Orphan Nuclear Bodies

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Orphan nuclear bodies are defined as nonchromatin nuclear compartments that have been less well studied compared with other well-characterized structures in the nucleus. Nuclear bodies have traditionally been thought of as uniform distinct entities depending on the protein “markers” they contain. However, it is becoming increasingly apparent that nuclear bodies enriched in different sets of transcriptional regulators share a link to the ubiquitin-proteasome and SUMO-conjugation pathways. An emerging concept is that some orphan nuclear bodies might act as sites of protein modification by SUMO and/or proteasomal degradation of ubiquitin-tagged proteins. By defining a specialized environment for protein modification and degradation, orphan nuclear bodies may increase the capacity of cells to survive under varying environmental conditions.

A nuclear body is a region within the nucleus that is morphologically distinct from its surroundings when observed by transmission electron microscopy (reviewed in Spector 2006). This excludes structures that are only detected upon overexpression of tagged nuclear proteins and most likely result from nonphysiological aggregation of excess protein.

On the basis of their structure observed at the electron microscopic level, nuclear bodies have been classified as either simple or complex (Bouteille et al. 1974). The simple nuclear bodies are small (0.2–0.5 μm), round, compact and finely fibrillar, whereas the complex nuclear bodies are larger (0.2–1.2 μm), heterogeneous in shape and texture, and enveloped by a peripheral capsule, which gives them a doughnut-shaped appearance. Orphan nuclear

bodies are primarily proteinaceous structures enriched in either transcriptional regulators or RNA-binding proteins (Table 1). Although it was initially thought that these structures might act as specialized compartments for gene expression, most genes and RNA species targeted by the protein factors enriched in nuclear bodies show a widespread distribution throughout the nucleoplasm. Moreover, nuclear bodies contain only a fraction of the total pool of transcriptional regulators and RNA-binding proteins in the nucleus, and many of these factors are in constant flux in and out of the bodies.

A breakthrough in this field was the recent discovery that transcription factors enriched in nuclear bodies are modified by SUMO and/or ubiquitin, and that SUMO-conjugation plays an important role in nuclear body assembly

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M. Carmo-Fonseca *et al.*

Table 1. Orphan nuclear bodies

Nuclear body	Description	Reference
Clastosome	Concentrates the proteasomal 20S and 19S complexes, and ubiquitin conjugates. Detected predominantly when the activity of the proteasome is stimulated, disassembles upon proteasomal inhibition. The number per cell ranges between 0 and 3, and the diameter is 0.2–1.2 μm .	Lafarga <i>et al.</i> 2002
Cleavage body	Enriched in cleavage factors CstF 64 kDa and CPSF 100 kDa, and DEAD box protein DDX1. Detected predominantly during S phase, is not affected by transcription inhibition. The number per cell ranges between 1 and 4, and the diameter is 0.2–1.0 μm .	Schul <i>et al.</i> 1996; Bléoo <i>et al.</i> 2001; Li <i>et al.</i> 2006
OPT domain	Enriched in transcription factors Oct1 and PTF. Partial colocalization with transcription sites. Detected predominantly during late G1 phase, disassembles upon transcription inhibition. The number per cell ranges between 1 and 3, and the diameter is 1.0–1.5 μm .	Pombo <i>et al.</i> 1998
Polycomb body	Enriched in PcG proteins in <i>Drosophila</i> embryos and human cells. Concentrates human RING1, BMI1, HPC, and some associate with pericentromeric heterochromatin. The number per cell ranges between 12 and 16, and the diameter is 0.3–1.0 μm .	Buchenau <i>et al.</i> 1998; Saurin <i>et al.</i> 1998
Sam68 body	Concentrates Sam68 and Sam68-like proteins SLM-1 and SLM-2. Disassembles upon transcription inhibition. Most likely enriched in RNA. The number per cell ranges between 2 and 5, and the diameter is 0.6–1.0 μm .	Chen <i>et al.</i> 1999
SUMO body	Enriched in SUMO-1 and SUMO- conjugating enzyme Ubc9. Concentrates transcription factors pCREB, CBP, c-Jun. The number per cell ranges between 1 and 3, and the diameter is 1–3 μm .	Navascués <i>et al.</i> 2007

(reviewed in Heun 2007). It is therefore possible that some nuclear bodies act as protein modification and degradation centers.

