lncRNA NORAD regulates the activity of Pumilio (PUM) proteins to prevent chromosome instability arising from the hyperactive repression of PUM targets[25–27]. PUM proteins are expressed at high levels in mammalian cells, reaching up to ~15,000 PUM1 and ~2,000 PUM2 molecules per HCT116 cell[25]. By contrast, the same cell expresses ~400 NORAD molecules, and yet NORAD effectively competes with other mRNAs with PUM response elements (PREs) that all together provide >300,000 PREs per cell[26] (Fig. 1a). Although NORAD shows high valency for PUM through 18 canonical PREs found in each NORAD molecule, the ~7,200 PREs provided by NORAD per cell are estimated to be more than 40-fold fewer than PREs from other cellular mRNAs. NORAD has been recently described to promote the formation of phase-separated PUM condensates called "NP bodies"[26] that accumulate around 50% of the PUM cellular pool in HCT116 cells. These condensates undergo liquid–liquid phase separation (LLPS) mediated by PUM intrinsically disordered regions (IDRs), and enable NORAD to sequester a super-stoichiometric amount of PUM that outcompetes other PRE-containing RNAs. This is achieved through two mechanisms of PUM recruitment. First, the stoichiometric multivalent interactions of NORAD with PUM allow a higher concentration of PUM in the areas occupied by NORAD than the surrounding areas. This process depends on NORAD PREs and the RNA-binding capacities of PUM and helps nucleate the NP bodies. Second, PUM–PUM interactions mediated by their IDRs further

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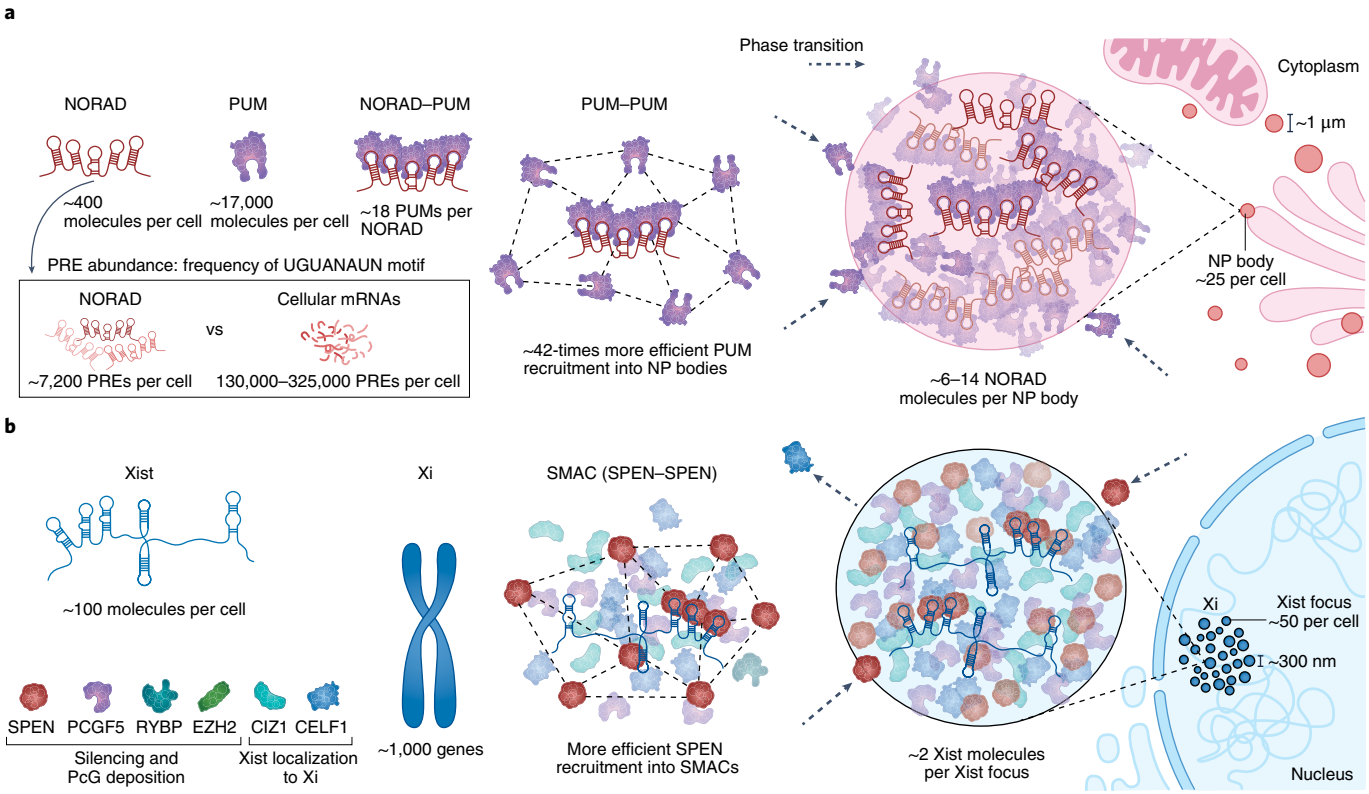

