

Review

Biogenesis of a Solid Condensate: Amyloid bodies

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Abstract: This year marks the 20th anniversary of the discovery that proteins with various cellular functions can be temporarily immobilized in the nucleolus, a process known as nucleolar sequestration. This review reflects on the progress made to understand the physiological roles of nucleolar sequestration and the mechanisms involved in protein immobilization. We discuss how nucleolar sequestration consists of a highly choreographed amyloidogenic liquid-to-solid phase transition that converts the nucleolus into Amyloid bodies (A-bodies). The study of solid condensates A-bodies will offer unique perspectives on cellular assembly of membrane-less compartments and provide alternative insights on pathological amyloidogenesis involved in neurological disorders.

Keywords: Nucleolar sequestration; nucleolus; physiological amyloids; ribosomal intergenic spacer; rIGSRNA; liquid-liquid phase separation; liquid-to-solid phase transition; complex coacervation; biomolecular condensates; beta-amyloid; MDM2; VHL; Cdc14; low complexity RNA; acidosis; heat shock

Nucleolar sequestration: Visitors to the nucleolus

The role of the nucleolus as the site of ribosome biosynthesis was established by the mid-1960's [1-3]. Nucleoli are built around tandem repeats of ribosomal DNA (rDNA) in nucleolar organizing regions (NORs) and structurally dependent on active transcription of rDNA [4,5]. The traditional role of the nucleolus as a hub of rRNA synthesis and ribosome assembly has been the subject of many excellent literature surveys [6-8]. This review focuses on a lesser known phenomenon originally coined "nucleolar sequestration", which describes the nucleolus' ability to sequester and release regulatory proteins in response to cellular cues [9-11]. 2019 will mark 20 years since nucleolar sequestration was first hypothesized by Bachant and Elledge [12] following the discoveries that cell-cycle regulator Cdc14 phosphatase and E3 ubiquitin ligase MDM2 could be temporarily localized in nucleoli to affect cell-cycle progression [13-17]. In *S. cerevisiae*, Cdc14 is sequestered in the nucleolus by Cfi1/Net1 until the onset of anaphase, when it is liberated to promote mitosis [15,16]. In mammalian cells, nucleolar sequestration of MDM2 prevents it from binding and exporting p53 into the cytoplasm for degradation [13,14,17]. This stabilizes p53 in the nucleus, where it acts as a transcription factor that promotes cell-cycle arrest or apoptosis. Another notable example of a mammalian protein is tumor suppressor VHL (von Hippel-Lindau protein), which is sequestered in the nucleolus in response to hypoxia-induced acidosis [18]. E3 ubiquitin ligase VHL degrades transcription factor HIF (hypoxia-inducible factor) under normal oxygen conditions. Nucleolar sequestration of VHL in hypoxic low pH settings enables HIF to evade degradation and activate its target genes involved in oxygen homeostasis. Cdc14, MDM2 and VHL represent three of the many examples reported over the last two decades. Table 1 summarizes the reports of nucleolar sequestration that have been observed for many other proteins under various cellular conditions,

including development [19-21], apoptosis [22,23], DNA damage [17,24,25], extracellular acidosis [18,26-28], heat shock [26-30], UV and ionizing radiation [31,32], alcohol [33], viral infection [34], transcriptional arrest [35], inhibition of rRNA synthesis [36] and proteasomal inhibition [29,37-39].

Nucleolar sequestration: A case of protein immobilization

Table 1. List of the proteins whose activities have been reported to be regulated by nucleolar sequestration.