THE CLASTOSOME: A NUCLEAR BODY LINKED TO THE UBIQUITIN-PROTEASOME SYSTEM

Proteins in cells and organisms exist in a dynamic state with individual half-lives ranging from minutes to years. This implies that protein degradation must be highly selective and tightly

controlled (reviewed in Ravid and Hochstrasser 2008). Eukaryotic cells have two major mechanisms for protein degradation: lysosomes and proteasomes. Although exogenous particles and endogenous cellular organelles are targeted for digestion in lysosomes, the majority of intracellular proteins are degraded by proteasomes (reviewed in Murata *et al.* 2009). Proteasomes degrade short-lived regulatory proteins and thereby control cellular processes such as signal transduction, cell cycle, and gene transcription. Proteasomes additionally clear misfolded and

aggregated proteins from the cell and produce some of the peptides to be displayed at the cell surface as part of the adaptive immune response.

A fundamental question about intracellular proteolysis is how specific proteins are recognized by the proteolytic machinery. In eukaryotic cells, most substrate proteins are targeted to the proteasome by the covalent attachment of many copies of the small protein ubiquitin. Once a protein has been tagged by polyubiquitination, it is recognized by the proteasome, unfolded and then degraded (Fig. 1). However, ubiquitination can function independently of the proteasome and proteasomes can degrade some proteins without their prior modification by ubiquitin (Ravid and Hochstrasser 2008).

The proteasome is an approximately 2.5-MDa (26S) protein machine composed of two

subcomplexes: a 20S barrel-shaped core that carries the catalytic activity, and a flanking regulatory 19S particle that recognizes and unfolds protein substrates (reviewed in Cheng 2009). The 20S core particle consists of four stacked rings that form a cavity at the center of the particle where the peptidolytic active sites are sequestered. Substrates gain access to this cavity through narrow pores present at either axial end of the core particle. These pores are normally closed and require a mechanism of gate opening carried out by the 19S regulatory particle. One regulatory particle may associate with either axial end of the core particle. The regulatory particle contains ATPases presumed to function in substrate unfolding, and ubiquitin chain receptors responsible for recognition of the substrate proteins (Fig. 1).

Proteasomes were first shown to localize to the nucleus by Franke and colleagues (Hugle

Figure 1. The 26S proteasome is composed of one core particle (20S) and one or two regulatory particles (19S). Proteins destined for degradation are initially attached to ubiquitin polymers. After this covalent modification, the substrate protein is able to bind (either directly or via adaptor proteins) to the 19S regulatory complex. Then, the protein is unfolded by ATPases that encircle the entrance of the 20S catalytic core, and the polyubiquitin chain is removed by proteasome-associated deubiquitylating enzymes. Finally, the unfolded protein is translocated into the central proteolytic chamber, where it is cleaved into short peptides.

M. Carmo-Fonseca *et al.*

et al. 1983; Kleinschmidt *et al.* 1983). Subsequent studies confirmed that proteasomes, as well as components of the ubiquitin-proteasome system occur both in the nucleus and in the cytoplasm. Although some nuclear proteins, such as p53, are first exported from the nucleus and then degraded in the cytoplasm, several lines of evidence indicate that proteins can be ubiquitinated and degraded by the proteasome within the nucleus (Tao *et al.* 2005; Rockel *et al.* 2005).

Immunofluorescence studies using antibodies specific for the 20S and 19S proteasomal subcomplexes reveal that although proteasomes are most often diffusely distributed throughout the nucleoplasm, they occasionally concentrate in discrete structures (Fig. 2). By electron microscopy, these structures correspond to previously described complex, ring-shaped

nuclear bodies (Bouteille *et al.* 1974). Double-labeling experiments further show the presence of ubiquitin-conjugates, c-Jun and c-Fos, two short-lived transcription factors degraded by the ubiquitin-proteasome system, in the proteasome-containing nuclear bodies (Lafarga *et al.* 2002). Proteasome-containing nuclear bodies form in response to stimuli that activate proteasome-dependent proteolysis and disappear when proteasome function is inhibited. Taken together, these results suggest that proteasome-containing nuclear bodies are sites of protein degradation, and the name clastosome (from the Greek *klastos*, broken and *soma*, body) was coined to refer to this nuclear domain (Lafarga *et al.* 2002).