Fig. 1 | The lncRNAs NORAD and Xist induce phase transitions with distinct regulatory functions. a, Sequestration and inhibition of activity. NORAD–PUM multivalent interactions together with PUM–PUM IDR-mediated interactions induce the formation of phase-separated NORAD–PUM condensates or NP bodies[26]. These enable NORAD to sequester a supra-stoichiometric amount of PUM, thereby outcompeting other RNAs with PREs and effectively repressing PUM activity[25,26]. **b**, Concentration or recruitment of interacting regulators. Xist interacts with several factors required for XCI[45] and can induce the formation of Xist foci that allow Xist to seed a phase transition into SMACs containing several XCI factors, including SPEN[32], which appear to be driven by IDR-mediated SPEN–SPEN interactions. Efficient SPEN recruitment is required for effective silencing of the entire Xi[32,35].

amplify the supra-stoichiometric recruitment of PUM and consolidate the phase-separated compartment (Fig. 1a). In this manner, LLPS provides each PRE in NORAD with an estimated 42-times higher efficiency of PUM binding than isolated PREs[26], which effectively titrates the activity of PUM from other targets, increases their expression and therefore prevents genome instability[25].

By engaging in a conceptually similar mechanism, the lncRNA Xist controls X chromosome inactivation (XCI) and dosage compensation. Xist mediates chromosome-wide silencing of genes located only on the inactivated X chromosome (Xi) to compensate for X chromosome dosage in female cells. Importantly, Xist can perform these tasks at a remarkable stoichiometric disadvantage of about one Xist molecule per ten target genes or per megabase of silenced genomic DNA[28–30] (Fig. 1b). Therefore, it is unsurprising that Xist utilizes multiple mechanisms for XCI. One of these strategies involves the creation of high concentration territories close to its transcription site[31] and the formation of Xist foci that help nucleate supramolecular complexes (SMACs)[32].

Similar to NORAD, which provides a high PUM valency for seeding NP bodies, Xist A-repeats probably provide multivalency for SPEN (also known as SHARP) and most of its functional interactors at the RNA–protein interface. The precise nature of RNA recognition by SPEN is less clear; thus, it is currently uncertain how many SPEN molecules can simultaneously bind the A-repeats[33,34]. According to a recent study[35], initial Xist–SPEN interactions support further homotypic SPEN–SPEN interactions to trigger SPEN concentration gradients in the nucleus (Fig. 1b). As in NORAD–PUM bodies, these Xist–SPEN assemblies appear to be driven by SPEN IDR interactions[32,35] and were described to be nucleated by an average of 2 Xist molecules and ~35 SPEN molecules[32]. Further homotypic and heterotypic protein–protein interactions enable the formation of ~50 Xist foci, which are confined to a Xi territory and are enriched in SPEN and in several other factors that help Xist localization to the Xi, Polycomb deposition and silencing propagation across the entire Xi[32] (Fig. 1b). Although the sharp increase in XCI factors at the boundary of Xist foci supports a phase transition, it is unclear whether Xist foci or SMACs exhibit features of liquid–liquid or other types of phase separation[32]. After foci nucleation, partition and maturation of SMACs, some components remain enriched at the Xi after Xist deletion[36]. Indeed, XCI was shown to be maintained independently of Xist in the late differentiation of mouse embryonic stem cells[37], and the molecular ratio of Xist to Xi genes suggests that XCI happens at regions not directly occupied by Xist[28–30]. This allows room for mutually dependent silencing mechanisms, including Polycomb deposition, a process previously described as essential to spread silencing across the Xi through various mechanisms (reviewed in ref. [38]), including chromatin reconfiguration and compaction[31,32,39]. Indeed, compaction-deficient clones show alterations in the reorganization of genes around Xist foci, which results in deficient clustering of SMACs and impaired silencing[32].

