The Nucleolus and Nucleolar Proteins of *Dictyostelium*

by

Andrew Joseph Catalano

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Cell and Systems Biology
University of Toronto

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Andrew Joseph Catalano  

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2011  

Abstract  

*Dictyostelium* is a model eukaryote for the study of a multitude of fundamental cellular processes as well as several human diseases. Despite its extensive study relatively little is known about its nucleolus. Only three nucleolar proteins have been identified. The nucleolus in *Dictyostelium* is different than that of other eukaryotes since it is neither bipartite nor tripartite, possessing no visible subcompartments at the ultrastructural level. Moreover, it exists as two to four patches adjacent to the inner nuclear envelope instead of within the nucleoplasm. The aim of this study was thus to identify and characterize novel nucleolar proteins in *Dictyostelium* in order to better understand the structure and function of its nucleolus. Previous work had shown that NumA1, a protein linked to cell cycle in *Dictyostelium*, localizes to similar intranuclear patches suggesting it may be nucleolar. NumA1-binding partners Ca$^{2+}$-binding protein (CBP) 4a and puromycin-sensitive aminopeptidase A may therefore also reside in the nucleolus. Based on the function of a potential NumA1 homologue in other organisms, BRG1-associated factor 60a homologue Snf12 and checkpoint kinase 2 (Rad53 in yeast) homologue forkhead-associated kinase (Fhk) A were chosen as potential nucleolar proteins in *Dictyostelium* that may also be involved in cell cycle events. Using a diversity of approaches, this study found that NumA1, CBP4a, Snf12, and FhkA are nucleolar proteins in *Dictyostelium* while puromycin-sensitive aminopeptidase A is
nucleoplasmic. Several nuclear localization signals (NLSs) were identified in these proteins some of which also act as nucleolar localization signals (NoLSs). These NLS/NoLSs (within NumA1 and Snf12) represent the first NoLSs and first NLS/NoLSs identified in *Dictyostelium*. Treatment with the rDNA transcription inhibitor AM-D led to the budding of nucleolar CBP4a, Snf12, and FhkA from the nucleus to the cytoplasm, a phenomenon not previously observed in any organism. This study also examined for the first time the redistribution of nucleolar proteins during mitosis, a time when the nucleolus disassembles into its component parts. The nuclear envelope was also shown to become permeable at this time. Finally, multiple nucleolar subcompartments were identified suggesting compartmentalization of different functions in the *Dictyostelium* nucleolus.
Acknowledgments

I would like to thank my supervisor and friend, Dr. Danton H. O’Day for everything that he has done for me over the past several years. He has always been encouraging and helpful and has a genuine enthusiasm for science which makes discussing data always a pleasure. I could not have asked for a better supervisor and I am incredibly thankful for everything.

I would like to thank my committee members, Dr. Joel Levine and Dr. Bryan Stewart for their guidance and input into my research throughout the years. Both have been very helpful and I am grateful to have their input into my research.

I would like to thank my parents who have always been encouraging over the years even if they don’t understand exactly what a nucleolus is.

I would like to thank my lab mates Katya, Rob, Andres, and Aldona for their support and encouragement over the years. I have been incredibly fortunate to have had the opportunity to work along side each of you and I find it hard to believe how well we all got along. We are a team in the lab and this thesis is in some ways a team effort. We troubleshooting together, spent long hours working together, and were always encouraging to each other in times of non-significant results. I could not have asked for better lab mates. Thank you all for everything you’ve done over the years.