Clastosomes are defined as nuclear bodies of irregular shape and size that contain both catalytic and regulatory subunits of the

Figure 2. The clastosome is a nuclear body enriched in proteasomes. (A,B) Colocalization of ubiquitin-conjugates (A, red staining) and 19S proteasomal complexes (A and B, green staining) in a human neuron mechanically isolated from dorsal root ganglia obtained from an autopsy of a patient without any diagnosed neurological disorder. Bar, 5 μ m. (C, D) Several clastosomes are observed in the nucleus of a neurosecretory neuron isolated from rat hypothalamus after osmotic stress; double-immunofluorescence with antibodies specific to 20S proteasomal complexes (C and D, red staining) and a nucleoporin (C, green staining). (E) Immunogold labeling with antibodies directed against the 20S proteasome reveals a doughnut- or ring-shaped nuclear body. Bar, 300 nm.

proteasome and ubiquitylated proteasome substrates. Clastosomes are normally absent or scarce in most cell types, indicating that these nuclear bodies are not essential for proteasome function. Most likely, protein substrates tagged for proteolysis are rapidly encountered, recognized and degraded by the diffusely distributed proteasomes in the nucleoplasm. Clastosomes become prominent when cells are exposed to stimuli that suddenly increase the levels of proteins targeted for degradation, for example, inducible expression of *c-fos* by serum, or stress. Clastosomes may therefore form as a result of an overload of protein substrates. Clastosomes assemble transiently and revert after the stimulus, consistent with the view that they accumulate substrates queuing up for proteolysis. Moreover, clastosomes are not detected in cells treated with specific proteasome inhibitors, arguing that clastosome assembly requires proteasomal activity. Clastosomes may also act by sequestering proteasomes, thereby controlling the availability of proteolytic machines for nuclear protein degradation.

Recent studies suggest that regulated protein degradation by the ubiquitin proteasome system is an important modulator of neuronal function (reviewed in Tai et al. 2008). In particular, the localization of proteasomes to synapses is controlled by synaptic stimulation, thus providing a mechanism for local protein turnover (Bingol and Schuman 2006). The observation that clastosomes are widespread in glial cells and many types of brain neurons (Ádori et al. 2006; Baltrons et al. 2008) raises the possibility that these nuclear bodies participate in distinctive features of protein turnover in the nervous system. The importance of proteolysis in neurons is further underlined by the presence in a wide spectrum of neurodegenerative diseases of pathological protein aggregates that lead to a severe impairment of the ubiquitin-proteasome system (Bennett et al. 2005). Although the presence of ubiquitin and proteasomes in inclusion bodies formed by disease-associated protein aggregates suggests a link with dysfunction of the ubiquitin-proteasome system (Janer et al. 2006), the mechanism by which accumulation of protein aggregates in inclusion bodies

contributes to disease remains unknown (see Orr 2010).

THE PROTEASOME IS A COMPONENT OF BOTH CLASTOSOMES AND PML NUCLEAR BODIES

The PML protein colocalizes with ubiquitin and the proteasome in nuclear bodies (Zhong et al. 2000; Lallemand-Breitenbach et al. 2001; Lafarga et al. 2002). Recent studies show that PML can be modified by SUMO and this triggers its polyubiquitylation and proteasomal degradation (Tatham et al. 2008; Lallemand-Breitenbach et al. 2008). According to the model proposed by De Thé and colleagues (Lallemand-Breitenbach et al. 2001), after PML protein modification, proteasomes are recruited to primary PML bodies giving rise to mature PML nuclear bodies, which have a characteristic shell- or ring-like morphology by electron microscopy and are therefore indistinguishable from clastosomes. An alternative view is that modified PML protein is recruited to clastosomes (Fig. 3). As clastosomes form independently of PML protein (Lafarga et al. 2002), we favor the later model. Indeed, clastosomes can be seen in the nucleus containing distinct proteasome substrates, suggesting diversity among this type of nuclear bodies (Fig. 3). Clearly, it is crucial to determine what nucleates de novo assembly of a clastosome: Is it the proteasome, the protein substrate or a complex of proteasomes with associated substrates? Moreover, it is becoming increasingly apparent that the cell's population of proteasomes may actually represent a diverse group of functionally distinct members (Hanna et al. 2007; Hanna and Finley 2007). An exciting speculation is that clastosomes result from particular proteasome configurations that display novel and unique properties, distinct from the "canonical" forms present throughout the nucleoplasm.