There are notable similarities between how NORAD and Xist bypass stoichiometric imbalances. Both seem to benefit from homotypic interactions of IDRs to nucleate phase transitions[7]. As with many other lncRNAs, NORAD and Xist sequences are partially repetitive, which facilitates multivalent interactions. Either through discrete binding motifs, as in the case of the PREs of NORAD, or clusters of the A[40], B[32] and E[36,41] repeats in Xist, repetitive sequences have proven essential for the functionality and phase transitions

seeded by these and other RNAs[42]. Emerging evidence also points to the contribution of the RNA structure to the function of NORAD and Xist. A recent preprint[43] suggests that NORAD folds in a way that brings some of the PREs into close spatial proximity within a module that could facilitate NP body formation. This is further supported by the observation of a critical number of four PREs being required for NP body formation, but only in specific contexts, as the 3′ untranslated regions of mRNAs with multiple PREs did not sustain efficient NP body formation[26]. Similarly, the module containing the approximately eight A-repeats found in Xist has been proposed to fold into four structural units necessary for the binding of SPEN and the nucleation of the XIST–SPEN complexes[34]. Notably, single copies of the A-repeat motif did not show specific recognition by SPEN[33], nor were they sufficient for mediating silencing[44]. This suggests that the structure and modular clustering of NORAD PREs and Xist A-repeats are relevant for the cooperative binding of PUM and SPEN, respectively, and are therefore relevant for the biomolecular condensates seeded by both lncRNAs.

Despite the remarkable analogies between both RNAs, it is important to highlight the differences as they use the same mechanism with drastically distinct outcomes. On the one hand, Xist recruits a supra-stoichiometric number of RNA-binding proteins (RBPs) to concentrate and spatially contain XCI[32]. On the other hand, NORAD sequesters PUM proteins to titrate and inhibit their activity[25,26]. Differences are also evident in the enrichment of interactors in each compartment. It is currently unknown whether other proteins besides PUM are enriched in NPs. Notably, the Xist binding proteome is remarkably diverse[45], with some factors being recruited during Xist foci maturation without showing accumulation, or even showing depletion, which suggests that there is finely regulated stepwise recruitment and eviction of interactors at discrete spatiotemporal windows[32,46] (Fig. 1b).

Phase separation-independent concentration gradients. Some biomolecular condensates form through LLPS; however, this is not the only mechanism by which they are formed. Several lncRNAs have been proposed to work as spatial organizers in the nucleus to induce the formation of discrete concentration gradients without forming phase-separated compartments[9]. In fact, RNA is thought to regulate the formation, physicochemical properties, composition and half-lives of these high concentration territories[7]. The recent development of RNA and DNA SPRITE (RD-SPRITE) has allowed the high-resolution three-dimensional (3D) mapping of the DNA–RNA and RNA–RNA interactions. This study suggests a model in which noncoding RNAs (ncRNAs) can modulate the formation of spatially and functionally delimited concentration gradients of the ncRNAs themselves, other diffusible RNAs and proteins, where distinct nuclear functions are favoured[47]. Many of these concentration territories could be maintained by lncRNAs that remain in 3D proximity to their loci, such as Xist, Airn, Kcnq1ot1, Pvt1 or Chaserr[47].

In this model, lncRNAs could help seed concentration gradients in specific spatial territories where they can act at substoichiometric ratios[9] (Fig. 2). These territories do not necessarily have a delimited phase-transition interface, but they probably share many features of membraneless compartments. They could grow, dynamically exchange contents with their surroundings and even coalesce[48]. They could also bridge distinct genomic regions into shared territories in the nucleus to control several processes underpinning successful gene regulation, RNA processing and heterochromatin assembly[47].

Some well-described lncRNAs that probably exploit this mechanism of action include Airn and Kcnq1ot1. Both lncRNAs can induce chromatin silencing over multi-megabase domains despite being expressed at fewer than ten copies per cell. Throughout the Airn domain, silencing is primarily defined by pre-existing genome architecture, the abundance of the RNA itself and the distance to the lncRNA locus, where specific CpG islands (CGIs) nucleate the spread of Polycomb repressive complexes (PRCs) after exposure to the lncRNA[49]. Airn was suggested to work in such a way to silence the imprinted genes *Slc22a3*, *Slc22a2* and *Igf2r* in mouse placenta[50]. Airn was shown to interact directly with the *Slc22a3* promoter and recruit the G9a H3K9 histone methyltransferase, inducing allele silencing. Paternal silencing of *Slc22a2* and *Igf2r* also depended on Airn in this context; however, they did not require a direct interaction with Airn or even G9a. Instead, it was suggested that Airn could create a repressive nuclear compartment similar to Xist. The chromatin environments surrounding Airn, Kcnq1ot1 and Xist suggest a common mechanism to engage and spread the epigenetic silencing machinery over megabase-sized domains[49]. This may be enabled by RBPs such as HNRNPK, which binds both lncRNAs and PRCs, as shown for Xist[51]. These RBPs may nucleate super-stoichiometric interactions with themselves and other proteins to form individual foci with high levels of PRCs, as previously shown for PRC-mediated repression of developmental loci[52]. Additionally, a lncRNA carrying a cargo of PRCs could initiate PRC spread in a 3D contact domain[49]. A similar model has been proposed for the lncRNA Firre. Deletion of *Firre* resulted in the loss of proximity of several *trans*-chromosomal loci to the *Firre* locus, and this is dependent on a physical interaction of Firre with HNRNPU. This study suggests that through such interactions with nuclear-matrix proteins such as HNRNPU, Firre could provide a 'zip code' for the formation of nuclear subcompartments[53]. Similar findings have been reported for HNRNPU and SAF-B in the regulation of the interphase chromosome structure together with chromatin-associated RNAs. Whereas HNRNPU seems to form high concentration territories through oligomerization[54], SAF-B has been proposed to do so by phase separation[23].