I would also like to thank Jean-Christophe Billeter for his guidance with the molecular work. He always had an answer to my question.
Finally, I would like to thank my future wife, Jade for her love and support over the past five years. You motivated me to work hard, comforted me when things didn’t go so well, and tolerated my “crazy hours”. Thank you for everything.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AM-D</td>
<td>actinomycin-D</td>
</tr>
<tr>
<td>asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-Bis(o-aminophenoxy)ethane-(N,N,N',N')tetraacetic acid</td>
</tr>
<tr>
<td>BAF</td>
<td>brahma-related gene 1-associated factor</td>
</tr>
<tr>
<td>BME</td>
<td>Bestatin Methyl Ester</td>
</tr>
<tr>
<td>BRCT</td>
<td>breast cancer carboxy terminus</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>CaMBD</td>
<td>CaM-binding domain</td>
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<tr>
<td>CaMBP</td>
<td>CaM-binding protein</td>
</tr>
<tr>
<td>CBP</td>
<td>Ca(^{2+})-binding protein</td>
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<tr>
<td>Chk</td>
<td>checkpoint kinase</td>
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<tr>
<td>COG</td>
<td>COG5531</td>
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<tr>
<td>DAPI</td>
<td>4',6'-diamidino-2-phenylindole</td>
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<tr>
<td>DFC</td>
<td>dense fibrillar component</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>eukaryotic translation initiation factor</td>
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<td>HPLC</td>
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<td>Hsp</td>
<td>heat shock protein</td>
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<tr>
<td>MBS</td>
<td>m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester</td>
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<tr>
<td>MDM</td>
<td>murine double-minute</td>
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<tr>
<td>MTOC</td>
<td>microtubule-organizing center</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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</tr>
<tr>
<td>NES</td>
<td>nuclear export signal</td>
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<td>nuclear localization signal</td>
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<td>NoSC localization signal</td>
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<tr>
<td>TRAP</td>
<td>tumour necrosis factor receptor-associated protein</td>
</tr>
<tr>
<td>WWIM</td>
<td>WW-interacting motif</td>
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Chapter 1
The nucleolus, previously identified, and putative nucleolar proteins in *Dictyostelium*

1.1 The nucleolus

The nucleolus is a multifunctional subnuclear compartment mainly responsible for the production of ribosomes. It has been studied for more than 200 years (Fontana, 1781; Wagner, 1835). More than 100 years ago a wealth of literature already existed including the idea of nucleolar disassembly during mitosis (Montgomery, 1898; Strasburger, 1893; 1897). We know today that within the nucleolus the transcription of rDNA genes results in the accumulation of rRNA processing machinery (Tschochner and Hurt, 2003). This ultimately leads to the assembly of ribosomal subunits which are exported to the cytoplasm (Tschochner and Hurt, 2003). These synthetic processes result in the formation of the nucleolus and its subcompartments, leading to either a bipartite or tripartite nucleolus. A tripartite nucleolus is composed of a fibrillar center (FC; site of transcriptionally inactive rDNA), a dense fibrillar component (DFC; site of transcriptionally active rDNA and rRNA processing), and a granular component (GC; site of ribosomal subunit assembly) whereas a bipartite nucleolus possesses a merged FC and DFC as well as a GC region (Boisvert et al., 2007; Thirty and Lafontaine, 2005). Upon entry into mitosis these structures are lost as the nucleolus disassembles (Boisvert et al., 2007).

The nucleolus is more than just a factory for ribosomes. The human nucleolar proteome contains more than 700 proteins which are highly conserved (90% have clear homologues in yeast) however only 30% are involved in ribosome biogenesis (Andersen et al., 2005; Boisvert et al., 2007). The remaining, majority of nucleolar proteins are involved in a multitude of cellular
events such as DNA replication, DNA repair, viral replication, molecular sequestering, chaperone activity, centrosome function, cell signaling, the regulation of stress response, and the control and regulation of cell cycle events (Boisvert et al., 2007; Okuda, 2002; Pederson and Tsai, 2009; Sirri et al., 2008; Visintin and Amon, 2000). This may explain why the nucleolar proteome is very dynamic and changes under different metabolic conditions (Andersen et al., 2005). Understandably, the nucleolus is linked to a multitude of human diseases, most notably those caused by viral infections and cancer (Andersen et al., 2005; Boisvert et al., 2007).

1.2 The nucleolus of *Dictyostelium* during growth

*Dictyostelium discoideum* exists as single-celled amoebae that phagocytose bacteria for food. When starved the cells enter a developmental stage during which growth stops and differentiation begins. They first aggregate into mounds via chemotaxis to cAMP, which they themselves secrete. The mound develops into a slug containing differentiated prespore and prestalk cells which form the spore and dead stalk cells of a fruiting body respectively. Under favorable conditions the spores germinate to restart the life cycle as single-celled amoebae. The easy maintenance, haploid genome, separated growth and developmental stages, and relatively simple differentiation make *Dictyostelium* an excellent model organism to use for the study of several fundamental cellular processes such as cell growth, cytokinesis, cell movement, chemotaxis, phagocytosis, as well as development and differentiation (Williams, 2010). Perhaps more importantly, *Dictyostelium* is also used for the study of several human diseases such as bacterial and viral pathogens as well as several diseases affecting the central nervous system including Huntington’s disease (Annesley and Fisher, 2009; Loomis, 2006; Myre et al., 2011; Saxe, 1999; Williams, 2010).
Despite the extensive study of this organism, the nucleolus of *Dictyostelium* is not a current area of research. Much of what is known comes from ultrastructural studies performed decades ago. Nevertheless, its structural features have been well documented and prove to be somewhat different from those of other organisms. For starters, it is composed of two to four dense patches/lobes which occupy 31-39% of the total nuclear volume and are attached to the inner nuclear envelope (Fig. 1.1; Benichou et al., 1983; Maclean et al., 1984; Roos et al., 1992; Sameshima et al., 1991). The number and position of these patches are fixed although the morphology varies between cells (Roos et al., 1992). This differs from the single, spheroid body nucleolus which occurs in most organisms that is not attached to the nuclear envelope (Fig. 1.1).

