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In search of nonribosomal nucleolar protein function and regulation

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The life of the nucleolus has proven to be more colorful and multifaceted than had been envisioned a decade ago. A large number of proteins found in this subnuclear compartment have no identifiable tie either to the ribosome biosynthetic pathway or to the other newly established activities occurring within the nucleolus. The questions of how and why these proteins end up in this subnuclear compartment remain unanswered and are the focus of intense current interest. This review discusses our thoughts on the discovery of nonribosomal proteins in the nucleolus.

Introduction

In December 1965, many of the world's cell and molecular biologists most keenly engaged in the nucleolus gathered in Montevideo, Uruguay, for a southern summer summit (Perry, 1966; Vincent and Miller, 1966). Built upon previous momentum (Swift, 1959; Perry, 1965), this conference conclusively established the role of the nucleolus as the site of ribosome biosynthesis, based on a confluence of form and function as compelling as any that cell biology had witnessed at the time. The key evidence was the demonstration that the nucleolus organizer locus contains DNA that hybridizes to ribosomal RNA (rRNA; Ritossa and Spiegelman, 1965; Birnstiel et al., 1966), that rapidly labeled precursors of rRNA are present in isolated nucleoli (Penman, 1966), and that anucleolate *Xenopus laevis* embryos fail to synthesize rRNA, arresting in development when their maternal stockpile of ribosomes becomes limiting (Brown and Gurdon, 1964). Moving fast forward, it is now known that the nucleolus has other functions (Pederson, 1998; Olson et al., 2000, 2002; Raska et al., 2006; Boisvert et al., 2007). Yet something remains unresolved: certain proteins accumulate in the nucleolus as apparently uninvited visitors with no identifiable roles in nucleolar functions, including the recently established ones. Does this signify a storage role of the nucleolus, keeping these proteins out of reach of their otherwise appropriate times and loci of function, or are these visitors moonlighting in other jobs while in the nucleolus?

Although molecular traffic in and out of the nucleus has come to be understood quite well in the past 20 years, springing from an initial finding (Kalderon et al., 1984) to a modern understanding as structural biology (Lim and Fahrenkrog, 2006; Alber et al., 2007), the same cannot be said for the question of how intranuclear bodies form by the accretion of various proteins or RNA, which has just begun to be investigated (Kaiser et al., 2008; Misteli, 2008). With the genome itself being a clear organizing principle of the nucleus, one can easily envision how various molecular machines assemble at sites of replication, repair, and transcription, as well as on chromosome-tethered structures such as nascent RNAs, because of their physical link to the genome. Wandering molecules encounter avid sites on the DNA or transcript and simply behave as dictated by the equilibrium association constants involved, operating as standard bimolecular (collision-dependent) reactions. But how then do intranuclear bodies form without any apparent physical connection with the genome, such as promyelocytic leukemia bodies and interchromatin granule clusters (also known as nuclear speckles) inter alia (Gall, 2000; Lamond and Spector, 2003; Handwerger and Gall, 2006; Borden, 2008)? At these sites throughout the nucleoplasm, certain molecules seem to become concentrated for no apparent reason; i.e., without a (known) fixed nucleating anchor. The formation of these protein-enriched bodies stands as a major unsolved issue in cell biology.

Although the nucleolus is often included in lists of "nuclear bodies," it is more properly viewed as a cytogenetic phenomenon in which the transcriptional activity of the rRNA genes and the co- and posttranscriptional recruitment of rRNA processing factors, ribosomal structural proteins, and assembly promoting factors generate a specific cytological entity. This view is reinforced by the observations that formation of micronucleoli or nucleolus-like structures can be induced by single copies of rDNA (Nierras et al., 1997; Oakes et al., 1998) or, even more remarkably, by a tandem array of DNA-binding sites for the upstream binding factor component of the RNA polymerase I

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Abbreviations used in this paper: APC/C, anaphase-promoting complex or cyclosome; NoLS, nucleolar localization signal; rRNA, ribosomal RNA; SRP, signal recognition particle.