Although low-to-moderate expression appears as a disadvantage to be compensated for, recent work suggests that the relatively low expression of Xist is important for constraining XCI to the Xi and avoiding silencing on other chromosomes. Indeed, finely tuned artificial overexpression of Xist from the Xi led to increased Xist localization at autosomal regions[35]. Similarly, overexpression of Airn from its endogenous locus led to the spreading of chromatin marks associated with epigenetic silencing beyond their regular boundaries[49]. These studies suggest that while lncRNAs can help nucleate functional spatial compartments within cells, their activity is spatially limited owing to their low abundance.

Additional mechanisms of substoichiometric action of lncRNAs

Biomolecular condensates are appealing as facilitators of substoichiometric action because ultimately, within the condensate, they enable the relative accumulation of factors that brings them closer to stoichiometric balance. Another possibility, akin to enzymes, is the ability to support the conversion of supra-stochiometric amounts of substrate into product at high enough rates that allows a few molecules to induce physiologically relevant changes to a much more abundant pool of targets. This type of mechanism has been described for the lncRNAs Cyrano (also known as OIP5-AS1) and SLERT, although with marked differences and outcomes.

Target-directed microRNA degradation. MicroRNA (miRNA) molecules are typically long-lived, with half-lives four times longer than those of mRNAs, sometimes reaching days[55]. This is mainly attributed to their loading into complexes with Argonaute (AGO) proteins that protect them from exonuclease degradation[56]. However, some physiological contexts require rapid miRNA turnover, sometimes induced by specific signalling cues[57]. The rapid decay of some miRNA molecules is regulated by a recently described mechanism called target-directed miRNA degradation (TDMD). Highly complementary base-pairing between a miRNA and a

