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Subject:
Arf, Cajal body, centaurin, GEF, nuclear GTPase, nucleolus, PIKE

ISSN:
1398-9219
Nuclear functions of the Arf guanine nucleotide exchange factor BRAG2

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Running title: Nuclear functions of BRAG2

Key words: Arf, GEF, Cajal body, centaurin, nucleolus, nuclear GTPase, PIKE

Length: 44,568 characters
Abstract

BRAG2 is a guanine nucleotide exchange factor for the GTPase Arf6 that cycles between the cytoplasm and nucleus in a CRM1/exportin1 dependent manner. Despite its presence in the nucleus, nuclear functions have not previously been described. Here we show that depletion of endogenous BRAG2 by RNAi leads to an increased number of Cajal bodies, and altered structure of nucleoli, as indicated by less focal fibrillarin staining. This result was surprising given that nuclear BRAG2 is diffusely distributed throughout the nucleoplasm and is not concentrated within nucleoli at steady state. However, we found that ectopic expression of the nuclear GTPase PIKE/AGAP2 causes both BRAG2 and the Cajal body marker coilin to accumulate in nucleoli. Neither the GTPase activity of PIKE nor the nucleotide exchange activity of BRAG2 is required for this nucleolar concentration. Increased levels of exogenous BRAG2 in nucleoli result in a redistribution of fibrillarin to the nucleolar periphery, supporting a role for BRAG2 in regulating nucleolar architecture. These observations suggest that, in addition to its role in endocytic regulation at the plasma membrane, BRAG2 also functions within the nucleus.
Introduction

ADP-ribosylation factors (Arfs) are a family of small Ras-like GTPases that regulate membrane trafficking by nucleating the assembly of coat protein complexes involved in carrier vesicle formation. The binding of GTP, which promotes Arf activation, is catalyzed by a family of guanine nucleotide exchange factors (GEFs) referred to as the Sec7 family [1]. Conversely, down-regulation of Arf activity is facilitated by a family of Arf-specific GTPase activating proteins (GAPs). Although there are only 6 mammalian Arfs, there are 15 recognized Arf GEFs and more than 20 Arf GAPs in the human genome, indicating that Arf activation is under extensive and complex regulatory control [2].

The Brefeldin Resistant Arf GEFs (BRAGs) constitute a subclass of Arf GEFs that have so far been poorly characterized. The three BRAG proteins (BRAG1, BRAG2/Arf-GEP100 and BRAG3) share a common domain organization consisting of an IQ domain followed by the catalytic Sec7 domain and an adjacent PH domain. We have previously shown that BRAG2 is expressed in at least two splice isoforms, BRAG2a and BRAG2b [3] that appear to act preferentially on Arf6 [4]. Depletion of endogenous BRAG2 by RNAi leads to increased surface levels of β1 integrins and stimulates both cell attachment and spreading, suggesting that BRAG2 acts at the plasma membrane to control integrin internalisation [3].
Like most Arf-GEFs, endogenous BRAG2 is expressed at low levels and is difficult to detect by morphological methods. However, expression of tagged constructs revealed that in addition to the expected cytosolic distribution, both BRAG2a and BRAG2b exhibited a distinct concentration in the nucleus at steady state and can exit the nucleus in a classical Crm1/exportin1 dependent manner [3]. In agreement with this observation, immunoprecipitation from subcellular fractions [3] and proteomic analysis of nuclear constituents [5] demonstrated that a fraction of endogenous BRAG2 is indeed nuclear.

Although the role of Arfs in carrier vesicle formation is well characterized, it is not clear whether these proteins have additional functions in the nucleus. Arfs are small enough to freely diffuse through nuclear pores and several Arf-like GTPases are reported to localize to the nucleus and/or nucleolus [6]. Indeed the BRAG2 substrate Arf6 has been detected in nuclei both biochemically and morphologically [7]. Moreover, both BIG1, a GEF for Arfs 1, 2 and 3 that is localized to the TGN at steady state, and centaurin α1, a GAP for Arf6, have been reported to enter the nucleus and interact with the nucleolar protein nucleolin, suggesting that they may be involved in rRNA processing and/or ribosome biogenesis [7,8]. Similarly, another centaurin family member PIKE (AGAP2/centaurin-γ1) has also been found within nuclei [9]. A splice isoform of PIKE lacking the Arf-GAP domain (PIKE-S) concentrates in the nucleus where it has been shown to activate PI3-kinase in a nucleotide-dependent manner [10].

Here we show that RNAi-mediated depletion of endogenous BRAG2 alters the organization of both nucleoli and Cajal bodies, suggesting that BRAG2 may be involved
in RNP biogenesis as suggested for the other Arf regulators BIG1 and centaurin-α1. Additionally, we demonstrate that co-expression of BRAG2 and the nuclear GTPase PIKE-S alters nucleolar morphology. Taken together, these findings suggest a functional link between regulators of membrane traffic and nuclear processes.
Results

BRAG2 depletion alters fibrillarin staining

We have previously demonstrated that both BRAG2a and BRAG2b are effectively depleted by transfection of cells with a single BRAG2 siRNA duplex [3]. To investigate if endogenous BRAG2 has nuclear functions we examined the distribution of several well-characterized nuclear markers in BRAG2-depleted HeLa cells. Because of the low level of endogenous BRAG2 expression, we could not use immunofluorescence microscopy to identify individual cells in which BRAG2 was depleted, however Western blot analysis indicates that we routinely depleted both BRAG2a and BRAG2b to undetectable levels in the cell population (Fig. 1D).