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transcriptional machinery (Mais et al., 2005). However, in the past decade, the nucleolus has taken on a broader life, with new functions and the revelation of many visiting molecules that have no apparent role in either ribosome production or in the recently discovered novel functions of the nucleolus. Thus, a paradox now looms before us: how and why do these molecules visit this subnuclear compartment? We do not have the answer, but we believe that a clear statement of the problem is an essential first step.

The open nucleolus

Two key points to be recognized from the start are that nucleoli are neither membrane-bound, which was never seriously entertained, nor are they as extremely compact as had once been assumed. Measurements of the refractive index of nucleoli reveal a mass per unit volume that is, surprisingly, only twice that of the nucleoplasm (Handwerger et al., 2005). Therefore, all diffusing nuclear molecules may permeate nucleoli, with their nucleolar residence times determined simply by their relative affinities for preexisting, anchored nucleolar elements. Although in this sense the nucleolus can be regarded as an open compartment for all nuclear proteins, here we will only address ones that concentrate in the nucleolus by increased retention.

The newly recognized nucleolar functions, and the visitors they beckon

When preribosomal particles (nucleolar ribonucleoprotein complexes containing pre-rRNA) were first characterized, it was noted that they had a higher protein:RNA ratio than mature cytoplasmic ribosomes (Pederson and Kumar, 1971; Kumar and Warner, 1972). More recent work has revealed a large number of proteins that bind to pre-rRNAs but do not remain with exported ribosomes (Fatica and Tollervey, 2002). In the context of this article, we do not regard these as provocative nucleolar visitors, as they have demonstrable affinity for pre-rRNA or its processed intermediates. However, the situation is entirely different with respect to the nucleolar transit of RNAs and proteins involved in assembly of the signal recognition particle (SRP; Jacobson and Pederson, 1998; Ciufo and Brown, 2000; Politz et al., 2000; Grosshans et al., 2001) and U2 and U6 spliceosomal small RNA modification (Ganot et al., 1999; Yu et al., 2001), which are the most clearly established nonribosomal functions of the nucleolus at present. There is no known molecular basis for these nucleolar visitations, and no obvious reason, notwithstanding speculation (e.g., Pederson and Politz, 2000), why SRP assembly or U2 and U6 RNA modification should occur in the nucleolus. To learn if the nucleolus is essential for these latter functions, one would need new experimental approaches such as using the *X. laevis* embryo homozygous for the anucleolate mutation (Elsdale et al., 1958) or cells in which the nucleoli are ablated by hyper-focused, thermally minimal laser irradiation (Berns et al., 2000).

New visitors to the nucleolus

Molecules that are present in the nucleolus but have no known roles in the biosynthesis of ribosomes, the SRP, or the modification of U2 or U6 RNAs have now come into the spotlight. They include several proteins that are known to be involved in cell growth control, telomere maintenance, and protein degradation, as well as certain microRNAs (Politz et al., 2006). This wave of information began to arise in the 1990s in a rather episodic fashion and mostly came in beneath the radar. A major advance ensued when two groups described a proteomic catalog of purified HeLa cell nucleoli, revealing that more than half of the 700 proteins in the nucleolar complex were unrelated to ribosome biosynthesis (Andersen et al., 2002, 2005; Scherl et al., 2002). The most provocative of these were ones with known roles in cell cycle regulation.

The nucleolus in cell cycle control and beyond

How then would a nucleolus-centered mechanism come to work on behalf of cell cycle control? A connection between the nucleolus and cell cycle had been described in early literature, where UV microbeam ablation of interphase nucleoli in grasshopper neuroblasts was observed to cause an arrest of mitosis that could not be explained simply on the basis of ribosome deficiency (Gaulden and Perry, 1958). This general notion has been more recently raised in connection with a human bone marrow failure disease (Pederson, 2007). Indeed, there is now a compelling body of evidence showing that eukaryotic cell cycle progression is not only associated with ribosome biosynthesis, but that the nucleolus may indeed be the central regulatory link between these two cellular activities. Such evidence includes a crosstalk between a nucleolar protein involved in ribosome biosynthesis and the p53 regulation of cell cycle progression (Pestov et al., 2001), cyclin-dependent kinases involved in nucleolar interphase maintenance (Sirri et al., 2002), and cell cycle arrest and p53-tiggered apoptosis caused by inactivation of an rDNA transcription factor (Yuan et al., 2005). These and other recent findings seem not to be as well known across the emerging nucleolus–cell cycle consolidated field as would be ideal. What is needed to further pursue this concept of a nucleolus–cell cycle link is a nucleolar protein that has no role in ribosome production but is nonetheless intimately connected to cell cycle progression or cell division. One such protein is the cyclin phosphatase Cdc14 and another is nucleostemin. Other examples exist, but Cdc14 and nucleostemin are particularly enabling cases that cogently illuminate the issue.