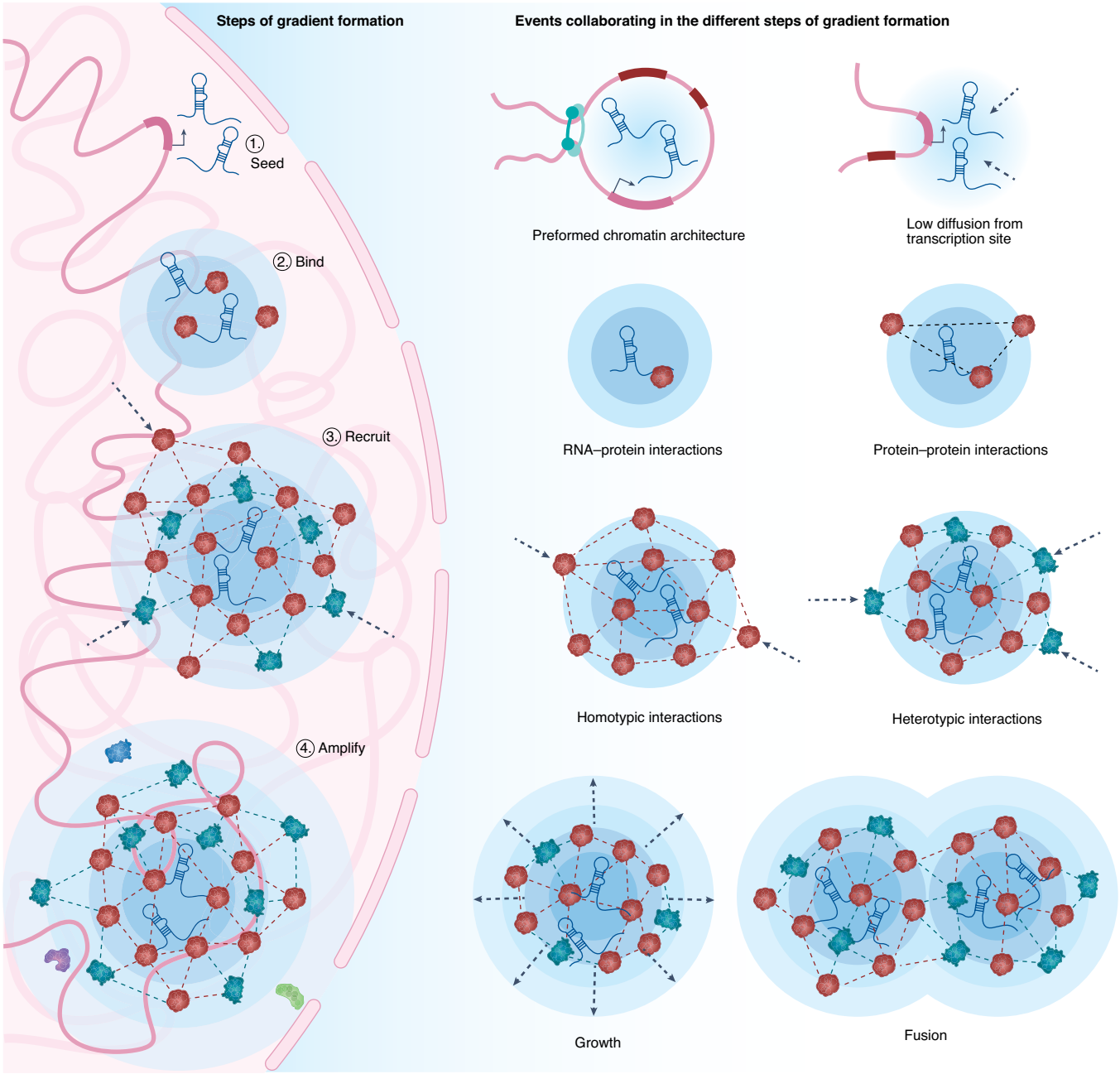

Fig. 2 | ncRNAs are essential for the formation and function of nuclear concentration gradients. In the proposed model, ncRNAs help seed local concentration gradients close to their loci, a process that is probably influenced by predefined genome architecture[49] and their low diffusion rate from transcription sites[47,94] (step 1). RNA–protein interactions and protein–protein interactions favour the concentration of otherwise diffusible factors such as proteins and other RNAs[9,47] (step 2). Further supra-stoichiometric recruitment of protein factors is enabled by homotypic and/or heterotypic interactions[49,54] (step 3). The size of these condensates can be further amplified by the levels of ncRNA expression[49] as in Xist[35] and perhaps even by the fusion of compartments that could bring distant genomic regions into high-concentration territories in the nucleus[47,53] (step 4).

target, which is rare in endogenous genes, promotes AGO proteolysis mediated by ZSWIM8 Cullin-RING E3 ligase, which exposes the miRNA for degradation[58,59]. This is the case for the lncRNA Cyrano, which reduces miR-7 levels through TDMD to de-repress miR-7 targets[60,61]. Cyrano-mediated TDMD of miR-7 does not affect Cyrano stability, which allows it to be recycled to degrade an estimated 16 additional miR-7 molecules, as revealed by recent quantitative analyses[60]. This mechanism has been observed at much lower efficiencies for viral RNAs[62], endogenous RNAs[63,64] and artificial sequences[65], thereby highlighting the remarkable 17–170-times higher capacity of Cyrano-mediated TDMD. Such TDMD efficiency allows ~100 copies of Cyrano per cell to limit miR-7 expression from ~1,800 copies to around 40 molecules per cell[60] (Fig. 3a). This prevents miR-7-dependent degradation of *Cdr1as*, a circular RNA with reported functions in neuronal activity[60,61,66].

High-throughput analysis of all putative miRNA:target pairs in mice predicts that less than 1% of target RNAs fulfil the high complementarity required to trigger TDMD[63]. Additionally, the probability of miRNA:target interaction is highly dependent on the expression levels of both components[63], the expression of the ZSWIM8 complex[58,59] and the TDMD base-pairing and surrounding sequences[67], which imposes significant context restrictions for