To determine if BRAG2 depletion altered nucleolar structure we used the well-characterized nucleolar marker, fibrillarin. Fibrillarin is a marker of the dense fibrillar component of nucleoli, and is the methyl-transferase subunit of box C/D snoRNPs that is responsible for 2'O-methylation of rRNA [11]. No obvious difference in fibrillarin staining was observed between mock transfected cells and cells receiving a control RNA duplex (not shown). However there was an obvious effect of BRAG2 depletion on the distribution of endogenous fibrillarin. In virtually all cells, the area of fibrillarin staining was increased, and the staining was no longer as punctate and compact compared to cells treated with control duplex (Fig. 1A). Quantification of the area of fibrillarin staining in three separate experiments demonstrates that cells depleted of BRAG2 exhibit ~30% increase in the area of fibrillarin staining compared to control cells (Fig. 1B). This increase was statistically significant (p<0.01), while measurements of DAPI staining
indicate that there was no significant difference in the size of nuclei. Since it is unlikely that BRAG2 was depleted to a similar extent in all cells, this effect on fibrillarin distribution is probably underestimated. (The largest area of fibrillarin staining observed after BRAG2 siRNA treatment was ~9.5 times larger than the average fibrillarin-positive area in control cells.) This observation suggests that the depletion of endogenous BRAG2 isoforms disrupts normal nucleolar architecture.

**Depletion of BRAG2 increases the number of Cajal Bodies**

In addition to its effects on fibrillarin, we observed that depletion of BRAG2 by siRNA also increases the number of Cajal bodies (CBs) in HeLa cells. Like nucleoli, CBs are nuclear compartments involved in RNP biogenesis. CBs are often associated with nucleoli or specific gene loci, and are implicated in both snRNA and snoRNA post-transcriptional modifications. Coilin is the most prominent marker of CBs, however it can also be found in the nucleoplasm and nucleoli [12, 13].

Immunolabeling of endogenous coilin revealed that there was no obvious difference in CB numbers between mock transfected cells and cells that received control RNA duplex (not shown). However there was an obvious increase in CB number in the BRAG2 depleted cells compared to mock or control cells (Fig. 1A). Counting CB numbers/nucleus in three separate experiments indicates that there was a significant decrease in the number of nuclei with no obvious CBs (p<0.01), and a significant increase in the number of nuclei with 2 or 3 CBs (p<0.01, Fig. 1C). Additionally, the CB in BRAG2 siRNA treated cells usually appeared larger and more pronounced (Fig. 1A).
Taken together these findings indicate that endogenous BRAG2 may regulate CB number and structure.

**Expression of the nuclear GTPase PIKE induces the accumulation of BRAG2 in nucleoli**

How does BRAG2 exert its influence on nucleolar structure and CB number? The only known function of BRAG2 is guanine nucleotide exchange activity, and the only documented substrates of BRAG2 nucleotide exchange activity are the Arf GTPases, principally Arf6 [3, 4]. However, Arf6 is not obviously localized to the nucleus and we were unable to conclusively demonstrate an effect of Arf6 mutants on any aspect of nuclear morphology (not shown). Hence, we began to consider the possibility that other GTPases may be the nuclear substrates of BRAG2.

One candidate is the nuclear GTPase PIKE-S [10]. PIKE-S is the short isoform of PIKE/AGAP2/GGAP2/centaurin-γ1. PIKE-S contains a Ras-like GTPase domain that is capable of hydrolyzing GTP, part of a bi-partite PH domain, and interaction domains for the cytoskeleton protein 4.1N and the adapter protein Homer [10] (Fig.5A). Two other isoforms of PIKE exist (PIKE-L and PIKE-A) that contain an extended C-terminus, which includes the second half of the bi-partite PH domain, an ARF-GAP domain and two ankyrin repeats [9] (Fig. 5A). The ARF-GAP domain of PIKE-A can stimulate GTP-hydrolysis on its own GTPase domain [14]. This suggests that, despite poor primary sequence identity, the tertiary structure of AGAP GTPase domains may be similar to Arf GTPase domains, and that they are possible substrates for Arf-GEFs such as BRAG2.
PIKE-S is primarily localized to the nuclei of HeLa cells (Fig. 2A). As we have previously described for BRAG2 [3] (also see Supplemental Fig. 1B-D), PIKE-S nuclear staining is typically diffuse and not obviously concentrated within a discrete sub-nuclear organelle. Occasionally cytoplasmic staining can also be observed which has a tubular appearance (Fig. 2A, arrow). The proportion of cells with cytoplasmic tubule staining varies greatly between experiments (values ranging from ~1-24%) and appears to be influenced by expression levels, cell confluence and cell-cycle stage.

To determine if PIKE-S and BRAG2 co-localized within nuclei we co-transfected HeLa cells with myc-PIKE-S and HA-BRAG2a. Surprisingly, we found that co-expression of PIKE-S dramatically altered the distribution of BRAG2a. Instead of a diffuse nuclear distribution (Fig. 2B), BRAG2 became concentrated in large nuclear patches resembling nucleoli, and there was a dramatic decline in BRAG2 cytoplasmic staining (Fig. 2C). The same effect was seen for BRAG2a-GFP and, importantly, untagged BRAG2a (Supplemental Fig. 1E, H). Co-expression of PIKE-S also induced BRAG2a to localize to nuclear patches in BHK cells (not shown) and PC12 cells (Supplemental Fig. 2D). The longer BRAG2 isoform (HA-BRAG2b) also accumulated in nuclear patches in the presence of PIKE (not shown). Despite the concentration of BRAG2 in nuclear patches, PIKE-S staining remained largely unchanged and only rarely co-localized with the BRAG2 foci (Fig. 2C).
Unfortunately, detection of BRAG2 in the nuclear patches by antibodies, particularly our polyclonal anti-BRAG2 antibody directed against residues 153-176 was difficult (compare HA-BRAG2a staining in Supplemental Fig. 1F and G). This observation suggests that antibody access may be impaired when BRAG2 is concentrated in nuclear patches, making it difficult to define the localization of endogenous BRAG2. Indeed, Sheval and colleagues recently provided evidence that an increase in antigen accumulation within the nucleolus may prevent antigens from being recognized by specific antibodies [15].