Protein symbol	Full name	Stimulus	Nucleolar response	Reference
Cdc14	Cell division cycle 14 (<i>S. cerevisiae</i>)	Anaphase	Release	Shou et al., 1999 Visintin et al., 1999
Pch2	Pachytene checkpoint 2 (<i>S. cerevisiae</i>)	Meiotic prophase arrest	Release	San-Segundo and Roeder, 1999
MDM2	Murine double minute 2 homolog	Ribosomal stress DNA damage	Capture	Weber et al., 1999 Lohrum et al., 2000 Bernardi et al., 2004
hTERT	Human telomerase reverse transcriptase	Transformation, DNA damage Ionizing radiation	Release Capture	Wong et al., 2002
c-Myc	Proto-oncogene c-Myc	Proteasomal stress	Capture	Arabi, 2003
ADAR2	Adenosine deaminase that acts on RNA 2	Inhibition of rRNA synthesis	Release	Sansam et al., 2003
VHL	von Hippel Lindau tumor suppressor	Extracellular acidosis	Capture	Mekhail et al., 2004
RelA	p65 subunit of transcription factor NF-κB	Aspirin, serum withdrawal, UV-C radiation	Capture	Stark and Dunlop, 2005 Chen and Stark, 2017
Polycomb	Polycomb (<i>D. melanogaster</i>)	Cell differentiation	Capture	Chen et al., 2005
Hand1	Heart and neural crest derivatives expressed 1	Cell differentiation	Capture/ Release	Martindill et al., 2007
Hsc70	Heat shock chaperone 70	Recovery from heat shock	Capture	Banski et al., 2010
Ulp1	Small ubiquitin-related modifier (SUMO) protease (<i>S. cerevisiae</i>)	Alcohol	Capture	Sydorsky et al., 2010
p53	Cell-cycle regulator; tumor suppressor	Proteasomal inhibition (MG132)	Capture	Krüger and Scheer, 2010 Latonen et al., 2011
Piwi	piRNA binding protein (<i>Drosophila</i>)	Heat shock	Capture	Mikhaleva et al., 2018

Much work has been devoted to study the high mobility of proteins in the cell, particularly within the nucleus [40,41]. Typically, proteins maintain their dynamic profiles as they rapidly exchange between affinity interactions and the cellular milieu, turning over within seconds to minutes [41-43]. The nucleolus is considered a dynamic droplet, assembled by liquid-liquid phase separation [44,45], whose proteins are highly mobile [35]. It is remarkable, then, that proteins sequestered in the nucleolus are immobile, as assessed by photobleaching analyses and heterokaryon fusion studies [18,27,28,46]. This has been demonstrated for Cdc14 [47], MDM2 [27], VHL [46], RNF8 [48], DNMT1 [27], and a recently reported visitor, piRNA binding protein Piwi in *Drosophila* under heat shock [49]. Even resident nucleolar proteins such as RNA polymerase I subunit RPA16, Pescadillo and SENP3 can undergo cycles of mobility/immobility [28]. Here, VHL is used to elaborate the dynamics involved: VHL switches from a highly dynamic, uniform distribution under standard growth conditions (21% O₂, pH 7.4) to an immobilized state in the nucleolus rapidly on exposure to extracellular acidosis (1% O₂, pH 6.0) [46]. Only upon neutralization of extracellular pH is VHL released from the nucleolus to return to its original distribution and mobility [46]. These immobile nuclear foci were originally called “nucleolar detention centers”, as targets are both localized and detained within the nucleolus, unable to freely diffuse elsewhere [28,46,50]. From this perspective, the function of nucleolar detention or immobilization is to temporarily inactivate relevant proteins, as they would be kept away from their normal targets. Just as possible, though, is that the clustering of detained proteins may render an enzymatic reaction more efficient. Reports suggest that immobilized nucleolar Cdc14 maintains Spo12 dephosphorylation [47] and Piwi switches from its canonical role in non-nucleolar transposable element (TE) repression to rDNA-specific TE repression when it is immobilized in the nucleolus [49]. From these studies, it is clear that cells have evolved a strategy to regulate molecular networks by reversibly switching proteins between a mobile and an immobile state. Whether nucleolar sequestration represents a loss- or gain-of-function might depend on which proteins undergo immobilization.