Cdc14

The Cdc14 phosphatase is a prototypic example of the nucleolus apparently acting as a storage site of cell cycle regulatory proteins. It was initially proposed that the nucleolar localization of Cdc14 prevents premature exit from mitosis. The notion is that Cdc14 is tethered by Net1 (also termed Cfi1) in a nucleolar complex called RENT (*regulator of nucleolar silencing and telophase*) from G1 to metaphase. Upon entry into anaphase, Net1 is phosphorylated and Cdc14 is released from the nucleolus (Shou et al., 1999; Azzam et al., 2004). The freed Cdc14 then dephosphorylates and activates Cdh1. The anaphase-promoting complex or cyclosome (APC/C)-bound Cdh1 (APC/C$^{\mathrm{Cdh1}}$) functions as an E3 ubiquitin ligase and degrades mitotic cyclins, thereby inactivating mitotic cyclin-dependent kinases and triggering mitotic exit (Visintin et al., 1998; Shou et al., 1999; Visintin et al., 1999). Afterward, the return of Cdc14 back to the nucleolus is promoted by APC/C^{Cdh1}-controlled degradation of Polo kinase Cdc5, which is also a key factor in the nucleolar release of Cdc14 (Visintin et al., 2008). This original concept of Cdc14 activation during mitosis has now been extended in two new directions. First, nucleoplasmic translocation of Cdc14 can also occur in response to stress and may play a role in solidifying the G2 DNA damage response checkpoint mechanism. This stress response role of Cdc14 is itself mediated by the APC/C^{Cdh1}-controlled degradation of Polo kinase (Bassermann et al., 2008). In addition, Net1, the same protein that holds Cdc14 in the nucleolus in preparation for mitotic exit, has been shown to stimulate RNA polymerase I transcription independently of its cell cycle role (Shou et al., 2001). The latter finding exemplifies the potential versatility of nucleolar proteins in both ribosome biosynthesis and cell cycle progression, a point to be revisited in the following paragraph.

Nucleostemin

A newcomer to this field is a nucleolar protein highly expressed by stem cells and cancer cells, hence named nucleostemin (Tsai and McKay, 2002). Nucleostemin is a GTP-binding protein whose intranuclear distribution is regulated by its state of GTP binding (Tsai and McKay, 2005). The pro–self renewal and cell cycle regulatory activities of nucleostemin may be related to its interaction with a multitude of proteins, including p53 (Tsai and McKay, 2002; Ma and Pederson, 2007), MDM2 (Dai et al., 2008; Meng et al., 2008), TRF1 (telomeric repeat binding factor 1; Zhu et al., 2006), ARF (*alternative reading frame*; Ma and Pederson, 2007), RSL1D1 (ribosomal L1 domain containing 1; also known as cellular senescence-inhibited gene or CSIG; Meng et al., 2006), and B23/nucleophosmin (Ma and Pederson, 2008a). The complexity of nucleostemin's activity is reflected not only by the variety of its interacting partners but also by how it influences each of these proteins. In the case of p53 for example, nucleostemin depletion leads to p53 activation via its interaction and regulation of MDM2 (Dai et al., 2008; Meng et al., 2008). However, overexpression of nucleostemin may also trigger similar p53 phenotypes (Dai et al., 2008; Ma and Pederson, 2008b). Based on data showing that nucleoplasmic mobilization of nucleostemin stabilizes MDM2 protein and promotes G2–M progression, a proposed idea is that the nucleolus operates as a counting device, which tallies the number of cell division by the loss of MDM2 protein during mitosis and signals cell cycle exit when MDM2 protein falls below a threshold level (Meng et al., 2008). In this model, nucleostemin plays a role in inactivating this counting mechanism to safeguard the proliferative potential of continuously dividing cells. Notwithstanding these findings, a potential function of nucleostemin within the nucleolus has just begun to emerge. Among the three nucleostemin-binding proteins in the nucleolus, ARF and B23 have been known to participate in both the cell cycle regulatory and ribosomal-processing pathways. The other protein, RSL1D1, has recently been shown to delay replicative senescence in human fibroblast cells by regulating PTEN (Ma et al., 2008). Could RSL1D1 be the primary nucleolar target of nucleostemin that confers its anti-senescence activity, as seen in mouse embryonic fibroblast cells (Zhu et al., 2006); or could nucleostemin turn out to be like ARF, Net1, and B23, and possess independent roles in rRNA transcription or processing (Shou et al., 2001; Sugimoto et al., 2003)? A recent study indicated a role of nucleostemin in pre-rRNA processing (Romanova et al., 2008). The relationships between the ribosomal and nonribosomal functions of nucleostemin and others are nonexclusive and await further clarification. Even if a ribosome-related phenotype is found in connection with these nonribosomal proteins, a major challenge still lies in how to dissect its direct versus indirect effects.