Triple transfections with HA-BRAG2a, myc-PIKE-S and GFP-tagged nucleolar markers (B23 or fibrillarin), confirmed that the nuclear patches are nucleoli. B23 is a nucleolar phosphoprotein that localizes to the granular compartment of nucleoli, and has been implicated in ribosome assembly and transport, nucleolar assembly, and centrosome duplication [16]. Although neither BRAG2 nor PIKE-S co-localized with the nucleolar markers when expressed alone (Fig. 3A-B and D-E), BRAG2 clearly co-localized with B23, and was closely associated with fibrillarin when co-expressed with PIKE. B23 and BRAG2 were usually concentrated deep within nucleoli (Fig. 3C) while fibrillarin surrounded the BRAG2 patches (Fig. 3F). The same results were obtained for nuclear patches containing BRAG2b (not shown). The co-localization of BRAG2 and B23, and the close proximity of fibrillarin indicate that PIKE-S expression induces an accumulation of BRAG2 in nucleoli. This indicates that under some circumstances BRAG2 does enter nucleoli, and that the effects of BRAG2 depletion on nucleolar structure may be direct.
In contrast to its dramatic effects on BRAG2 localization, PIKE-S expression had no obvious effect on the distribution of several other nuclear markers including endogenous Ran, or GFP-tagged SF2/ASF, histone2B, Nup98 (not shown), B23 (Fig. 3A), and fibrillarin (Fig. 3D). Additionally nuclear SMN staining was not affected by PIKE-S (see Supplemental Fig. 4C).

However, importantly we found that the Cajal body marker, coilin, also became concentrated in nucleolar structures upon expression of exogenous PIKE-S (see Supplemental Fig. 3). Together with our observation that depletion of endogenous BRAG2 led to alterations in Cajal body numbers, these findings suggest that BRAG2 and coilin are functionally linked.

Nucleoli are composed of fibrillar centers surrounded by dense fibrillar components, and interspersed by granule components. However, due to the limited resolution of light microscopy distinct dense fibrillar component/granule component boundaries cannot be seen. B23 is found in the granular component of nucleoli, and fibrillarin in the dense fibrillar component. GFP-tagged B23 typically appears to be evenly and diffusely distributed through out the nucleolus in HeLa cells (Fig. 3A-B). GFP-fibrillarin has a more punctate staining pattern, but these puncta are evenly distributed throughout the entire nucleoli and are not obviously segregated from the granular components (Fig. 3D-E and Supplemental Fig. 2A-B).
The presence of exogenous BRAG2 within nucleoli clearly altered fibrillarin staining, just as depletion of the endogenous protein had. As noted above, neither BRAG2 nor PIKE had any effect on the distribution of nucleolar markers when expressed alone. However when BRAG2 was induced to accumulate in nucleoli by PIKE-S, fibrillarin and B23 were no longer evenly distributed throughout these nucleoli. In the presence of nucleolar BRAG2, GFP-B23 accumulated deep within nucleoli with BRAG2 such that it was less concentrated at the nucleolar periphery (Fig. 3C). In contrast, both GFP-tagged (Fig. 3F) and endogenous (Supplemental Fig. 2C) fibrillarin were excluded from the nucleolar interior and instead became concentrated in the nucleolar periphery. The presence of the N-terminus of BRAG2 alone is sufficient to restrict fibrillarin to the nucleolar periphery (Fig. 3G). A similar phenotype was observed in PC12 cells (Supplemental Fig. 2D). Hence, both depletion of endogenous BRAG2 and exogenous nucleolar BRAG2 alter nucleolar morphology.

**Accumulation of BRAG2 in nucleoli does not require the catalytic activity of either PIKE or BRAG2**

The ability of PIKE-S to bind GTP is necessary for its capacity to activate nuclear PI 3-kinase and increase cyclin-D1 levels [10]. To determine if PIKE nucleotide binding correlates with its ability to promote nucleolar BRAG2, we made use of a PIKE mutant containing two point mutations in the nucleotide binding pocket, which fails to bind GTP (PIKE [K413A, S414N], [10]). Surprisingly, co-transfection of BRAG2 and the nucleotide deficient mutant of PIKE-S still promoted the concentration of BRAG2 in nucleoli (Fig. 4A), indicating that this process does not require PIKE-S GTPase activity.
This observation further suggests that the accumulation of BRAG2 in the nucleolus is not the result of PIKE-induced alterations in nuclear PI 3-kinase or cyclin D1 activity.

Because BRAG2 has been shown to catalyze Arf nucleotide exchange both in vitro [4] and in vivo [3], it was possible that the observed changes in nuclear architecture were the result of enhanced Arf activity. To test this possibility, we utilized a catalytically inactive BRAG2 mutant in which the conserved catalytic glutamate residue was changed to lysine (BRAG2a(E498K), [3]). Despite the lack of catalytic activity, we found that co-expression of PIKE-S induced the nucleolar accumulation of BRAG2(E498K) (Fig. 4B) where it is surrounded by the nucleolar marker fibrillarin (Fig. 4C). Taken together, these data indicate that neither PIKE GTPase activity nor BRAG2 GEF activity are required for the nucleolar concentration of BRAG2.