Mechanisms of nucleolar sequestration

MDM2, Cdc14 and VHL are examples where further work has yielded mechanistic insights as to how proteins are sequestered in the nucleolus. MDM2 exhibits flexibility in how it becomes localized in the nucleolus. Under conditions where tumor suppressor p^{14/19}ARF is induced, ARF binding MDM2 unmasks a cryptic nucleolar localization signal (NoLS) within its C-terminal RING domain, which is essential for MDM2 nucleolar sequestration [14,17,51,52]. Nucleotide binding, specifically ATP, to MDM2 behaves similarly, independent of ARF [53]. Alternatively, during DNA damage, e.g. doxorubicin, and acidosis, MDM2 is shuttled into the nucleolus by direct binding to PML (promyelocytic leukemia protein) [13], itself a target of nucleolar sequestration [54]. Cdc14 is anchored in the nucleolus for most of the cell cycle through its association with Cfi1/Net1 [15,16]. The timing of its release during cell division is regulated by a signaling cascade of phosphorylation events that directly weaken the interaction of Cdc14 with Net1, releasing Cdc14 to act as a mitotic exit activator [55]. Whether these targeting signals also promote protein immobilization is unclear. Mapping analysis of VHL identified an approximately 30 amino acid fragment referred to as a nucleolar detention signal (NoDS) that is necessary and sufficient to immobilize proteins in nucleoli [48]. The NoDS is composed of an arginine/histidine-rich sequence followed by two or more hydrophobic LXV motifs where X can be any hydrophobic residue e.g. LWL, LLV, LFV, LQV [48]. The NoDS is highly predictive and was identified in many other candidates including DNA methyltransferase I DNMT1, promyelocytic leukemia protein PML and DNA polymerase delta catalytic subunit POLD1, all shown to be immobilized in nucleoli under extracellular acidosis [27,48]. The identification of the NoDS in many proteins provided evidence that nucleolar sequestration is a common cellular strategy to regulate protein function.

Transformation of nucleoli into Amyloid bodies (A-bodies)

Proteins with limited mobility have been observed to display amyloid-like properties in physiological settings [56,57]. Amyloidogenesis is the process whereby soluble proteins assemble into aggregates known as amyloid fibrils [58]. Nucleolar detention centers composed of proteins such as VHL, MDM2, POLD1, etc share many properties associated with the amyloid state beyond immobility. First, these nuclear foci stained positive with amyloidophilic dyes such as Congo red, Thioflavin S/T, Amylo-Glo, and ANS [26]. Second, the fibrillar organization of these nuclear foci observable by electron microscopy (EM) are as predicted for the amyloid-fold [26]. Third, proteins found in these nuclear foci displayed biochemical properties associated with amyloids, including resistance to Proteinase K and insolubility in common detergents [26]. Consequently, the term “nucleolar detention centers” was replaced with “Amyloid bodies” (A-bodies) to reference the transformation of the nucleolus into an A-body, a molecular prison of proteins in their amyloid-like state [26].

