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Evidence for nucleolar subcompartments in *Dictyostelium*

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ABSTRACT

The nucleolus is a multifunctional nuclear compartment usually consisting of two to three subcompartments which represent stages of ribosomal biogenesis. It is linked to several human diseases including viral infections, cancer, and neurodegeneration. *Dictyostelium* is a model eukaryote for the study of fundamental biological processes as well as several human diseases however comparatively little is known about its nucleolus. Unlike most nucleoli it does not possess visible subcompartments at the ultrastructural level. Several recently identified nucleolar proteins in *Dictyostelium* leave the nucleolus after treatment with the rDNA transcription inhibitor actinomycin-D (AM-D). Different proteins exit in different ways, suggesting that previously unidentified nucleolar subcompartments may exist. The identification of nucleolar subcompartments would help to better understand the nucleolus in this model eukaryote. Here, we show that *Dictyostelium* nucleolar proteins nucleomorphin isoform NumA1 and Bud31 localize throughout the entire nucleolus while calcium-binding protein 4a localizes to only a portion, representing nucleolar subcompartment 1 (NoSC1). SWI/SNF complex member Snf12 localizes to a smaller area within NoSC1 representing a second nucleolar subcompartment, NoSC2. The nuclear/nucleolar localization signal KRKR from Snf12 localized GFP to NoSC2, and thus also appears to function as a nucleolar subcompartment localization signal. FhkA localizes to the nucleolar periphery displaying a similar pattern to that of Hsp32. Similarities between the redistribution patterns of *Dictyostelium* nucleolar proteins during nucleolar disruption as a result of either AM-D treatment or mitosis support these subcompartments. A model for the AM-D-induced redistribution patterns is proposed.

1. Introduction

The nucleolus is a multifunctional nuclear compartment involved in a multitude of cellular processes and human diseases [1–6]. In addition to its role in certain viral infections and cancer, it has been linked to Alzheimer's and other neurodegenerative diseases [5,7]. The nucleolus typically consists of three subcompartments, a fibrillar center (FC), a dense fibrillar component (DFC), and a granular component (GC). Although these subcompartments are associated with various stages of ribosomal subunit production the majority of nucleolar proteins (about 70% of the 700+ in humans) are involved in non-ribosomal processes [1].

Dictyostelium is a model eukaryote for the study of several fundamental cellular processes and human diseases however, compared to other organisms, little is known about its nucleolus [8,9]. The *Dictyostelium* nucleolus consists of 2–4 patches adjacent to the inner nuclear envelope [10,11]. Unlike other organisms no nucleolar subcompartments are visible ultrastructurally but rather, granular and fibrillar material are interspersed throughout the nucleolar matrix [12]. rDNA (which cannot be distinguished from the remaining nucleolar material by EM) exists not in the center but around the periphery of each nucleolar patch [12–14]. Although other lower eukaryotes such as *Polysphondylium* possess a nucleolus similar in morphology, *Dictyostelium* is the only genus for which such a nucleolus has been studied.

Despite the lack of visible subcompartments from EM studies, recent work suggests that the *Dictyostelium* nucleolus may contain functionally distinct regions. Calcium-binding protein 4a (CBP4a) and forkhead-associated kinase A (fhkA) are two recently identified proteins in *Dictyostelium* that localize to different nucleolar locales; FhkA localizes around the nucleolar periphery while CBP4a resides within the nucleoplasm [15]. BAF60a/SMARCD1 homologue Snf12 and NumA1, two nucleolar proteins linked to cell cycle in

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Dictyostelium, localize differently since Snf12 localizes to a smaller area of the nucleolus than NumA1 [16,17]. If, as this work suggests, functional subcompartments do exist, these and other nucleolar proteins should localize to different regions of the nucleolus, thereby delineating some of the nucleolar subcompartment locales. Despite the recent identification of several nucleolar proteins in *Dictyostelium* no two have been colocalized.

Here, we examined the localization of nucleolar NumA1, CBP4a, Snf12, eukaryotic translation initiation factor 6 (eif6), and Bud31 (identified as a nucleolar protein in this present study). We have identified two novel nucleolar subcompartments which we have called nucleolar subcompartment 1 (NoSC1; containing CBP4a) and nucleolar subcompartment 2 (NoSC2; containing Snf12). NoSC2 is contained within NoSC1 and exhibits high morphological variability. Given these new findings, we present a model for the actinomycin-D (AM-D)-induced redistribution of nucleolar proteins, a recently discovered yet poorly understood phenomenon.