The N-terminus of PIKE is sufficient to induce the nucleolar accumulation of BRAG2

To determine which region of PIKE may promote the nucleolar localization of BRAG2, several partial PIKE constructs were utilized (Fig. 5A). All PIKE-S constructs (PIKE 268-273, PIKE 1-669 and PIKE 1-384) exhibited a localization similar to that of full-length PIKE-S; they were largely localized to nuclei with occasional cytoplasmic tubule staining (not shown) which was not obviously altered by co-expression with BRAG2 (Fig. 5B-D). In contrast, a construct containing the C-terminus of PIKE-L (PIKE 670-1186) had a much more cytoplasmic distribution (Fig. 5E). PIKE 670-1186 contains the full PH domain, ARF-GAP domain and ankyrin repeats of PIKE, which are only found in
PIKE-L or PIKE-A but not in PIKE-S. This construct was unable to induce BRAG2 nucleolar localization (Fig. 5E). Similarly, the C-terminus of PIKE-S (PIKE 268-753), which contains the GTPase domain, was also unable to promote BRAG2 nucleolar accumulation (Fig. 5D), confirming that PIKE GTPase activity is neither necessary nor sufficient for this process. In contrast, the two N-terminal constructs, PIKE 1-669 and PIKE 1-384, were still able to promote nucleolar accumulation of BRAG2 (Fig. 5B-C), indicating that the N-terminus of PIKE-S is both necessary and sufficient for this process. This region of PIKE-S is also found in PIKE-L, but not PIKE-A. Indeed, PIKE-L can also promote nucleolar accumulation of BRAG2, but in keeping with its more cytoplasmic distribution, does so less efficiently (not shown). The same results were obtained for the effect of PIKE-S expression upon coilin localization (see Supplemental Fig.3).

The N-terminus of BRAG2 is sufficient for nucleolar localization

To determine which region of BRAG2 may be required for PIKE-induced nucleolar accumulation, three HA-tagged partial BRAG2 constructs were designed (Fig. 6A). The N-terminus of BRAG2a (1-250) contains a consensus IQ domain and one proline rich domain (PR), but otherwise remains poorly defined. The mid-region of BRAG2 (251-596) contains a second PR, the catalytic Sec7 domain, two predicted nuclear localization signals (NLS) and a serine-rich region. The C-terminus of BRAG2 (597-841) contains the PH and coiled-coil domains and the third putative NLS. When expressed in HeLa cells all three HA-tagged constructs exhibited at least a partial nuclear localization (Fig. 6B, D and F). Despite the presence of the PH domain, which would be predicted to bind
plasma membrane phospholipids, the C-terminus of BRAG2 had the most prominent nuclear localization (Fig. 6F).

When co-transfected with myc-PIKE-S the localization of BRAG2a 251-596 and BRAG2a 597-841 did not appear to change (Fig. 6E and G). However when the N-terminus of BRAG2a (1-250) was co-expressed with PIKE-S prominent nucleolar staining was observed (Fig. 6C). These observations indicate that the N-terminus of BRAG2 is both necessary and sufficient for nucleolar retention, and confirms that catalytic GEF activity is not required.

A predicted calmodulin binding IQ domain is located within the N-terminus of BRAG2a (residues 16-30). However, a mutant (BRAG2a I19E,R24E) lacking key residues previously reported to be vital for calmodulin binding [17] had a similar distribution to wild-type BRAG2a (Fig. 6H) and accumulated in nucleoli in response to PIKE-S, suggesting that calmodulin binding is not necessary for this localization (Fig. 6I).
Discussion

BRAG2 is an Arf guanine nucleotide exchange factor with apparent specificity for Arf6. It has been reported to localize to early endosomes [4] and the plasma membrane, where it appears to regulate β1-integrin internalization [3]. We have also reported that a significant pool of BRAG2 localizes to the nucleus, and cycles between the nucleus and cytoplasm in a Crm1/exportin dependent manner [3]. To determine if BRAG2 has nuclear functions in addition to its roles in the cell periphery, we depleted cells of endogenous BRAG2 and examined the effects of this treatment on nuclear architecture. Depletion of both BRAG2 isoforms by siRNA results in an altered pattern of fibrillarin staining and increased numbers of Cajal bodies (CB). Additionally, we found that expression of the nuclear GTPase, PIKE-S, specifically induces accumulation of both BRAG2 isoforms in nucleoli, where the presence of BRAG2 restricts fibrillarin to the nucleolar periphery. Surprisingly, these effects of PIKE-S and BRAG2 on nuclear/nucleolar architecture required neither the catalytic activity of BRAG2 nor nucleotide binding to PIKE, as the N-termini of both proteins are sufficient to induce this response.

The effect of BRAG2 on nucleolar structure

The nucleolus has recently been the subject of several proteomic studies that highlight its complexity [18]. It is comprised of fibrillar centers surrounded by dense fibrillar components, and around this the granule components. Fibrillarin is a marker of the dense fibrillar components, while B23 is a granule component marker. In BRAG2-depleted cells, the area of endogenous fibrillarin staining is increased and has a more dendritic,
less focal punctate pattern. Conversely, the concentration of BRAG2 in nucleoli that occurs in the presence of PIKE leads to an altered distribution of B23 and fibrillarin, such that fibrillarin localizes to the nucleolar periphery, and B23 to the core along with BRAG2. Collectively these results suggest that BRAG2 may have a role in nucleolar structure and function.

**The effect of BRAG2 on Cajal Bodies**

CB are compact nuclear bodies often associated with nucleoli or specific gene loci. Sequence specific guide RNAs accumulate in CB where they are thought to direct post-transcriptional modification of snRNAs and snoRNAs. The snRNAs subsequently direct pre-mRNA splicing, while the snoRNAs are involved in rRNA biogenesis within nucleoli [12]. The numbers of CB tend to increase in highly proliferative cells where more transcription and translation is taking place. However, in some cells no CB may be apparent. For example CB are present in all fetal tissues, while they are not present in all adult tissues [19].