Amyloid bodies are distinct from liquid-like membrane-less compartments

Amyloid-like biophysical characteristics make A-bodies distinct from other membrane-less compartments that display liquid-like properties [44,45,59-64], such as stress granules, nucleoli and germ granules, and from other structures that originate from the nucleolus (e.g. nucleolar caps and nucleolar aggresomes). Liquid-like membrane-less compartments are dynamic, their constituents are mobile and they do not typically stain with amyloidophilic dyes [41,59]. Thus, A-bodies are better categorized as solid-like structures as they are static and harbor immobile proteins that form strong intermolecular bonds associated with the amyloid-fold. Several authors have suggested that the formation of A-bodies represents a physiological liquid-to-solid phase transition rather than a liquid-liquid phase separation implicated in the formation of liquid condensates [65,66]. The term liquid-to-solid phase transition had, thus far, been reserved to describe the formation of pathological aggregates [67-72]. Other physiological examples of solid-like structures include Balbiani bodies observed in *Xenopus* [73] and pH-regulated fluid-to-solid transition of the cytoplasm in yeast [74]. Table 2 summarizes the differences between liquid-like and solid-like condensates in the cell. By similar criteria, A-bodies may be contrasted from other stress-induced nucleolar structures, namely nucleolar caps [75] and nucleolar aggresomes [37,38]. By EM, A-bodies have a unique fibrillar organization characteristic of amyloids that has yet to be observed in the cell [26] (Fig. 1). Nucleolar caps appear as electron-dense amorphous structures and nucleolar aggresomes are cavernous, occupying a large electron-light central space of the nucleolus [76] (Fig. 1). Each structure exhibits significant remodeling of the nucleolus [28,75,76]. Interestingly, while the formation of nucleolar caps and A-bodies is accompanied by a redistribution of nucleolar components and subsequent arrest of ribosomal biogenesis [28,75,77], nucleolar aggresomes require transcriptionally active nucleoli to form [37,38] with all nucleolar compartments intact and visible by EM. Additionally, proteins are dynamically sorted into nucleolar caps and remain mobile [75] while proteins in A-bodies are immobile [26-28,46]. Photobleaching experiments have not been reported for proteins of nucleolar aggresomes. Therefore, A-bodies are unique structures with amyloidogenic properties distinct from other cellular bodies.

Table 2. Contrast between liquid-like and solid-like condensates in the cell.

	Liquid-like condensates	Solid-like condensates
Examples	Cytoplasm: <ul style="list-style-type: none"> Stress granules P-bodies Nuclear: <ul style="list-style-type: none"> Stress granules Cajal bodies Speckles Paraspeckles Nucleoli PML bodies 	Amyloid bodies Balbiani bodies
Protein mobility	Proteins are mobile; continuously exchanging with the structure and the surrounding milieu	Proteins are immobile; engaged in strong intermolecular interactions
Shape	Spherical	Spherical
Characteristics	Structure is dynamic; exhibiting properties of water droplets: <ul style="list-style-type: none"> Fluid Cycles of fusion (coalescence) and fission Wetting behavior Flows under shear force 	Structure is non-dynamic; exhibiting properties of amyloids: <ul style="list-style-type: none"> Static Positive staining with amyloidophilic dyes (e.g. Congo red) Resistant to Proteinase K Insoluble in common detergents Cross-β diffraction pattern Fibrillar organization
Material properties	Viscous	Elastic
Function	Biochemical reactions	Cell dormancy
Mechanism	Liquid-liquid phase separation	Liquid-to-solid phase transition

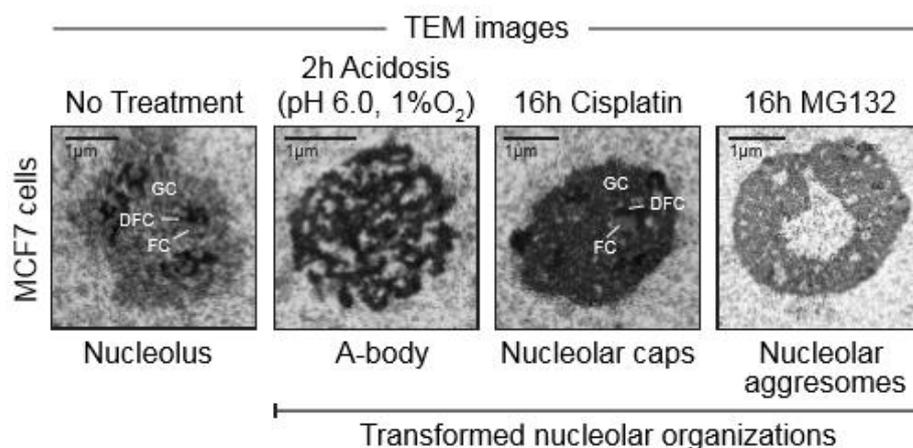


Figure 1. Contrasting A-bodies to other transformed nucleolar organizations, namely nucleolar caps and nucleolar aggresomes. Nucleoli can be transformed into A-bodies, nucleolar caps or nucleolar aggresomes under acidotic conditions, transcriptional arrest (16h cisplatin) and proteasomal inhibition (16h MG132), respectively. By transmission electron microscopy (TEM), A-bodies appear

fibrillar and distinct from the amorphous, electron-dense nucleolar structure observed when nucleolar caps form or from the electron-light nucleolar cavity that is a nucleolar aggresome. Far right image taken from [67].