2. Materials and methods

2.1. Materials

For all experiments *Dictyostelium discoideum* AX3 cells were used. Cells were grown in HL-5 at 21 °C shaking at 180 rpm, as previously described [18]. The QIAquick® PCR Purification Kit (Qiagen), QIAquick® Gel Extraction Kit (Qiagen), and QIAprep® Spin Miniprep Kit (Qiagen) were used for all PCR purifications, gel extractions, and plasmid isolations respectively. Restriction enzymes were purchased from New England BioLabs® Inc.

2.2. Antibodies and GFP-fusion protein-expressing cell lines

Anti-NumA1, anti-CBP4a, and anti-Snf12 were produced and verified as previously described [15–17,19]. Cell lines expressing either C-GFP-Snf12 or GFP-NLS-3 (GFP-KRKR) from Snf12 were made and verified as previously described [17]. Cell lines expressing either C-GFP-eIF6 or C-GFP-Bud31 were made by amplifying full length eIF6 or Bud31 via PCR using total cDNA as template as previously described [16]. Primers used to amplify eIF6 were CAGAGCTCAAAATGGCTACAAGATTAC (forward) and CAACTAGTTA CATTATCAACAATTGAATTTC (reverse). Primers used to amplify Bud31 were CAGAGCTCAAAATGCCAAAAATAAAA (forward) and CAACTAGTACTTTCATTTTCGCTTTCA (reverse). Restriction sites (underlined) and kosak site were incorporated. Full length eIF6 and Bud31 were ligated into pDM-323 (for expression of C-terminally-fused GFP) as previously described [16]. Constructs for GFP-eIF6, GFP-Bud31, and pDM-317 (for expression of GFP alone) were transformed into *Dictyostelium* as previously described [16]. Colonies were selected for and maintained in 10 μg/mL G418.

2.3. Live cell viewing, treatment, fixation, and immunolocalization

GFP-fusion protein-expressing cells were viewed live after adhering to a cover slip for 30 min prior to slide-mounting. Fixation in ultracold methanol and immunolocalization was performed as previously described [20]. Cells were allowed to adhere to coverslips (in HL-5). After blocking, cells were incubated with either anti-NumA1 (1:40), anti-Snf12 (1:40), or anti-CBP4a (1:10) for 60 min followed by Alexa Fluor® 555 goat anti-rabbit (1:40) for 45 min. For some experiments cells were then probed with anti-GFP (1:500; Santa Cruz®) for 60 min followed by Alexa Fluor® 488 goat anti-mouse (1:100) for 45 min. All secondary antibodies were purchased from Invitrogen™. 5 μL ProlongAntifade (Invitrogen™) containing DAPI was placed on the slide prior to mounting and cover slips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera using Nikon Imaging Software Elements Basic Research 3.0.

3. Results

3.1. Snf12 localizes to a portion of the nucleolus

GFP-Snf12 was recently shown to localize predominately to the nucleoplasm and, in ~20% of cells, also to the nucleolus [17]. In order to determine if Snf12 localizes throughout the entire nucleolus C-GFP-Snf12 was colocalized with the nucleolar marker NumA1. The edge of the nucleolar area occupied by NumA1 colocalized with the edge of the DAPI-stained nucleoplasm (Fig. 1A). This demonstrates that NumA1 occupies the entire nucleolus, since DAPI does not stain nucleoli [13]. C-GFP-Snf12 was detected in a smaller region within the NumA1-occupied area (Fig. 1A). Anti-Snf12 detected C-GFP-Snf12 throughout the nucleoplasm but only in a portion of the nucleolus (Fig. 1B).

3.2. Nucleolar C-GFP-Snf12 localization pattern varies in size, shape and position

The nucleolar region occupied by C-GFP-Snf12 varied in size from a small area to two large nucleolar regions filling most of the nucleolus as demonstrated by colocalization of C-GFP-Snf12 with DAPI counterstaining (Fig. 2A). The location and shape of this region varied as well. It was located either adjacent to the nuclear envelope region, adjacent to the nucleoplasm, or as two regions, one adjacent to the nuclear envelope region and the other adjacent to the nucleoplasm (Fig. 2B). Its shape varied from globular to linear (Fig. 2B).