Coilin is a prominent marker of CB, but can also be found in the nucleoplasm and in nucleoli. The function of coilin remains to be elucidated but it is known to interact with several snRNP and snoRNP biogenesis components [12,13]. Our observation that BRAG2 and coilin co-localize in nucleoli in response to PIKE-S expression, and that depletion of BRAG2 affects the distribution of coilin suggests that the functions of these two proteins are inter-related.
Depletion of endogenous BRAG2 from HeLa cells by siRNA resulted in a significant increase in the number of CB. Due to the constant cycling of CB and nucleolar constituents between these two nuclear bodies, and also the nucleoplasm, it is difficult to determine if the effect of BRAG2 depletion on CB numbers is secondary to its disruption of nucleolar architecture, or a more direct effect on CB constituents. Similarly, effects on CB could lead to changes in nucleolar architecture by disrupting the appropriate trafficking and processing of snoRNPs and other constituents of the rRNA/ribosome processing machinery.

**How does PIKE cause BRAG2 to accumulate in nucleoli?**

Several GTPases have now been characterized which regulate nucleolar architecture and ribosome biogenesis including Nug1, Nug2, Nog1 and Kre35 [20]. Additionally, Arfs and Arf-like GTPases have been reported in nuclei or nucleoli, but this localization is still debated. In particular the nuclear/nucleolar localization of the Arf-like proteins Arl4, 5 and 7 remains controversial, since previous studies have utilized proteins epitope tagged at the N-terminus, which would eliminate N-terminal myristoylation and could alter both their localization and function [6].

PIKE is another recently described nuclear GTPase. Despite enhancing PI 3-kinase activity, which would lead to changes in nuclear phosphoinositide composition, PIKE has not previously been shown to affect nuclear structure [10]. We demonstrate that PIKE-S expression results in the accumulation of both BRAG2 and coilin in nucleoli. This effect appears to be specific since the localization of several other nuclear markers was not
obviously affected by PIKE-S over-expression (not shown). Surprisingly, this effect is independent of the GTPase domain of PIKE, ruling out the possibility that PIKE affects BRAG2 and coilin localization by altering nuclear phospholipid composition or increasing levels of cyclin D1. Rather, the N-terminus of PIKE-S (which is also present in PIKE-L) is sufficient to promote BRAG2 and coilin nucleolar localization. This region of PIKE is unique and contains three proline rich domains as well as the binding sites for the cytoskeleton protein 4.1N and the adaptor protein Homer [9]. Interaction with protein 4.1N could link PIKE-S to NuMa and the nuclear matrix, the actin cytoskeleton and/or microtubules [21]. Indeed we find that PIKE, BRAG2 and the RNP biogenesis protein SMN can co-localize on α-tubulin labeled cytoplasmic tubules (see Supplemental Fig. 4). However, it remains unclear if these cytoplasmic tubule structures are involved in the promotion of BRAG2 nucleolar accumulation.

We have also defined the region of BRAG2 required for its PIKE-induced nucleolar accumulation. We found that nucleolar targeting did not require the catalytic Sec7 domain, the PH domain, or the C-terminal coiled-coil domain. Instead our data indicate that the N-terminus of BRAG2a is both necessary and sufficient for accumulation in nucleoli. This domain is also present in BRAG2b, which similarly accumulates in nucleoli after PIKE expression. The N-terminus of BRAG2a contains a predicted calmodulin-binding domain that is well conserved in all the BRAGs. However, we found that that calmodulin binding is not required for nucleolar localization.
Live imaging studies have so far indicated that diffusion rather than directed (motor driven) movement is responsible for the intranuclear movement of proteins [22]. ATP regulation of movement has been reported, but is thought to represent energy dependent release or retention of molecules from intranuclear sites, rather than directed movement via motors [23, 24]. This suggests that cell signalling may regulate energy dependent release or retention of proteins at specific nuclear loci, but is unlikely to regulate active trafficking of proteins to their nuclear binding sites. This evidence makes it unlikely that PIKE actively translocates BRAG2 (and coilin) to nucleoli. Rather it suggests that PIKE may alter the retention of proteins within nucleoli, or their release from other nuclear sites of retention. This regulation could be achieved by altering nuclear/nucleolar architecture, or by changing the conformation of BRAG2 itself such that its affinity for specific binding sites within nuclei is altered. In the case of coilin, phosphorylation and methylation may be important post-translational modifications that regulate Cajal body retention [12]. If PIKE induces BRAG2 accumulation in nucleoli through increasing nucleolar retention this would suggest that at steady state BRAG2 is cycling through this nuclear compartment. Further studies are required to determine whether nucleolar retention or release from other subcellular locations is occurring. However, since PIKE does not appear to localise to nucleoli itself, we suggest that PIKE may compete with BRAG2 for binding to another intranuclear site. Thus PIKE over-expression may release BRAG2 from an anchoring site within the nucleus and allow it to concentrate in nucleoli. It is unlikely that PIKE affects BRAG2 localization by inhibiting it’s nuclear export since blocking nuclear export with leptomycin B increases nuclear BRAG2 levels, but does not result in detectable nucleolar localization [3].
Conclusion

We have demonstrated that depletion of BRAG2 alters the distribution of the nucleolar component fibrillarin and increases the number of Cajal bodies, suggesting that BRAG2 may regulate RNP biogenesis. Additionally, expression of the nuclear GTPase, PIKE-S, causes BRAG2 to accumulate in nucleoli raising the possibility that at steady state BRAG2 may cycle through this nuclear compartment. Mechanisms of this regulation remain unknown, but do not include PIKE GTPase activation of PI3 kinase or BRAG2-mediated activation of Arfs. Together these data suggest that BRAG2 does have nuclear functions and that its activities are not restricted to the cytoplasm. Since two other Arf regulators, BIG1 and centaurin-α1, have been reported to bind another nucleolar component, nucleolin, this raises the intriguing possibility that crosstalk may exist between regulators of membrane trafficking and RNP biogenesis.
Materials and Methods