Protein sequestration into A-bodies: an example of physiological liquid-to-solid phase transition

At any one time, under the microscope, a plethora of membrane-less compartments populate the cell. How compartments maintain their unique identities and how proteins and/or RNA discriminate in which compartment they will be found has yet to be clarified. New insights into the biophysical properties of membrane-less compartments have demonstrated unexpected links between the sequence-encoded information in protein and RNA components of compartments and the material properties they impart, in dictating function [59,78-86]. A flurry of recent studies demonstrated the importance of RNA-binding proteins with prion-like, low complexity domains (LCDs) in forming membrane-less compartments. Equally as exciting has been the work done to uncover the specific sequences or structural elements embedded in architectural RNA that dictate the biophysical properties of membrane-less compartments [87,88]. With these new tools, we have begun to understand how specific elements within essential RNA and protein components are involved in A-body biogenesis.

A multi-step model of A-body biogenesis

Time-lapse imaging of A-body formation revealed that the solid-state organization is achieved through what appears to be a liquid-to-solid phase transition, in which proteins are initially found in liquid-like droplets that mature into a solid-like structure. These liquid-like droplets are distinct from the established nucleolar compartments involved in ribosome biosynthesis [89]. It was Woodruff et al. [90] who first proposed that solid-like structures such as A-bodies and Balbiani bodies originate from a liquid-liquid phase separation event that matures over time into a solid-like state. Fig. 2 is a step-wise reference guide of A-body formation and disassembly, showcasing A-body biogenesis as a precisely choreographed routine rather than simply a means to store misfolded aggregates.

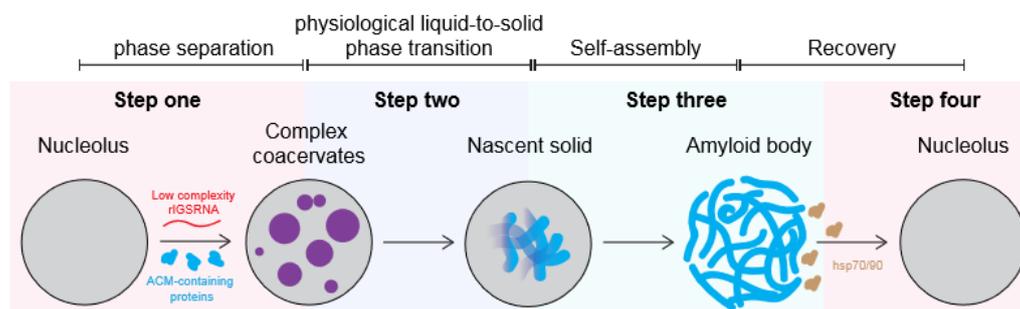


Figure 2. Amyloid body biogenesis is a precisely choreographed routine. We propose that, on stress, low complexity rIGSRNA derived from the rDNA intergenic spacer accumulate in the nucleolus. Step one: Low complexity rIGSRNA interact with short cationic peptides, such as the R/H-rich sequence of the ACM (formally NoDS), to form nucleolar liquid-like foci. Step two: Local concentration of proteins with amyloidogenic propensity in the liquid droplets triggers physiological amyloidogenesis and generates nascent A-bodies. Step three: Once seeded, nascent A-bodies self-assemble into fibrillar, solid-like A-bodies. A-bodies enable cells to rapidly and reversibly store a large array of proteins and enter cellular dormancy in response to stress. Step four: Upon recovery/stimulus termination, A-body disaggregation is mediated by heat-shock protein (hsp) chaperones 70 and 90. Through these steps, A-body biogenesis represents a physiological liquid-to-solid phase transition.