3.3. Bud31 can be used as a nucleolar marker in Dictyostelium

In yeast and humans Bud31 is an RNA splicing factor and DNA transcription factor suggesting that it may function in the nucleolus in *Dictyostelium* [21] (dictyBase.org). This was confirmed as GFP-Bud31 localized throughout the nucleolus and to a lesser extent throughout the nucleoplasm in both live and fixed cells (Fig. 3A–C).

3.4. NumA1, eIF6, and Bud31 colocalize throughout the entire nucleolus

eIF6 is a *Dictyostelium* nucleolar protein involved in translation initiation [22]. As previously observed by Balbo and Bozzaro (2006), GFP-eIF6 was detected predominately in nucleoli of both live and fixed cells (Fig. 3A–C) [22]. GFP-Bud31 and GFP-eIF6 colocalized with anti-NumA1 throughout the entire nucleolus (Fig. 3B and C). Western blots confirmed that both GFP-Bud31 and GFP-eIF6 were properly expressed (data not shown).

3.5. CBP4a localizes to a portion of the nucleolus and Snf12 localizes to a smaller area within this region

CBP4a is a nucleolar binding partner of NumA1 [15,23]. Colocalization of either GFP-eIF6 or GFP-Bud31 with anti-CBP4a revealed that CBP4a occupies only a portion of the nucleolar area occupied by GFP-eIF6 and GFP-Bud31 (Fig. 3C). Colocalization of C-GFP-Snf12 with anti-CBP4a revealed that C-GFP-Snf12 occupies a smaller area within the area occupied by CBP4a (Fig. 3D). Thus C-GFP-Snf12 colocalizes with only a portion of CBP4a and CBP4a occupies only a portion of the nucleolus.