DNA constructs

For details of BRAG2a-GFP, HA-BRAG2a, HA-BRAG2b and HA-BRAG2a (E498K) constructs see [3]. The HA-BRAG2a vector was used as a template for site directed PCR mutagenesis to produce the IQ domain mutant of BRAG2a (BRAG2a I19E,R24E). PCR primers used to generate BRAG2a I19E, R24,E; CCCGCACCGAGCAGACGCGCGTTTGAGCAGGTACCAG and its complement (substituted nucleotides underlined). BRAG2a 1-250, 251-596, and 597-841 were also produced by PCR using pRC-HA-BRAG2a as a template, and were subcloned into the EcoRI/NotI sites of pRC-HA. PCR primers as follows, for 1-250; GGGAAATTCAGATGCTAGAACGAAAGTATGGG and ATATATATGCAGCAGCCCGGA GCCGCTGCTCCTGC, for 251-596; GGAATTCTGCGGGTGGAGCATCTGCCGCTGC and ATATATATGCAGCAGCCCGGA GCCGCTGCTCCTGC, and for 597-841; GGAATTCTGCGGGTGGAGCATCTGCCGCTGC and ATATATATGCAGCAGCCCGGA GCCGCTGCTCCTGC.

For details of PIKE constructs see Rong et al. [9]. The remaining constructs were obtained from Eric Griffiths and Maureen Powers (Emory University) and details can be found in the following references; GFP-fibrillarin [25], GFP-B23 [26], and GFP-coilin [27].
Cell culture and transfections

HeLa cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% FBS and antibiotics, at 37°C, 5% CO₂. PC12 cells were maintained in 5% FBS, 10% horse serum DMEM at 37°C, 10% CO₂. HeLa cells were transfected using Fugene6 as instructed by the manufacturer (Roche, Florence, SC, USA). PC12 cells were transfected using 8μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and ~2.4μg DNA / 35mm plate. RNAi was performed as described previously [3].

Immunofluorescence microscopy and image analysis

Cells were grown on glass coverslips and fixed with 4% paraformaldehyde 20h (HeLa), 24h (PC12) or 48h (siRNA) post-transfection. For PC12 cells coverslips were coated with 50μg/ml polyD-lysine (Sigma, Saint Louis, MO, USA). After fixation cells were treated with 0.1%SDS/PBS for 5 minutes and indirect immunofluorescence microscopy performed with the following antibodies as described previously [28]. Mouse anti-HA antibody 16B12 (1/800, Covance, Princeton, NJ, USA), mouse anti-myc antibody 9E10 (1/100), DAPI (100ng/ml, Sigma), mouse anti-fibrillarin (1/500, EnCor Biotechnology, Gainesville, FA, USA), mouse anti-coilin (1/300, Sigma). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA, USA) as follows; donkey anti-mouse or rabbit Cy2 (1/400), donkey anti-mouse or rabbit Cy3 (1/500). For details of rabbit anti-PIKE antibody (PIKE-N) see Rong et al. [9]. HeLa cells were imaged using an Eclipse E800 microscope (Nikon, 60 x objective) equipped with a RETIGA digital camera (Q-Imaging, Surrey, BC, Canada) and using Adobe Photoshop software. ImageJ
software (NIH) was used to measure the area of fibrillarin and DAPI staining in images of RNA duplex treated cells. T-tests were used to determine the significance of data. For deconvolution, serial images (0.2μm steps) of cells were collected with a Zeiss Axioplan2 microscope (100x objective), equipped with a Hamamatsu C4742-95 digital camera, using Openlab software (Improvison, Lexington, MA, USA). These images were deconvolved in Openlab and then processed in Photoshop.

**Immunoprecipitation and immunoblotting**

Polyclonal rabbit anti-BRAG2 antibody, raised against residues 153-176 of BRAG2a as described by [4], was obtained from Vladimir Marshansky (Massachusetts General Hospital) and used for immunoprecipitation (1/500). At least ~1.8mg of total HeLa cell protein was required to detect BRAG2 isoforms by immunoprecipitation. Immunoprecipitation and immunoblotting was performed as described by Hansen and Casanova [29]. For immunoblotting a 1/1000 dilution of rabbit anti-BRAG2 antibody and 1/10,000 of mouse anti-tubulinα (DM1 Sigma) were used.
Acknowledgments

The authors acknowledge the following colleagues for constructs: Michael Griffiths and Maureen Powers (Emory University), Michael Hebert and Greg Matera (Case Western Reserve University), Danyang Chen and Sui Huang (Northwestern University), Jennifer Lippincott-Schwartz (NIH), Tom Misteli (NIH), and Angus Lamond (University of Dundee). We also thank Ian Macara (University of Virginia) for leptomycin B and control siRNA duplex, and Vladimir Marshansky (Massachusetts General Hospital) for rabbit anti-BRAG2 antibody. We gratefully acknowledge Kim Yasutis for technical expertise, Martin Schwartz, Ian Macara, David Castle and members of the Casanova lab, for numerous discussions, and Richard Day for critical reading of the manuscript. This work was supported by NIH grants DK58536 and GM66251 to JEC.
References


Figure legends

Figure 1: BRAG2 depletion alters the morphology of nucleoli and CBs

HeLa cells were transfected with a control RNA duplex or BRAG2 siRNA duplex. ~ 48h post-transfection cells were fixed and indirect immunofluorescence used to detect endogenous fibrillarin and coilin (A). Bar is 2μm. After BRAG2 depletion the fibrillarin staining was less discrete and more dendritic (A, inset), and there was a significant increase (~30%) in the area of fibrillarin staining (B, p<0.01). The number of CB also increased after treatment with the BRAG2 siRNA duplex, and the CB were more pronounced (A, inset). After BRAG2 depletion there was a significant increase in the number of CB per nucleus (C, p<0.01). Western blot analysis of immunoprecipitates (D) indicates that both BRAG2a and b are greatly reduced by the BRAG2 siRNA duplex (lane B) when compared to mock (lane M) or control duplex treated cells (lane C). Anti-tubulinα was used to demonstrate even total protein levels.