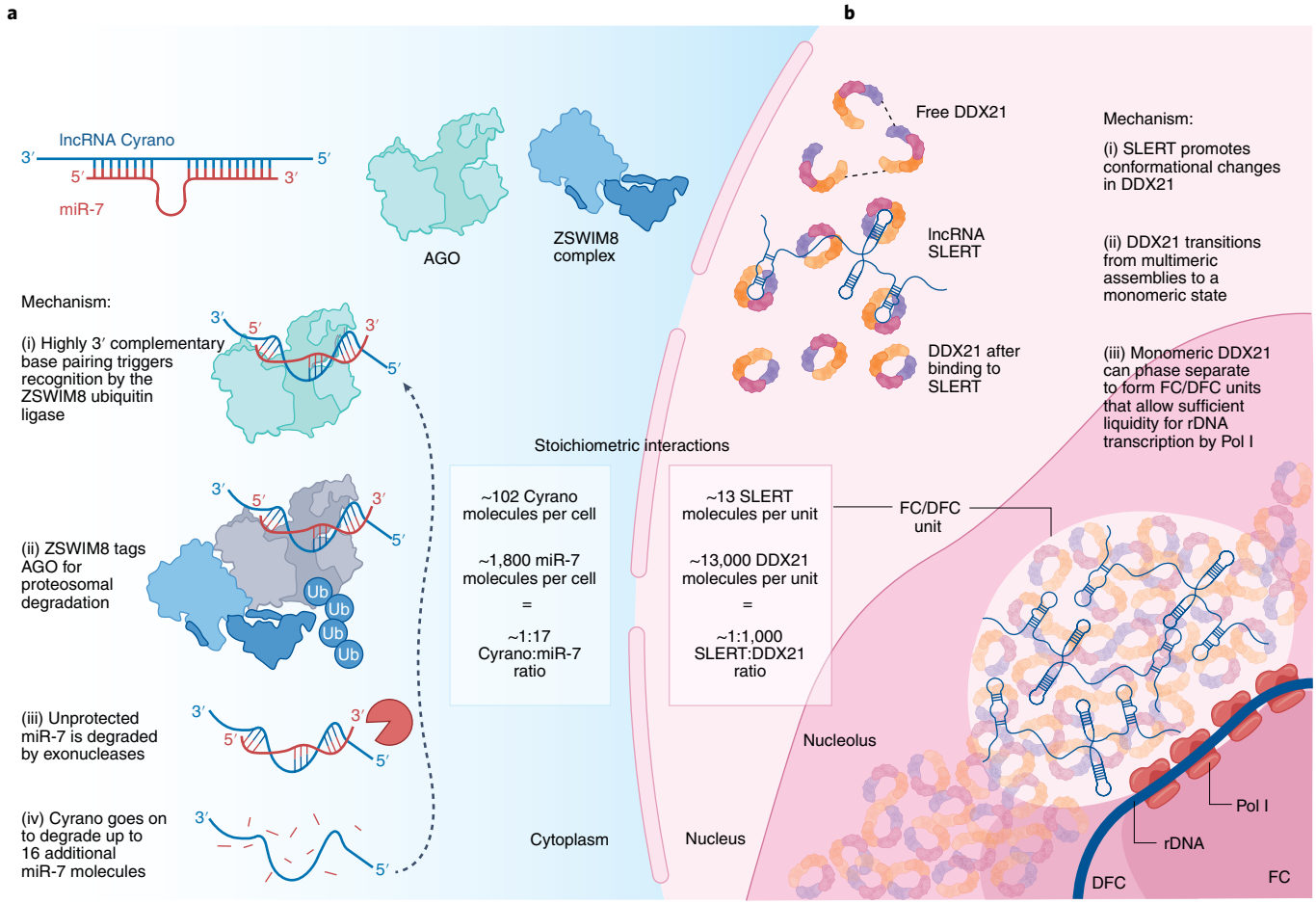

Fig. 3 | The lncRNAs Cyrano and SLERT are recycled to modify a supra-stoichiometric number of targets. a, Target-dependent miRNA degradation. The lncRNA Cyrano binds with high complementarity to AGO-loaded miR-7, which triggers TDMD[60]. Degradation of AGO mediated by ZSWIM8 leads to the exonuclease degradation of miR-7 without affecting the stability of Cyrano, which enables it to be recycled for the degradation of additional miR-7 molecules[58,60]. **b**, Chaperone lncRNA. The lncRNA SLERT acts as a chaperone to induce a conformational change of DDX21. This change in DDX21 prevents multimer formation, which allows monomeric DDX21 to induce the formation of phase-separated compartments at the interface between the FC and the DFC, thereby promoting the Pol I transcription of rDNA[69,70].

TDMD engagement[63]. These features of TDMD are probably advantageous for fine-tuning miRNA activity.

lncRNAs acting as protein chaperones. Recently, it has been demonstrated that the lncRNA SLERT, much like protein chaperones, can induce a conformational change of DDX21, a DEAD-box RNA helicase involved in multiple steps of ribosome biogenesis[68]. Using super-resolution structured illumination microscopy (SIM), DDX21 was found to form ~60 subnucleolar clusters per cell at the border between the fibrillar centre (FC) and the dense fibrillar component (DFC)[69]. These clusters surround polymerase I (Pol I) and dampen FC and DFC liquid properties and size, which effectively inhibits Pol I transcription[70]. However, this process can be counteracted by the nucleolar lncRNA SLERT, which induces a closed conformation of DDX21 and multimer-to-monomer transitions that favour a loose distribution of DDX21 that increases cluster liquidity and transcription by Pol I[69] (Fig. 3b).