THE POLYCOMB BODY: A TRANSCRIPTIONAL REPRESSOR DOMAIN?

Polycomb group (PcG) proteins are well-conserved, essential regulatory factors that bind

M. Carmo-Fonseca *et al.*

Figure 3. Clastosomes concentrate protein substrates for proteasomal degradation. (A, B) The panels depict a human neuron mechanically isolated from dorsal root ganglia obtained from an autopsy of a patient without any diagnosed neurological disorder, double-labeled with anti-PML (B, green staining) and anti-20S proteasomal complexes (A and B, red staining). Note that only one of the multiple PML bodies concentrates proteasomes (arrowhead) and that one body enriched in proteasomes does not contain PML (arrow). (C, D) Colocalization of 19S proteasomal complexes (C and D, red staining) and c-Fos (D, green staining) in the nucleus of a neurosecretory neuron isolated from rat hypothalamus after osmotic stress. Bar, 5 μ m. (E) Hypothetical model for PML protein traffic through PML bodies and clastosomes. PML (blue spheres) distributes diffusely in the nucleoplasm and associates transiently with primary PML bodies. Upon modification by SUMO and ubiquitin, the modified PML proteins (red spheres) are recruited to clastosomes for degradation.

to specific DNA regions in target genes and repress their transcription. PcG proteins maintain the silenced state of developmental genes, including homeotic genes and genes involved in mammalian embryonic stem cell differentiation (Schuettengruber *et al.* 2007). PcG proteins were first described in *Drosophila* as factors responsible for maintaining the

transcriptionally repressed state of Hox/homeotic genes throughout development. A growing number of vertebrate genes related to the *Drosophila* PcG proteins have recently been identified. PcG proteins exist in at least two distinct complexes termed Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). Members of PRC1, including

the HPH1/HPH2, Bmi1, HPC2 and RING1 gene products are diffusely distributed in the nucleus and in addition colocalize in both *Drosophila* and mammalian cells as large nuclear domains termed Polycomb or PcG bodies (Buchenau et al. 1998; Saurin et al. 1998). In *Drosophila*, several genes that are repressed by PcG proteins localize to Polycomb bodies, suggesting that these domains correspond to sites of gene silencing (Grimaud et al. 2006). However, the number of Polycomb bodies is orders of magnitude smaller than the number of genetic loci targeted by PcG proteins. This implies that either multiple gene targets are associated with each Polycomb body or that gene silencing by PcG proteins can occur outside Polycomb bodies. Although endogenous homeotic genes appear to cluster at PcG bodies by a mechanism that requires components of the RNA interference machinery (Grimaud et al. 2006), it remains unknown whether assembly of a Polycomb body is necessary for the functional association of PcG proteins with chromatin.

POLYCOMB AND PML NUCLEAR BODIES: THE SUMO CONNECTION

The small ubiquitin-like modifier (SUMO) is an ubiquitin-related protein that can be covalently conjugated to a variety of protein substrates, altering the properties of the modified proteins. SUMO conjugation is essential for cell and organism viability, from yeast to mammals (Nacerddine et al. 2005), and affects many biological processes, including cell cycle progression, maintenance of genome integrity, and transcription (reviewed in Hay 2005). SUMO substrates include protein components of both PML and Polycomb nuclear bodies and recent evidence implicates the SUMOylation pathway as an important player in the assembly of these domains (Heun 2007).

The PML protein and the PML body components HIPK2, Daxx, and Sp100 are modified by SUMO. Moreover, SUMO conjugation is necessary for formation of PML bodies and for recruitment of HIPK2, Daxx, and Sp100 into the bodies (reviewed in Hay 2005). SUMO-

modified HIPK2 represses the activity of bound transcription factors by associating with Groucho corepressor and HDAC1, and SUMO modification of Sp100 enhances its interaction with heterochromatin protein 1 (HP1), a well known initiator of repressive domains in chromatin.