Figure 2: BRAG2 localizes to nuclei and concentrates in nucleoli after PIKE expression

HeLa cells were transiently transfected with myc-PIKE-S (A), HA-BRAG2a (B), or myc-PIKE-S and HA-BRAG2a (C). Asterisks indicate untransfected cells. The position of nuclei was determined by DAPI staining (shown in Merge). PIKE-S staining is usually nuclear (A) with occasional cytoplasmic tubule staining (A, arrow). Diffuse nucleoplasmic and cytoplasmic staining was observed for BRAG2a (B). Co-transfection with PIKE induces nuclear patches containing BRAG2 (C). Images are representative of seven independent experiments. Bar is 1μm.
Figure 3: Exogenous BRAG2 alters nucleolar architecture

HeLa cells were transiently transfected with GFP-B23 (A-C), GFP-fibrillarin (D-G), HA-BRAG2a (B-C, E-F), HA-BRAG2a 1-250 (G) and myc-PIKE-S (C-D, F-G) PIKE is not shown in triple transfections. Neither BRAG2a nor PIKE alone have an obvious effect on B23 (A-B) or fibrillarin staining (D-E). However, when BRAG2a accumulates in nucleoli due to co-expression of PIKE, it results in a concentration of B23 deep within nucleoli (C), and fibrillarin re-distributes to the nucleolar periphery (F). The same phenomenon is seen when the N-terminus of BRAG2a alone accumulates in the nucleoli of HeLa cells (G). Images are representative of at least three separate experiments. Bar is 1μm.
Figure 4: Neither PIKE GTPase, nor BRAG2 GEF activity are required for nucleolar accumulation of BRAG2

HeLa cells were transiently transfected with wild-type myc-PIKE-S (B-C), or nucleotide binding deficient myc-PIKE-S K413A, S414N (A) and GFP-fibrillarin (C), wild-type BRAG2a (A) or catalytically inactive BRAG2a E498K (B-C). PIKE is not shown in the triple transfection (C). Expression of the nucleotide binding deficient PIKE-S mutant is still able to induce nucleolar accumulation of BRAG2a (A). Catalytically inactive BRAG2a concentrates in nucleoli when PIKE-S is expressed (B), where it is surrounded by fibrillarin (C). Hence, neither PIKE GTPase nor BRAG2 GEF activity are required for PIKE induced BRAG2 nucleolar accumulation. Bar is 1μM. Images are representative of at least three separate experiments.

Figure 5: The N-terminus of PIKE induces accumulation of BRAG2 in nucleoli

Diagrams of 3 PIKE isoforms (-L, -S and -A), and 4 partial PIKE constructs are shown in A. HeLa cells were transiently transfected with BRAG2a-GFP and the different PIKE constructs, myc-PIKE1-384 (B), 1-699 (C), 268-753 (D) or 670-1186 (E). Only constructs containing the N-terminus of PIKE-S (B-C) were able to promote nucleolar accumulation of BRAG2a. Bar is 1μm. Images are representative of at least three separate experiments.
Figure 6: The N-terminus of BRAG2a alone can localize to nucleoli

Diagrams of 2 BRAG2 splice isoforms and 3 partial BRAG2 constructs are shown in A. HeLa cells were transiently transfected with HA-BRAG2a 1-250 (B-C), 251-596 (D-E), 597-841 (F-G), or an IQ calmodulin binding mutant (HA-BRAG2a I19E R24E, H-I), and myc-PIKE-S (C, E, G, I). All BRAG2 constructs had at least partial nuclear localization (B, D, F, H). The localization of the BRAG2a mid-region (E) and C-terminus (G) was not obviously altered by co-expression of PIKE. However, the co-expression of PIKE promoted nucleolar accumulation of the N-terminus of BRAG2a (C). PIKE expression also promoted the nucleolar concentration of the BRAG2 IQ mutant (I). Bar is 1μm. Images are representative of at least three separate experiments. Asterisks indicate non-transfected cells.
Supplemental Material

Expression of PIKE also induces accumulation of coilin in nucleoli

CB are small nuclear bodies implicated in snRNP/snoRNP biogenesis. Coilin is usually diffusely distributed in the nucleoplasm as well as in punctate nuclear foci representing CB, and sometimes faintly in the nucleolus (Supplemental Fig. 3A). Upon expression of PIKE-S however, we found that GFP-coilin staining became restricted to nuclear patches that bore a striking resemblance to the BRAG2 nucleolar staining (Supplemental Fig. 3B). A triple transfection with HA-BRAG2a, GFP-coilin and myc-PIKE-S demonstrated that BRAG2 and coilin co-localize in the nuclear patches (Supplemental Fig. 3C). Hence, both coilin and BRAG2 can specifically accumulate in nucleoli in response to PIKE-S while other proteins found in the nucleoplasm are not affected. As for BRAG2 nucleolar accumulation, the N-terminus of PIKE was both sufficient and necessary for PIKE to stimulate accumulation of coilin in nucleoli (Supplemental Fig. 3D-G).

PIKE, BRAG2 and SMN co-localize on cytoplasmic tubules

In a subset of HeLa cells (< 24%) PIKE-S was consistently seen on cytoplasmic tubules. PIKE-L exhibits a more cytoplasmic distribution than PIKE-S [9] and localizes to cytoplasmic tubules with higher frequency (~50-97%). PIKE-L can also promote nucleolar accumulation of BRAG2, but in keeping with its more cytoplasmic distribution, does so less efficiently. Instead PIKE-L preferentially induces BRAG2 to concentrate on cytoplasmic tubules (Supplemental Fig. 4A, plus inset).
We also observed the co-localization of the PIKE N-terminus and BRAG2 N-terminus on cytoplasmic tubules. In many cells the N-termini of both proteins co-localized on cytoplasmic tubular structures similar to those observed for PIKE-L (Supplemental Figure 4B, plus inset). Neither full length BRAG2 nor its N-terminal fragment were found on tubules in the absence of PIKE, suggesting that PIKE is responsible for BRAG2's localization to tubules.