The mechanism of action of SLERT is remarkable for several reasons. First, the chaperone-like function of SLERT has been characterized with unprecedented detail. A similar mechanism has only been previously suggested for a lncRNA in the regulation of CCND1 by $ncRNA_{CCND1}$ (ref. [71]), and it is currently unknown whether other lncRNAs can work in a similar manner. Second, SLERT is estimated to accumulate at only 13 copies per FC/DFC unit in PA1 cells, ~1,000 times less abundant than DDX21, which means that SLERT can counteract DDX21 multimerization at a 1:1,000 substoichiometric disadvantage[69]. This is substantially above average compared to canonical protein chaperones that act at substoichiometric ratios, for example, to chaperone FUS (1:50)[72] or α-synuclein (1:200)[73]. Remarkably, it was observed that conformational transitions of DDX21 clusters were time-dependent, with higher DDX21 transitions requiring only longer incubation times. And third, SLERT is required for the phase separation of the FC/DFC units. However, there is no known recruitment or eviction of factors from the FC/DFC units. This is different from the previously mentioned concentration gradients and phase transitions that increase the local concentration of effector proteins as in the case for Airn, Kcnq1ot1 or Xist and also different from NORAD, for which phase transitions sequester and inactivate PUM. In this case, phase separation only appears to provide proper liquidity to ribosomal DNA (rDNA) transcribing units to achieve higher Pol I processivity[69], which showcases the versatility of phase separation as an organizing principle for the cell.

The exact mechanisms that enable SLERT and other RNAs to act as molecular chaperones require further investigation. Whether RNA enables similar changes as protein chaperones and the relative contributions of RNA sequence, size, structure and condensate formation for the chaperone activity remain open questions. Emerging

in vitro evidence supports that nucleic acids, especially RNAs, may have potent chaperone activity by themselves or in cooperation with canonical chaperones[74]. Remarkably, this activity could reach up to 300-fold higher efficiency than protein chaperones, similar to SLERT, which opens an exciting possibility for lncRNAs and RNA in general with far-reaching implications in proteostasis[75].

Determinants of lncRNA substoichiometric activity

Although the examples detailed above certainly show that some lncRNAs are capable of substoichiometric actions, it is probable that this ability is restricted to lncRNAs with specific features. Understanding these features will require detailed experimentation, including the establishment and characterization of the minimal functional units.

Abundance and localization of lncRNAs. Quantification of lncRNA molecules is a new standard for lncRNA studies, and their levels can be readily estimated in whole-cell extracts or subcellular compartments. However, as the field dives into molecular-level interactions, it has become evident that these estimates often lack the spatial resolution to solve precise accumulation across subcellular scales. This is especially relevant for RNAs working at substoichiometric ratios through the formation of biomolecular condensates. Considering the recent literature, although many lncRNAs have been proposed to act through phase separation, a precise and comprehensive account of their mechanism of action is still lacking. Specifically, without quantitative assessment, it is difficult to estimate the actual contribution of phase separation to the proposed lncRNA function. This gains relevance in systems with multiple components, as in the case of Xist SMACs. Threshold concentrations for partition and functionality depend on RNA stoichiometry and features of the interacting proteins such as IDRs, assembly cooperativity and trafficking[76]. Importantly, data on accumulation kinetics of specific components also support functionally driven phase transitions against the high stochasticity of cell aggregates[77]. In the study of Xist SMACs, live-cell 3D-SIM, fluorescence recovery after photobleaching microscopy and kinetic modelling were used to characterize the accumulation and exchange rates of SMAC components[32]. 3D-SIM substantially helps solve resolution issues of smaller condensates. Coupling SIM with activity-dependent fluorescent probes could allow simultaneous analysis of localization, stoichiometry, substructural organization and, potentially, regulatory activity. This tool was also instrumental in the mechanistic study of SLERT[69], which enabled the observation of immiscible coexisting liquid phases driving nucleolar substructure[78]. Substructural organization could be a widespread feature of other RNA-driven biomolecular condensates as it can enable the modulation of reaction rates both by concentrating molecules and by physically organizing them[8,79].