The mechanism of SUMO conjugation involves a cascade of events catalysed by an activating enzyme E1, a conjugating enzyme E2 (known as Ubc9), and a protein ligase E3 (Hay 2005). There are at least three types of SUMO E3 ligases in cells: the protein inhibitor of activated signal transducer and activator of transcription (PIAS) family, the nuclear pore complex protein RanBP2/Nup358, and the Polycomb group protein Pc2. One of the substrates of Pc2 is the carboxy-terminal binding protein (CtBP) transcriptional corepressor (Kagey et al. 2003). It has been proposed that Pc2 functions to recruit Ubc9 and CtBP to PcG bodies, where SUMO modification of CtBP occurs (Kagey et al. 2004). More recently, the zinc finger protein CTCF was also shown to be recruited to Polycomb bodies and to be modified by SUMO (MacPherson et al. 2009).

Intriguingly, only a small proportion of the total cellular pool of a given transcription factor is modified by SUMO. Yet, the entire population is functionally repressed. The following model was proposed to accommodate these observations: Upon conjugation to SUMO, a transcription factor is incorporated into a repression complex located in a nuclear body; even if SUMO is removed from the transcription factor at a later stage by specific proteases, the protein would still be retained in the repression complex (Hay 2005). Consistent with this view, PML and Polycomb nuclear bodies might result from the assembly of SUMO-dependent repression complexes. A variation of this hypothesis postulates that a SUMO-modified transcription factor recruits an enzyme capable of posttranslationally modifying chromatin. SUMO could then be removed from the transcription factor, whereas transcriptional repression would be maintained by the chromatin modification (Hay 2005). According to this alternative view, PML and Polycomb nuclear bodies could be sumoylation centers, from

M. Carmo-Fonseca *et al.*

which SUMO-modified transcription factors would be released. Consistent with the model that nuclear bodies can act as sites for SUMO-conjugation, the SUMO-conjugating enzyme Ubc9 is highly enriched in these structures (Navascués *et al.* 2007) (Fig. 4).

SEVERAL TRANSCRIPTIONAL REGULATORS LOCALIZE TO NUCLEAR BODIES

Many transcription factors associated with RNA polymerase II and III activity appear uniformly distributed throughout the nucleoplasm. In addition, some of these factors are found concentrated in larger nuclear domains, at least in some cell lines. A well characterized example is the so-called OPT domain, a region of approximately 1.3 μm in diameter that concentrates transcription factors Oct1 and PTF

(Pombo *et al.* 1998; Grande *et al.* 1997). The OPT domain is dynamic during the cell cycle: It appears during G1 phase and disappears in S phase. The OPT domain is also transcription-dependent: the transcription inhibitor DRB inhibits its formation and hastens its disappearance (Pombo *et al.* 1998). Although it has been proposed that the OPT domain might play a role in clustering genes on different chromosomes to a region where the appropriate transcription factors are concentrated (Pombo *et al.* 1998), as yet there is no evidence to support this view. In particular, genes that contain PTF and Oct1-binding sites have not been observed to colocalize in the domain. Alternatively, the OPT domain may contain intermediary complexes of transcription factors that form independently of their binding to regulatory sequences in target genes. Consistent with this

Figure 4. SUMO-1 localizes to nuclear bodies in neuron-like UR61 cells. (A) Electron microscopy reveals the presence of a round body (arrow) in the nucleus (nu, nucleolus). Bar, 1 μm . (B) Immunogold labeling of a nuclear body using antibodies specific for SUMO-1 (an arrow points to an adjacent doughnut-shaped structure that is most probably a clastosome). Bar, 300 nm. (C) The distribution of SUMO-1 is detected in green by immunofluorescence; the nucleolus is blue (immunofluorescence with antifibrillarin antibody) and the cell periphery is red (Rhodamine-Phalloidin Staining). (D) Co-localization of GFP-SUMO-1 (green) and SUMO E2 conjugating enzyme DsRed-Ubc9 (red) in nuclear bodies; the cytoplasm is stained blue (immunofluorescence with anti-SMN antibody). Bar, 5 μm .

view, nuclear domains enriched in transcription factors could form as a result of protein modification, namely SUMOylation, as suggested for PML bodies and more recently, for a novel type of nuclear body that concentrates KRAB-zinc finger transcriptional regulators (Briers et al. 2009).