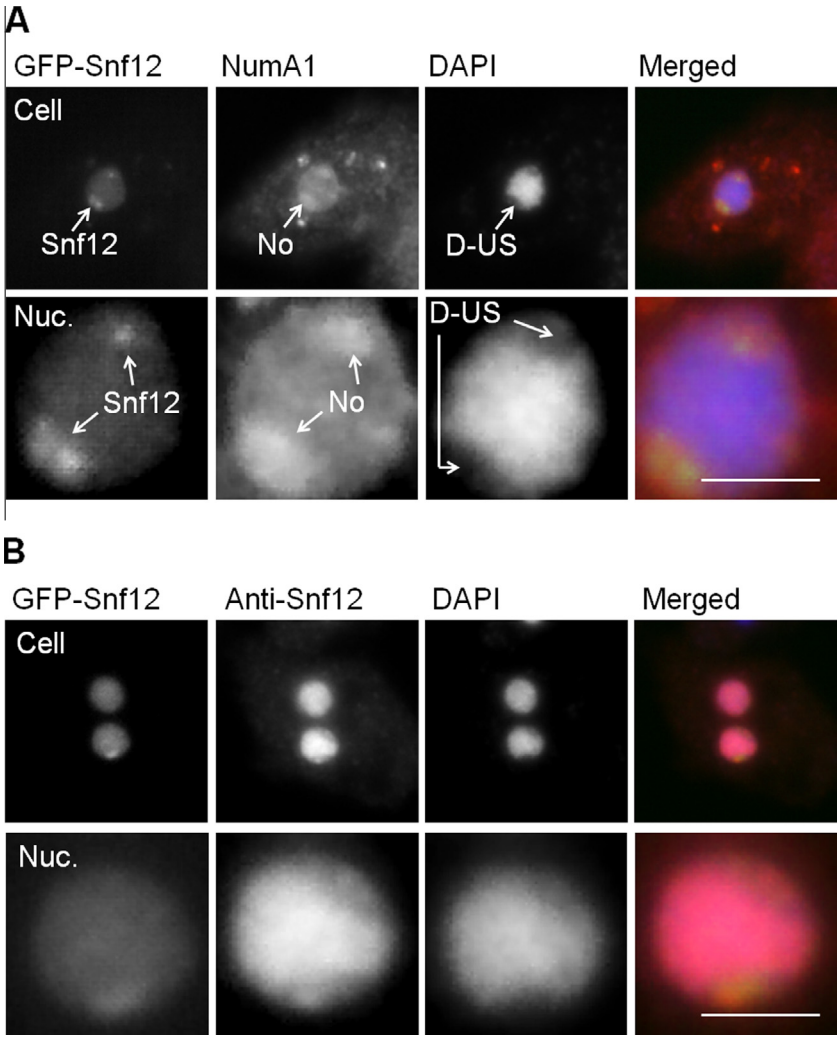

Fig. 1. C-GFP-Snf12 localizes to a portion of the nucleolus. (A) Cells expressing either C-GFP-Snf12 or GFP-NLS-3 probed with anti-NumA1. For both proteins, nucleolar NumA1 (No) was contained exactly within the unstained DAPI region (D-US) since the edge of the nucleolar NumA1 region colocalized with the edge of the DAPI stained region. Nucleolar C-GFP-Snf12 (Snf12) was detected in a portion of this nucleolar region Entire cell (Cell) and enlarged image of nucleus (Nuc.) are shown. Only C-GFP-Snf12 is shown. Scale bar represents 2 μm. (B) Cells expressing either C-GFP-Snf12 or GFP-NLS-3 probed with anti-Snf12. For both proteins, Snf12 was detected throughout the nucleoplasm (as demonstrated by colocalization with DAPI) but only in a portion of the DAPI counterstained region. Entire cell (Cell) and enlarged image of nucleus (Nuc.) are shown. Only C-GFP-Snf12 is shown. Scale bar represents 2 μm.

4. Discussion

Most eukaryotes possess nucleolar subcompartments which house different functions however no such compartments had been identified in *Dictyostelium* [24,25]. Here, we have shown that different nucleolar proteins localize to different areas within the nucleolus, arguing for the existence of nucleolar subcompartments in *Dictyostelium*. Subcompartmentalization of the nucleolus is also supported by the differential exit of proteins during mitosis and upon treatment with AM-D. NumA1, eIF6, and Bud31 localize throughout the entire nucleolus, CBP4a localizes to a portion of this region, and Snf12 localizes to an even smaller region within. This is the first time Bud31, a nucleolar protein in yeast, has been shown to localize to the nucleolus in *Dictyostelium*.

C-GFP-Snf12 localized differently in different cells. The localization pattern varied in size, shape, and position. It is unlikely that this variation was due to a variation in C-GFP-Snf12 expression level since no such variation was observed for the other nucleolar GFP-fusion proteins, GFP-Bud31 and GFP-eIF6. The localization of several GFP-Snf12 deletion constructs (GFP-Snf12-ΔSWIB, GFP-COG-NLS, GFP-Snf12-ΔNLS-1, and -2) also displayed the same variation (data not shown) [17]. Since the size and shape of the entire nucleolus changes with varying conditions and developmental stage it is not unreasonable to suggest that different nucleolar subcompartments may also undergo morphological changes under different conditions [11,26]. Together, this evidence suggests that either the size, shape, and position of this region are variable or perhaps this region may represent several different subcompartments.

The eight nucleolar proteins that have been identified in *Dictyostelium* do not all exhibit the same localization pattern. Some localize to the nucleolus and nucleoplasm (NumA1, eIF6, and Bud31), some to the nucleolus only (TRAP-1), some to subcompartments (Snf12 and CBP4a), and some to the nucleolar periphery (Hsp32 and FhkA) (Fig. 4A). Moreover, during mitosis these proteins each exhibit a unique pattern of redistribution [15–17]. *Dictyostelium* undergoes a semi-open mitosis and the nucleocytoplasmic translocation of all known nuclear proteins, including those in the nucleolus, has recently been reviewed [27]. This diversity in localization patterns suggests diversity in function. Accordingly, most of these eight proteins are each thought to function differently; Hsp32 is involved in heat shock response, TRAP-1 in