Additionally, the RNP biogenesis factor SMN could be found to co-localize with PIKE on cytoplasmic tubules. SMN is required for snRNP and snoRNP biogenesis. In nuclei SMN co-localizes with coilin in CB, or is found in associated foci called "gems" (gemini of CB, Supplemental Fig. 4C. [19]). SMN can also be found in the cytoplasm, either diffusely or in discrete foci (cytoplasmic gems). Despite the dramatic effect of PIKE-S on coilin localization, nuclear SMN was not obviously affected by PIKE expression (Supplemental Fig. 4C). The discrete nuclear SMN foci (gems/CB) were not noticeably affected by PIKE-S, even when coilin or BRAG2 clearly accumulated in nucleoli (not shown). Additionally, expression of BRAG2a alone had no obvious effect on nuclear SMN foci (not shown). Although PIKE-S expression did not obviously affect the localization of GFP-SMN in most cells, in cells where cytoplasmic PIKE tubules were observed GFP-SMN clearly co-localized with PIKE-S on these tubules (Supplemental Fig. 4C). No obvious tubular SMN staining was observed in the absence of PIKE. This observation suggests that cytoplasmic PIKE-S alters the distribution of cytoplasmic SMN and promotes the localization of SMN to tubular structures. It remains to be determined
if the tubular structures are the same cytoplasmic compartment to which PIKE induces 
BRAG2 localization, but this seems likely.

Additionally, co-labeling with tubulin antibodies indicated that these structures are 
aligned along microtubules, but are not coincident with them (Supplemental Fig. 4D, plus 
inset), suggesting that they may represent distinct structures that move along microtubule 
tracks.

**Supplemental Figure Legends**

**Supplemental Figure 1: Detection of nucleolar BRAG2a**

HeLa cells were transiently transfected with BRAG2a-GFP (B and E), HA-BRAG2a (C, 
F-G and J-K), or untagged BRAG2a (D and H), with (A and E-H) or without (B-D) myc-
PIKE-S. Asterisks indicate untransfected cells. The position of nuclei was determined by 
DAPI staining (shown in Merge). Nuclear staining was observed for BRAG2a, 
regardless of N-terminal HA-tag, C-terminal GFP tag, or the absence of a tag (B-D). 
PIKE-S staining is usually nuclear (A) with occasional cytoplasmic tubule staining (A, 
arrow). Co-transfection with PIKE induces BRAG2 nuclear patches, which are more 
pronounced for GFP than HA tagged or un-tagged BRAG2 (E-H). This appears to be at 
least partly due to poor antibody penetration. In particular, nucleolar HA-BRAG2a 
appears to be more easily detected when using the anti-HA antibody than the anti-
BRAG2a antibody (compare F to G). Bar is 1μm. Images are representative of at least 
three separate experiments.
**Supplemental Figure 2: Nucleolar BRAG2 alters the localization of endogenous fibrillarin**

HeLa cells were transfected with GFP-fibrillarin (A) or stained for endogenous fibrillarin after mock transfection (B), or transfection with BRAG2a-GFP and myc-PIKE-S (C). Endogenous fibrillarin is also shown in a PC12 cell after transient transfection with BRAG2a-GFP and myc-PIKE-S (D). PIKE is not shown in triple transfections and the PC12 image is deconvolved. Both GFP-fibrillarin (A) and endogenous fibrillarin (B) normally show a punctate pattern of staining that is evenly distributed throughout nucleoli. However, after co-transfection with BRAG2a-GFP and myc-PIKE-S endogenous fibrillarin in both HeLa (C) and PC12 cells (D) becomes restricted to the periphery of nucleoli. Each image is representative of at least three separate experiments. Bar is 1μm.

**Supplemental Figure 3: Expression of the N-terminus of PIKE induces concentration of coilin in nucleoli**

HeLa cells were transiently transfected with GFP-coilin (A-G), and either full-length PIKE-S (B-C) or the partial PIKE constructs, myc-PIKE 1-384 (D), 1-669 (E), 268-753 (F), and 670-1186 (G). Where triple-transfection with HA-BRAG2a was performed (C) PIKE is not shown. GFP-coilin alone (A) localizes to the nucleoplasm, CB (B, inset) and faintly to nucleoli. PIKE expression promotes nucleolar accumulation of coilin (B) where coilin co-localizes with BRAG2 (C). The N-terminus of PIKE alone is sufficient (D) and required (F-G) to induce this localization of coilin. Bars are 1μm. Images are
representative of at least three separate experiments. Asterisks indicate non-transfected cells.

**Supplemental Figure 4: PIKE, BRAG2 & SMN co-localize on cytoplasmic tubules**

HeLa cells were transiently transfected with myc-PIKE-L (A), PIKE–S (C-D) or PIKE 1-384 (B), and BRAG2a-GFP (A), HA-BRAG2a 1-250 (B), or GFP-SMN (C). BRAG2a and PIKE-L were often found to co-localize on cytoplasmic tubules (A, plus inset). A similar phenomenon was observed with the N-termini of PIKE (1-384) and BRAG2a (1-250; B plus inset). Expression of PIKE-S did not obviously alter the nuclear localization of SMN (C). However the presence of cytoplasmic PIKE-S appeared to induce co-localization of SMN with PIKE on cytoplasmic tubules (C, plus inset). Staining for endogenous tubulin-α suggests that the cytoplasmic PIKE tubules are aligned along microtubules (deconvolved images shown in D, plus inset). Bars are 1μm. Each image is representative of at least three separate experiments. The GFP-SMN construct was obtained from Angus Lamond (University of Dundee, [30]).