Beyond cell cycle control, nucleolar compartmentalization has also been linked to other biological events. One example is found in *Drosophila melanogaster* spermatogenesis, where nucleolar sequestration of polycomb factors allows primary spermatocytes to differentiate into mature spermatids (Chen et al., 2005). More recently, the role of the nucleolus in the phased retention–release of a specific transcription factor has been demonstrated to play a key role in cell fate determination (Martindill et al., 2007). During placentation, the bHLH transcription factor Hand1 is stored in the nucleolus of trophoblast stem cells. It is released into the nucleoplasm when trophoblast stem cells become committed to differentiation into giant cells. The mechanism underlying these two observations may reside in cell type or developmental stage–specific expressions of nucleolar anchors for these proteins, and this therefore broadens the regulatory landscape of the nucleolus to not only cell cycle progression but also developmentally regulated events (Martindill and Riley, 2008).

How do nucleolar proteins make their moves?

Like most if not all nucleolar proteins, nucleostemin shuttles between the nucleolus and the nucleoplasm at an astonishingly fast pace. The unsolved mystery, which has perhaps not been emphasized enough to date, is how these proteins cycle so rapidly between the nucleolar-bound and unbound states, and why they act in this way. In the case of nucleostemin, although a nucleolar localization signal (NoLS) comprised mainly of a stretch of basic residues is sufficient to mediate nucleolar accumulation when fused to GFP, the NoLS-tagged GFP does not display the same dynamic and regulated features as full-length nucleostemin (Tsai and McKay, 2005). The nucleolar residence time of full-length nucleostemin is longer than that of the NoLS-fused GFP, and involves a nucleolar retention signal localized in the separate GTP-binding domain of nucleostemin. Furthermore, its steady-state accumulation and dynamic cycling to and from the nucleolus is controlled additionally by a domain that favors its nucleoplasmic localization and acts essentially as a nucleoplasmic localization signal in a GTP-dependent manner (Tsai and McKay, 2005; Meng et al., 2007). Although structural biology studies of nucleostemin in the GTP-bound or unbound state have yet to be undertaken, it can be plausibly anticipated that a conformational switch occurs and that this may be a key determinant of the equilibrium binding affinity constant of the two forms for nucleolar or nucleoplasmic proteins.