*Dictyostelium mucoroides*, *Dictyostelium minutum*, and *Polysphondylium pallidum* also have a multi-patched nucleolus similar in morphology to that of *Dictyostelium discoideum* (Filosa and Dengler, 1972; Schaap et al., 1981; Hohl et al., 1970). However other slime molds such as *Myxomycetes*, Dinoflagellates, and Ascomycetes possess a nucleolus of the usual, singular spheroid sort (Garrison et al., 1979; Hohl and Hamamoto, 1969a; Hung and Wells, 1977; Kubai and Ris, 1969; Rae, 1970).

Several lines of evidence show that these patches are in fact nucleoli. First, their protein content is different from that of the nucleoplasm and they therefore represent a subnuclear compartment (Parish et al., 1980). Second, they are not densely packed DNA since *Dictyostelium* heterochromatin is located in a single patch adjacent to the centromere (Kaller et al., 2006). The final and most compelling evidence comes from studies using actinomycin-D (AM-D) which preferentially inhibits RNA polymerase I at low concentrations thereby inhibiting rDNA transcription (Huang et al., 1998; Perry, 1963; Perry and Kelley, 1970). Since the recruitment of nucleolar material is dependent on rDNA transcription AM-D treatment results in nucleolar dissolution (Benichou et al., 1983; Kawashima et al., 1979; Perry and Kelly, 1970). Accordingly,
AM-D treatment results in the diminishment of these patches, indicating that they are nucleoli (Kawashima et al., 1979).

The Dictyostelium nucleolus is neither bipartite nor tripartite since the FC, DFC, and GC subcompartments are not evident. This suggests that rRNA modification and ribosomal subunit assembly, which normally occur in the DFC and GC regions respectively, may both occur throughout the entire nucleolus. Accordingly, the Dictyostelium nucleolus consists of a uniformly distributed fibrous matrix and overlapping ribosome-like granules (fairly uniform in size, ranging from 10-50 kDa) which may be the functional equivalent of the DFC and GC regions respectively (Kanda et al., 1974; Maeda and Takeuchi, 1969). If true, the region equivalent to the DFC occupies less volume than that equivalent to the GC since the Dictyostelium nucleolus contains only 16 % RNA, the remainder most likely protein (Kawashima et al., 1979). Interestingly, AM-D treatment results in the separation of the granule-
like material from the rest of the nucleolus, suggesting they are functionally different from the fibrous material (Benichou et al., 1983).

Despite the homogeneous composition of the nucleolus the rDNA (equivalent to the FC region), although not visible as a nucleolar subcompartment, exists as a beaded ring-like structure (15-20 beads per ring) around the periphery of each nucleolar patch (Moerman and Klein, 1998; Simon and Olins, 1994). *Dictyostelium* rDNA is mostly extrachromosomal while some is also present on telomeres (Cockburn et al., 1978; Williams, 2010). Although not commonly observed, extrachromosomal rDNA also occurs in *Tetrahymena thermophilia* and *Naegleria gruberi* (Clark and Cross, 1987; Cockburn et al., 1978; Kapler, 1993; Williams, 2010).