RNA-BINDING PROTEINS IN NUCLEAR BODIES

Members of three major classes of RNA-binding proteins localize to orphan nuclear bodies. These include Sam68 and the related SLM-1 and SLM-2 proteins of the signal transduction and activation of RNA (STAR) family, two subunits of the cleavage stimulation factor (CstF) and the cleavage and polyadenylation specificity factor (CPSF) components of the mRNA 3'-end processing machinery, and DDX1, a member of the DEAD box protein family of RNA helicases.

Sam68 belongs to the STAR family of proteins characterized by a KH (hnRNP K homology) domain embedded in a highly conserved region called GSG (GRP33/Sam68/GLD1) domain, which is required for homodimerization and sequence-specific RNA binding. Sam68 is a substrate for Src tyrosine kinases and is therefore considered a strong candidate to integrate signal transduction pathways and RNA metabolism, particularly alternative splicing (Paronetto et al. 2007). Although Sam68 can translocate to the cytoplasm and associate with specific RNAs in polysomes (Grange et al. 2009; Paronetto et al. 2009), the protein is predominantly detected in the nucleus (Fig. 5). Sam68 localizes diffusely in the nucleoplasm but additionally concentrates in a few prominent structures termed Sam68 nuclear bodies or SNBs (Chen et al. 1999). SNBs are dynamic structures that disassemble during mitosis and after treatment of cells with transcriptional inhibitors. Occasionally they appear adjacent to the nucleolus (Fig. 5) or to a Cajal body. Electron microscopic studies show that SNBs contain nucleic acids, presumably RNA, and targeting of Sam68 to these structures involves the highly conserved GSG protein domain (Chen et al. 1999). Thus, SNBs might form as a result of

Figure 5. The RNA-binding protein Sam68 localizes to nuclear bodies in both HeLa cells (A) and rat neurons (B, C). Sam 68 is detected in green by immunofluorescence labeling. Nucleoli and cytoplasm are detected in red (propidium iodide staining). RNA splicing speckles are detected in blue (immunofluorescence with an antibody directed against the 2, 2, 7-trimethylguanosine cap structure of snRNAs). Bar, 10 μ m.

Sam68 self-assembly and binding to specific RNAs in the nucleus. Although the identity of such potential scaffold RNAs remains to be determined, they are expected to be short-lived because SNBs disassemble within a few hours after transcription inhibition (Chen et al. 1999). Recently, Sam68 was reported to be modified by SUMO (Babic et al. 2006), and a pending question is whether SUMOylation is related to formation of SNBs.

CPSF and CstF are multiprotein complexes involved in formation of the 3'-ends of most mRNAs (reviewed in Danckwardt et al. 2008). The CPSF 100 kDa subunit and the CstF 64 kDa subunits are diffusely distributed throughout the nucleoplasm and additionally concentrate in a few bright foci termed cleavage bodies (Schul et al. 1996). Cleavage bodies are primarily observed during S phase, apparently do not contain RNA, and are not affected

M. Carmo-Fonseca *et al.*

by inhibitors of RNA transcription (Li *et al.* 2006). Cleavage bodies often concentrate the DEAD box protein DDX1 (Bléoo *et al.* 2001) and are frequently found adjacent to Cajal bodies (Li 2006).

CONCLUDING REMARKS

Unlike the nucleolus, most types of orphan nuclear bodies are not detected in all cell types. It is therefore unlikely that these structures play an essential role in the nucleus. In general, orphan nuclear bodies are dynamic structures that assemble and disassemble at specific stages of the cell cycle or in response to changes in environmental conditions. A fundamental question is whether bodies form under these specific conditions as a result of aggregation of excessive protein that is not used, or contribute to increase the capacity of cells to survive under varying environmental stresses. Noteworthy, SUMOylation of the *Caenorhabditis elegans* PcG protein SOP-2 is required for both its localization to nuclear bodies and its physiological repression of Hox genes (Zhang *et al.* 2004). In further agreement with the view that nuclear bodies are functionally relevant, several lines of evidence implicate SUMOylation in assembly of PML nuclear bodies and maintenance of cell viability (Heun 2007).

Taken together, these results suggest that some nuclear bodies might represent sites of SUMO-conjugation and/or protein degradation by the ubiquitin-proteasome system, whereas others are more likely associated with transcription regulation and RNA metabolism. Further studies are needed to understand precisely what roles these different types of bodies play in the nucleus.

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