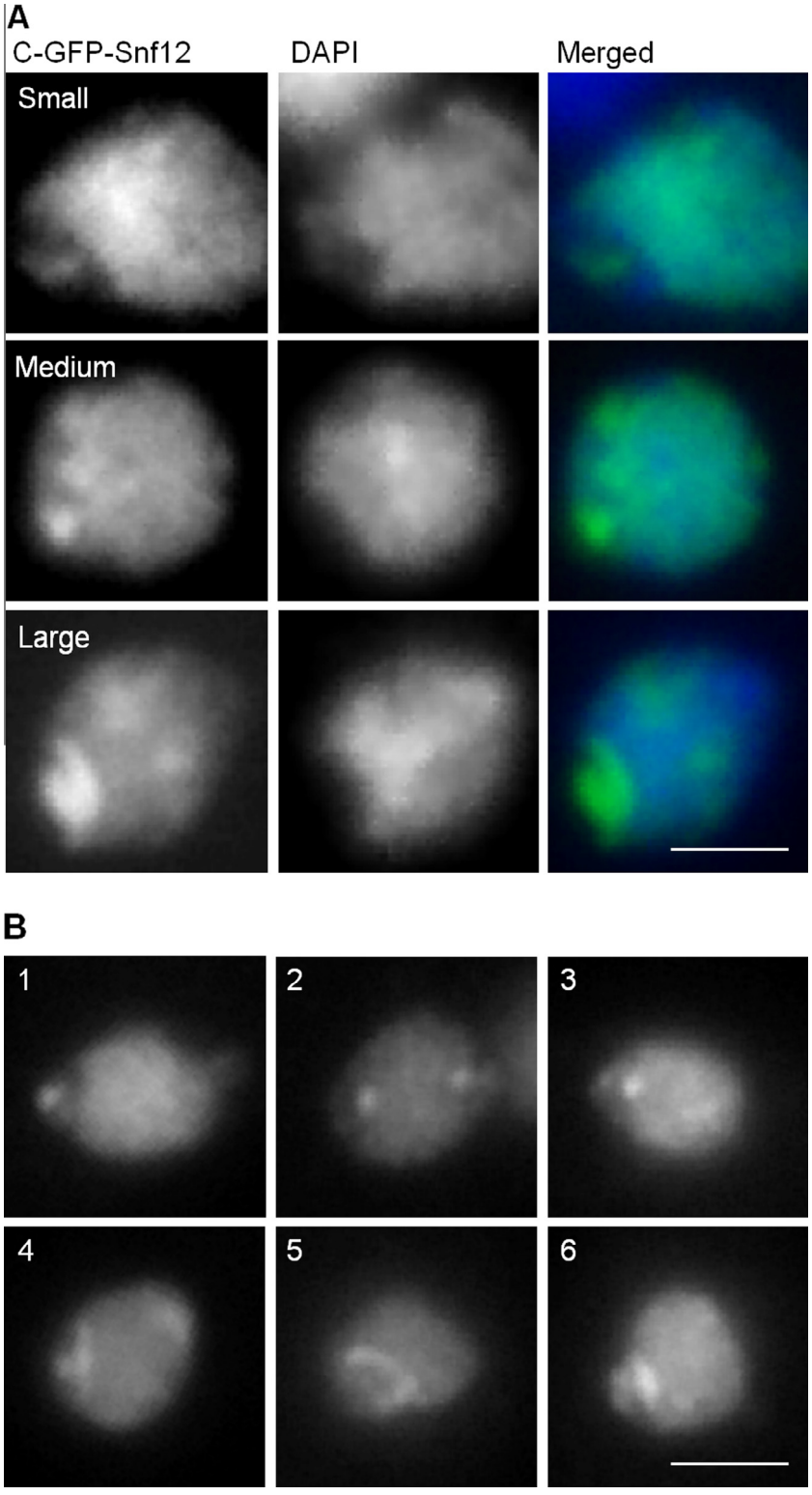

Fig. 2. Snf12-occupied nucleolar region varies in shape, size, and position. (A) In some cells C-GFP-Snf12 was detected in either a small, medium, or large portion of the nucleolus as demonstrated by colocalization with DAPI counterstain. (B) C-GFP-Snf12 was detected in nucleoli as a spot, a line, or a combination of both, either adjacent to the nucleoplasm or adjacent to the nuclear envelope region.

the formation of the spore coat, NumA1 and CBP4a have ties to cell cycle regulation, and Snf12 is involved in stress response [13,15–17,22,28,29]. As more nucleolar proteins are identified it will be interesting to determine if each nucleolar subcompartment houses proteins with similar functions and if additional subcompartments exist.