It should be noted that GTP-mediated binding is not the only mechanism that can control the nucleolar–nucleoplasmic cycling behavior of proteins. For example, a parallel case of an intracellular signal that regulates protein localization to the nucleolus is the hydrogen ion. Increases in pH have been shown to promote the nucleolar sequestration of the von Hippel-Lindau tumor suppressor protein, an E3 ubiquitin ligase, thereby eliciting a transient and reversible inhibition that eventually stabilizes hypoxia-inducible factor (HIF) and allows transcriptional activation of a wide range of genes in the hypoxic response of cells (Mekhail et al., 2004). It is equally important to note that some nucleolar molecules can vacate en masse upon cellular stress or during mitosis. Stress signals mobilize several nucleolar proteins that are known to stabilize or destabilize p53 proteins via their interaction with MDM2, a major player in the negative regulation of p53 (Tao and Levine, 1999; Zhang et al., 2003; Bernardi et al., 2004; Dai et al., 2004; Jin et al., 2004; Kurki et al., 2004; Dai et al., 2008; Meng et al., 2008). This nucleolar mechanism presumably allows cells to initiate or dampen stress responses via the MDM2–p53 pathway in a regulated fashion. The nucleolus undergoes disassembly and reassembly during mitosis as well. In this cell cycle window, most nucleolar proteins in the granular component are temporarily released into the prophase nucleoplasm and mitotic cytoplasm, which allows them to interact with other proteins. This adds a temporal dimension to the "interactomology" of nonribosomal nucleolar proteins and is another aspect of this field that warrants further investigation. Finally, an insightful analysis of DNA damage in relation to nucleolar integrity has indicated that the nucleolar structure itself may act as a stress sensor that modulates p53 levels (Rubbi and Milner, 2003).

An enigmatic aspect of the overall problem of nucleolus-concentrated proteins is that many lack a well-defined NoLS. It is thus logical to assume that these proteins gain their foothold inside the nucleolus by complexing with already anchored proteins. The fact that many nucleolus-concentrated proteins display dynamic shuttling into the nucleoplasm and back (Phair and Misteli, 2000; Chen and Huang, 2001; Pederson, 2001; Misteli, 2005; Tsai and McKay, 2005) means that their interactions with nucleolus-anchored molecules are reversible and relatively brief. Equally intriguing is the nucleolar dynamics of ribosomal proteins themselves. One would have thought, a priori, that they would arrive in the nucleolus in amounts stoichiometric with the ongoing tempo of rRNA synthesis, and that, after combining with rRNA, they would leave the nucleolus in assembled ribosomes, all neatly balanced on the ledger sheet. This, however, is not the case. Ribosomal proteins shuttle into and out from the nucleolus (Chen and Huang, 2001; Pederson, 2001), and the level of unassembled ribosomal proteins in the nucleolus actually exceeds what is needed for a one-to-one assembly with nascent rRNA (Lam et al., 2007). In fact, a demonstrated role of ubiquitin-mediated proteasome action on the ribosome biosynthesis pathway suggests that excess ribosomal proteins in the nucleolus and nucleus are monitored and degraded to create a set point for ribosome assembly stoichiometry (Stavreva et al., 2006). This is itself an intriguing issue that seems to suggest that the process of ribosome assembly is protein concentration driven, which is compatible with early studies that revealed a surprising immediacy of protein synthesis inhibition feeding back instantly on the rate of ribosome production (Pederson and Kumar, 1971), and yet also points to a surveillance mechanism that destroys excess ribosomal proteins.

Conclusions

Although the idea that nucleoli serve only as a passive, functionally inert hideout for certain proteins has been the conventional thinking for years, a different school of thought is gaining momentum; i.e., these proteins may be moonlighting in other jobs within the nucleolus. Of course, a question left hanging is why these functions would necessarily require a nucleolar siting as opposed to some discrete domain or body forming elsewhere in the nucleoplasm. It would seem that the most likely explanation is that there is some fundamental cross-regulatory link between ribosome biosynthesis and cell cycle progression, an idea that first arose many years ago in prokaryotic systems and remains a perfectly plausible scenario in eukaryotes as well (Rudra and Warner, 2004; Bernstein et al., 2007; Pederson, 2007). Not to be discounted is the possibility that this may occur simply because the nucleolus houses some enzymatic activities that are shared by the processing pathways of multiple proteins. As the production of ribosomes becomes the dominant event in cells and draws those utilitarian enzymes in and around the nucleolus, so go the other proteins whose maturation depends on them. Though this is speculation, all these intriguing possibilities can now be addressed one by one with the new methodologies available, and, most importantly, the always trustworthy catalyst of progress: an open mind. We expect the list of nucleolar functions to grow in the future, and so will our view of this beguiling nuclear domain be changed again, with pleasure.

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