Low complexity rIGSRNA form complex coacervates with the R/H-rich domain of the NoDS

sequencing, RT cloning and RNA-FISH. This is in contrast to other ncRNA that display high complexity, i.e. possess secondary structure, such as pRNA.

Amyloidogenic properties of the NoDS trigger the liquid-to-solid transition

In principle, solid-like structures should be composed of proteins that possess fibrillation propensity, i.e. increased likelihood of forming fibrils. Bioinformatic analysis of the consensus NoDS revealed the hydrophobic LXV motifs make up a region of high fibrillation propensity, as predicted by ZipperDB based on a Rosetta energy score of less than -23 kcal/mol. As a whole, the NoDS bears a striking resemblance to that of the prototypical pathological β -amyloid associated with Alzheimer's disease, in which a R/H-rich sequence is in close proximity to a highly amyloidogenic stretch referred to in literature as the P3 fragment. The fibrillation propensity of the NoDS was validated by demonstrating it exhibited classic amyloidogenic properties previously observed for β -amyloid, namely a cross- β x-ray diffraction pattern and formation of fibrils *in vitro*. For these reasons, the NoDS has been renamed the Amyloid-Converting Motif (ACM) (Fig. 4A).

It has been proposed that the function of the initial liquid state is to locally concentrate proteins with fibrillation propensity that would otherwise be at levels below the critical threshold in the cell [58]. Concentration-dependent activation of protein fibrillation has been well-documented *in vitro* [58,103]. Indeed, markers of early or nascent A-bodies included nuclear ANS and A11 staining [89], typically used *in vitro* to indicate accumulating pre-amyloidogenic structures [104-106]. Once seeded, the amyloidogenic process can be spontaneously triggered. Nascent A-bodies have been observed to expand by self-assembly, in which soluble proteins are added directly and autonomously to the growing amyloid structure [89].

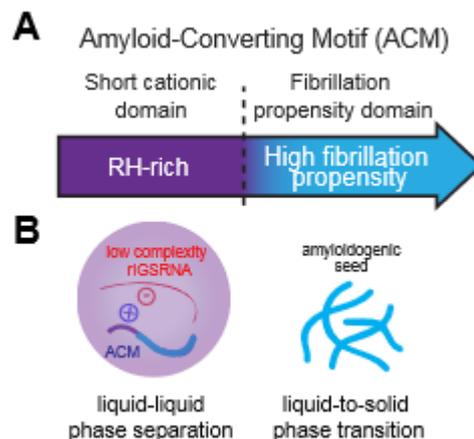


Figure 4. The Amyloid-Converting Motif (ACM). A: The ACM is necessary and sufficient to target and immobilize proteins in A-bodies. It consists of a R/H-rich short cationic domain flanking a high fibrillation propensity domain (determined by Rosetta energy of < -23kcal/mol). B: Its bipartite nature allows the ACM to traverse phase boundaries. Complex coacervation of short cationic domains and low complexity rIGSRNA (liquid-liquid phase separation) concentrates fibrillation propensity domains to activate a liquid-to-solid phase transition to form A-bodies.

The ACM traverses across phase boundaries to confer A-body identity

As described above, low complexity RNA sequences are important in conferring A-bodies their unique identity [89], adding to the list of architectural determinants, which includes sequence-specific RNA [107], mRNA secondary structure [70,79] and short unstructured RNA *in vitro* [108]. However, not every protein containing positively charged domains will be found in A-bodies. The low complexity rIGSRNA-mediated coacervates themselves appear immiscible from the three canonical compartments of the nucleolus [44,89], suggesting they exhibit distinct biophysical properties that may exclude mixing. Ultimately, it is the bipartite nature of the ACM that confers A-body identity

and differentiates the ACM from other motifs. The R/H-rich “short cationic domain” mediates liquid-liquid phase separation while the “fibrillation propensity domain” initiates the liquid-to-solid phase transition [26,89] (Fig. 4B). In this model, proteins without a region of high fibrillation propensity are unlikely to be incorporated into A-bodies. The balance of positive to negative charge ratio, presence of polar and/or aromatic residues, and modifications [109,110] will affect how proteins engage in intermolecular interactions with other proteins and RNA. Therefore, the ACM is versatile as it can physiologically transition proteins across phase boundaries.