AM-D treatment in higher eukaryotes leads to the condensation and subsequent separation of the FC and GC nucleolar regions [30]. Accordingly, in *Dictyostelium* different proteins leave the nucleolus in different ways after AM-D treatment which is consistent with the existence of nucleolar subcompartments [15–17]. The NumA1-occupied area decreases in size whereas CBP4a, Snf12,

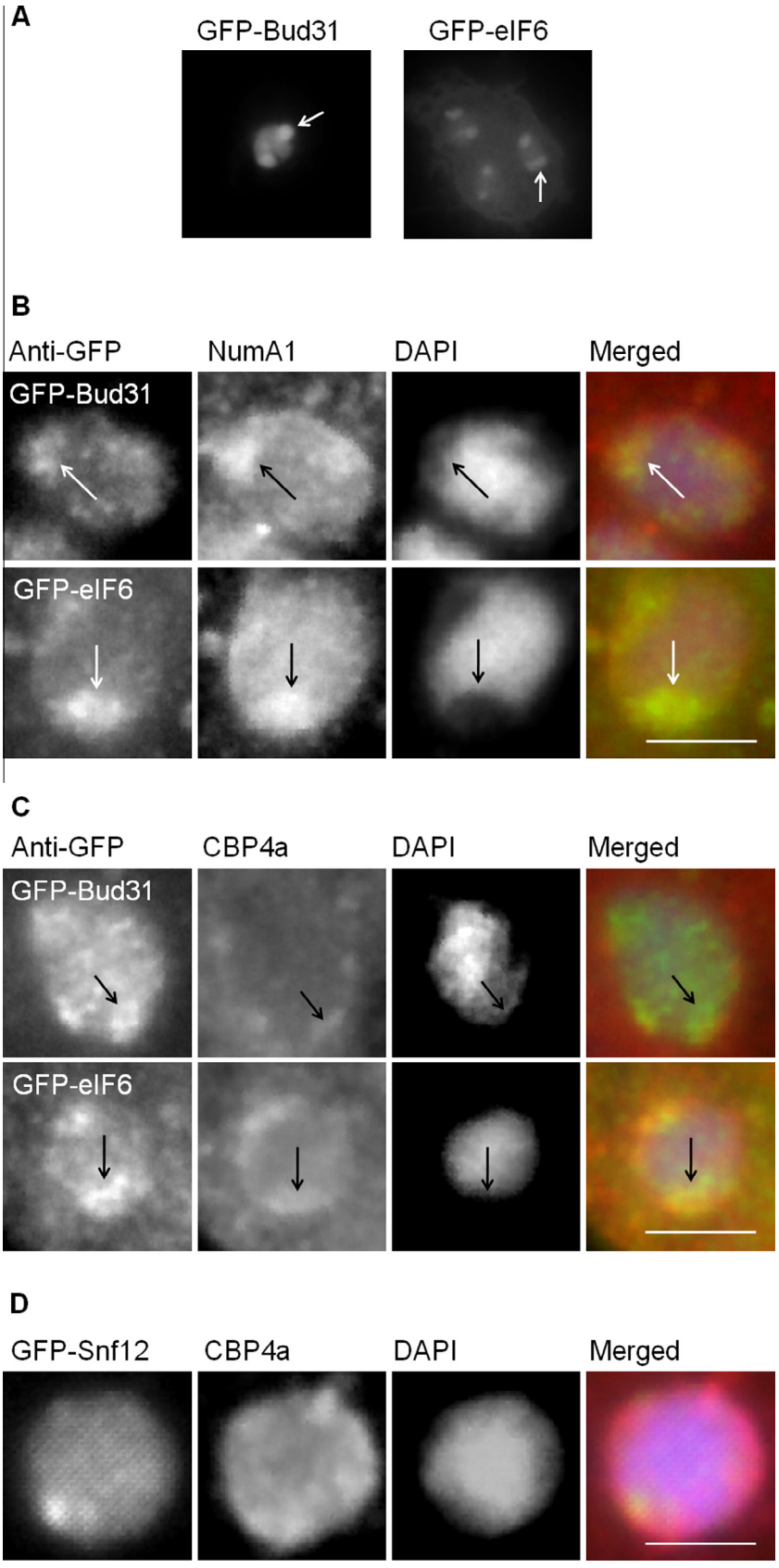

Fig. 3. Colocalization of Bud31, eIF6, NumA1, CBP4a, and Snf12. GFP-Bud31 or GFP-eIF6-expressing cells live (A) or fixed probed with either anti-NumA1 (B) or anti-CBP4a (C). NumA1 colocalized with GFP-Bud31 and GFP-eIF6 throughout the entire nucleolus (arrows) while CBP4a colocalized with only a portion of nucleolar GFP-Bud31 and GFP-eIF6. Scale bar represents 2 μm. (D) C-GFP-Snf12-expressing cells probed with anti-CBP4a. C-GFP-Snf12 colocalized with only a portion of nucleolar CBP4a. Scale bar represents 2 μm.