RNA sequences as determinants of function. The first facet of lncRNAs to consider as the determinant of function is their primary sequence. Many lncRNAs contain repetitive sequences[80], which are particularly relevant in the context of multivalency. Still, these repeats typically account for only a subset of the lncRNA sequence. The length of a typical lncRNA is around 1,000 nucleotides, and many functional lncRNAs are >10,000 nucleotides long. This poses significant challenges to their genetic and biochemical analyses, which could be greatly expedited through the use of either functional subsequences or synthetic mini-lncRNAs. For example, short linear or circular RNA sequences were recently shown to recapitulate the function of full-length NORAD in inhibiting PUM activity[26,43], and a mini-NEAT1 was shown to support paraspeckle formation[18].

lncRNA spatial structures. lncRNAs fold into dynamic structures, which modulates the likelihood of sequence motifs to establish interacting interfaces with other cellular factors[7,81]. The functionality of many lncRNAs probably depends on their spatial structures, which can include either specific RNA folds or an assembly of possible folds that position different parts of the lncRNA in particular orientations. Characterizing these properties requires both methods rooted in molecular biology, which can report on differential accessibility of RNA bases to solvent or on the formation of RNA–RNA interactions, and microscopy-based methods that can report on the larger-scale organization of the RNA molecules. Structural probing of RNAs by in-cell SHAPEmap[82] or COMRADES, as done recently for NORAD[43], can inform new biological hypotheses and focus downstream analyses to probe for sequence or structural motifs that may be important for RNA interactions in cells.

On the microscopy front, exciting technological developments are now enabling tools such as atomic force microscopy to study RNAs[83]. Atomic force microscopy was recently used to evaluate the structure of the lncRNA MEG3 (ref. [84]) and RBP interactions by the lncRNA NIHCOLE[85]. Single-molecule techniques such as fluorescence resonance energy transfer microscopy and total internal reflection fluorescence microscopy were used in a study of SLERT to track the conformational changes of DDX21 molecules and rDNA–DDX21 cluster interactions, respectively[69]. Moreover, fluorescence confocal microscopy has become instrumental for probing phase-separation dynamics in recent literature. It has now been used to study LLPS-induced replication factories by RNA viruses[86] and paraspeckle assembly by NEAT1 (ref. [18]). Exceptional detail was obtained in the recent work on NORAD[26]. Notably, most of the variables studied in vitro for NORAD or SLERT are supported by in vivo studies in cells, for example, by coupling confocal microscopy with fluorescence recovery after photobleaching, which provides valuable information on the liquidity of compartments by measuring the mobility, dwell times and exchange rates of individual components.

Outlook

The mechanisms discovered so far are not a comprehensive list, and additional alternatives will probably emerge, perhaps modelled after or drawing inspiration from proteins that have evolved mechanisms to function despite substoichiometric imbalances. For example, substoichiometric concentrations of inhibitors were recently found to activate their targets instead of causing partial inhibition[87]. The AGO phosphorylation cycle limits AGO time on the target[88], which may allow AGO to navigate an expanded target landscape, usually outnumbering miRNAs by ~10:1 (refs. [89,90]). These types of mechanisms could be enabled by lncRNAs and other RNAs expressed at low concentrations. Additionally, lncRNAs could promote other types of partitions beyond LLPS. Low-complexity lncRNAs have been described to seed liquid–solid phase transitions and amyloidogenic protein deposition[13], which provides support for the versatility of lncRNAs for functional compartmentalization. Currently, we know very little about the physicochemical cues for the containment of phase transitions and condensate homeostasis. From the examples highlighted here, phase transitions appear to be self-contained, and this is important to regulate their function. In the case of Xist, it appears to have its own regulatory feedback loop, which regulates its initial upregulation[91] and rates of transcription and turnover[92], and prevents silencing from spreading to autosomes[35]. Interestingly, the relatively low expression of many lncRNAs may be important for the regulatory containment of their associated phases. Recently, a mechanism was suggested whereby condensates of positively charged peptides can interact with low-concentration negatively charged RNAs to promote phase separation. If RNA concentrations increase and their negative charges outnumber the positive charges, the repulsion between negative charges will cause the dissociation of the phase-separated compartment[6]. This principle could underlie a similar observation with prion-like RBPs, in which high RNA:protein ratios, like those found in the nucleus, prevented phase

transitions, whereas low ratios found in the cytoplasm promoted them[93]. If this is a widespread phenomenon in biomolecular condensates, it could potentially explain the substoichiometric action of multiple lncRNAs. However, further experimental confirmation of this concept is required.

The recent advances spearheaded by studies highlighted here and others lay the groundwork for exciting research directions in the lncRNA field. By continuing to challenge classical notions of lncRNA biology, these recent discoveries on specific lncRNAs have the potential to uncover generally applicable functional principles and assist with assigning mechanisms of action to many lncRNAs, a task of paramount challenge and importance. These principles could dramatically increase the number of putatively functional lncRNAs, encouraging a rethinking of current pipelines and revisiting datasets to make room for the possibility of challenging stoichiometries being a driving force of lncRNA functionality.

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Competing interests

The authors declare no competing interests.

Additional information

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