Physiological amyloids promote cell dormancy

Endogenous A-bodies have been found in primary cultures and cell lines exposed to various stimuli, the cores of human tumors, and subsets of cells in normal human and mouse tissues [26]. So, while A-bodies share structural features with pathological amyloids, their ubiquitous and reversible nature is indicative of a physiological function. Pathway enrichment analysis determined that many of the proteins sequestered in A-bodies are involved in cell cycle progression and DNA synthesis, such as CDK1, POLD1, DNMT1 [26]. By temporarily detaining key factors from their sites of activity into A-bodies, major molecular networks are disrupted, resulting in metabolic inactivity [77] and arrested proliferation/DNA synthesis [26]. This is different from a quiescent state (G0) in the cell cycle where cells arrest proliferation but remain metabolically active. Therefore, the biological role of A-body formation is to promote cellular dormancy as an adaptive response to environmental stressors. These findings are consistent with reports that show Balbiani bodies [73] and fluid-to-solid transitions in yeast [74] promote dormancy, reinforcing the concept that cells utilize different states of matter to perform various biological functions (Table 2). The material properties of solid-like systems – non-dynamic, amyloid-like – result in loss of metabolic activity and promote cellular dormancy while those of liquid-like systems – dynamic, fluid-like – concentrate biochemical reactions.

A-bodies as a target for therapeutic discovery

Implications for pathological amyloids

A-bodies that are physiologically produced remain confined within the nucleus. How the nuclear environment alters the interaction properties of aggregation-prone proteins to prevent toxicity [111,112] compared to the cytoplasm is unclear. These results imply the cell tightly regulates the induction and degradation of low complexity rIGSRNA within the nucleolus in response to stimuli. Whether aberrant production or localization of low complexity RNA are involved in amyloid pathologies such as Alzheimer’s and Huntington’s Diseases will be the subject of future studies.

Implications for tumor cell dormancy and metastasis

Given the evidence that A-bodies promote cell dormancy, perhaps there is a link between A-body formation and tumor cell dormancy, that allow cancer cells to adapt to the harsh hypoxic/acidotic conditions of the tumor microenvironment. Such a connection has pertinent implications for chemotherapeutic resistance and metastasis [113,114]. With major metabolic pathways shut down and key drug targets stored away in A-bodies, a small population of dormant tumor cells may be resistant to treatment, survive for a prolonged period of time and contribute to disease recurrence or metastasis upon their metabolic reactivation. Preventing cancer cells from forming A-bodies and going dormant or, more realistically, preventing cancer cells from reactivating may become a viable treatment option.

An interesting avenue of research that may offer a more effective cancer treatment strategy involves manipulating the proteins that evade capture into A-bodies. Interestingly, proteins that remain active to sustain basal metabolism and viability under stress tend to be devoid of fibrillation propensity domains and evade capture by A-bodies. These proteins would be more susceptible to manipulation and serve as better chemotherapeutic targets than proteins captured in A-bodies.

Conclusion

Twenty-years after its discovery, the study of nucleolar sequestration has led to the discovery of a physiological amyloidogenic program that converts the liquid nucleolus into the solid A-body. The study of A-bodies has provided a rare opportunity to interrogate the functional and regulatory interactions necessary to form amyloids in a cellular setting. Why exactly nucleolar coacervates differ from other liquid condensates in their ability to transition to the solid state remains a fundamental question that will surely offer alternative insights into how we understand and treat diseases related to age, neurodegeneration and cancer.

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