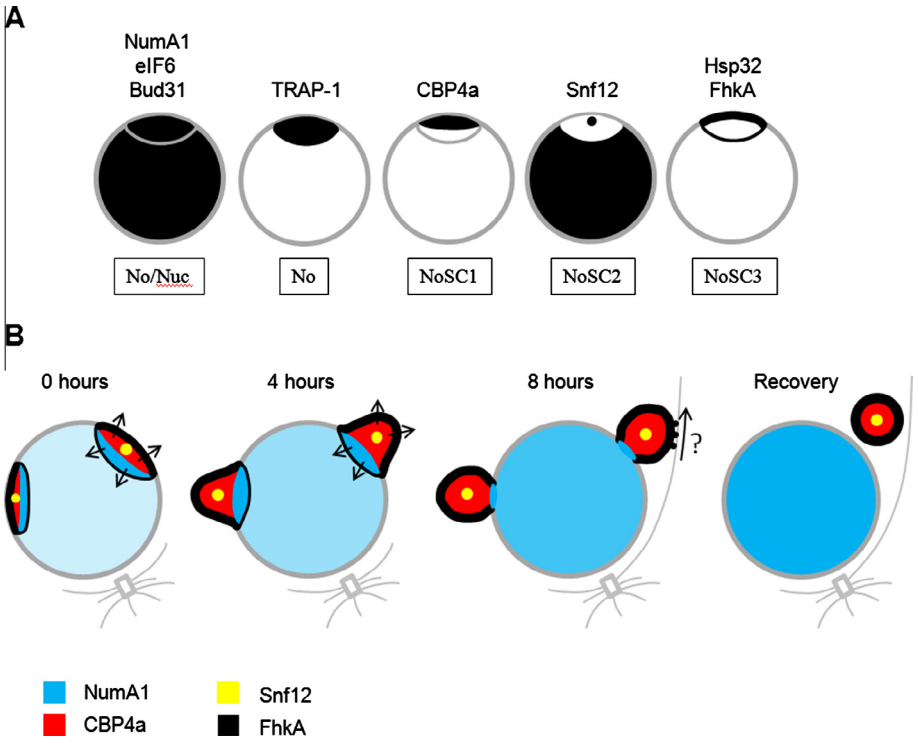

Fig. 4. Localization of all known *Dictyostelium* nucleolar proteins and model for AM-D-induced redistribution. (A) Different *Dictyostelium* nucleolar proteins localize differently. NumA1, eIF6, and Bud31 localize to both the nucleolus and nucleoplasm (No/Nuc), TRAP-1 localizes only to the nucleolus (No), CBP4a localizes only to NoSC1, Snf12 localizes to NoSC2 as well as the nucleoplasm, while Hsp32 and FhkA localize to the nucleolar periphery. Black represents protein localization and gray represents the nuclear envelope. (B) After AM-D treatment NumA1leaves the nucleolus presumably redistributing throughout the nucleoplasm. CBP4a, Snf12, and FhkA bud off, presumably as one unit, and enter the cytoplasm. Proteins that bud off do not relocate to the nucleolus after AM-D recovery. The centrosome is present on the opposite side of the nucleus. The nuclear envelope, centrosome, and microtubules are shown in gray. Given the relationship between centrosome position, microtubules, and the nucleolus, microtubules may help guide the budding nucleolus in the proper direction (represented by "?" in figure) [13,15–17,22,29].

and FhkA appear to bud off from the nucleus [15–17]. This phenomenon is strikingly similar to the mitotic arrest-induced nuclear envelope extensions that occur in budding yeast [31]. These extensions are thought to result from the accumulation of excess phospholipids during mitotic arrest [31]. This also fits with recent work demonstrating that nucleoli behave like liquid-like droplets rather than solids [32].

The identification of nucleolar subcompartments has allowed us to create a model of the nucleolus and nucleolar proteins during AM-D treatment in *Dictyostelium* that would explain the redistribution patterns observed with NumA1, CBP4a, Snf12, and FhkA (Fig. 4B). The model suggests that during nucleolar budding proteins in the body of the nucleolus (such as CBP4a and Snf12) remain in the center while proteins at the nucleolar periphery (such as FhkA) remain at the periphery. Even after the process is complete, these nucleolar proteins remain in their respective locations.

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