1.3 The nucleolus of *Dictyostelium* during development

Starvation of *Dictyostelium* amoebae triggers entry into their developmental stage. This transition is accompanied by a number of functional and morphological changes in both the cell and nucleolus. In other organisms development also leads to changes in nucleolar morphology (Lafarga et al., 1985). Not only does *Dictyostelium* require a different set of proteins during development, but it also requires new ribosomes that are functionally different from those used during growth (Cocucci and Sussman, 1970). Naturally, these changes are reflected in the composition of the nucleolus, where at least 75 % of the rRNA used during growth is degraded during morphogenesis and replaced with newly synthesized rRNA (Cocucci and Sussman, 1970). Although the need for functionally different ribosomes is unclear, these newly synthesized ribosomes are required for the progression from late aggregation to fruiting body stage (Ishida et al., 1974). Evidence suggests that during this time only developmental ribosomes can either translocate to the cytoplasm or translate developmental mRNA effectively (Cocucci and Sussman, 1970).
Accordingly, a mutant which possessed a nucleolus only during growth was not capable of full development (Ishida et al., 1974). This nucleolus was diminished during aggregation and lost during the slug stage (Ishida et al., 1974). It is thought that these mutant cells are capable of degrading vegetative rRNA but not capable of forming developmental rRNA, a transition induced not by starvation but by cell aggregation (Ishida et al., 1974). This indicates that developmental rRNA/ribosomes are required for proper cell-cell adhesion and spore/stalk cell differentiation but not for proper slug migration (Ishida et al., 1974). Moreover, the presence of wildtype cells in chimeras can not compensate for the absence of these ribosomes (Ishida et al., 1974). Accordingly, when these aggregating mutant cells were allowed to revert back to the growth stage the nucleolus reappeared (Ishida et al., 1974).

Along with the functional changes that occur during the growth/development transition the nucleolus also undergoes a number of morphological changes. For instance aggregating cells possess on average only two nucleolar patches (Sameshima et al., 1991). One of these patches protrudes towards the front of the nucleus (i.e., oriented towards the direction of travel) extending into a nozzle-like structure surrounded by nuclear membrane (Sameshima et al., 1991). Further on in aggregation however evidence suggests that only one patch (with a nozzle) remains (Sameshima, 1985). Other changes include a decreased amount of granules (Maeda and Takeuchi, 1969).

1.4 The relationship between the *Dictyostelium* nucleolus and the microtubule-organizing center

A tight relationship exists between the nucleolar patches and the microtubule-organizing center (MTOC) in *Dictyostelium*, the functional significance of which remains unknown. A similar relationship exists in *Saccharomyces* (Yang et al., 1989). The *Dictyostelium* MTOC is a
multilayered, matchbox-shaped structure from which microtubules polymerize (Omura and Fukui, 1985). It associates with the nucleus during both growth and aggregation at a location where nucleolar patches are absent (Kuriyama et al., 1982; Moens, 1976; Omura and Fukui, 1985; Sameshima et al., 1991). This absence may be due to the presence of an intra-nuclear structure, perhaps involved with connecting the MTOC to the nuclear envelope (Kuriyama et al., 1982; Moens, 1976; Sameshima et al., 1991).

In aggregating cells microtubules organize at the MTOC, which is at the rear of the nucleus (with the front pointing towards the direction of chemotaxis), and run alongside the nuclear envelope to which they are attached via crossbridges (Sameshima et al., 1991). The position of the nucleolar patch (directly opposite the MTOC, at the front of the nucleus) and the presence and direction of the protruding nozzle structure all depend on the presence of microtubules (Sameshima et al., 1991). The nozzle is therefore at the front of the nucleus/cell in line with the orientation of microtubules and directly opposite the MTOC at the rear (Sameshima, 1985). Microtubules however do not influence the shape of the protruding nucleolar patch which in their absence may protrude anywhere along the nuclear envelope (except around the MTOC; Sameshima et al., 1991).

### 1.5 An introduction to the previously identified nucleolar proteins of *Dictyostelium*

Given the hundreds of nucleolar proteins that exist in other organisms and the high degree of conservation between nucleolar proteomes of different species it naturally follows that *Dictyostelium* also possesses a large number of nucleolar proteins. Despite this assumption, only three have been previously identified, heat shock protein (Hsp) 32, eukaryotic translation initiation factor (eIF) 6, and tumour necrosis factor receptor-associated protein (TRAP) 1 (Balbo
and Bozzaro, 2006; Moerman and Klein, 1998; Yamaguchi et al., 2005). Interestingly, null mutations for each of these proteins have proven to be lethal suggesting a need for alternative approaches in determining the function of nucleolar proteins in Dictyostelium. In keeping with the multifunctionality of the nucleolus each protein is involved in different cellular processes.

1.6 Nuclear and nucleolar localization signals

Nuclear proteins must enter the nucleus through the nuclear pore complex (NPC). Although smaller proteins (less than 40 kDa) can freely diffuse through the NPC, most nuclear proteins bind to α-importin which leads to the active transport of the nuclear protein across the NPC (Stewart, 2007). Nuclear proteins must thus possess a nuclear localization signal (NLS) which is responsible for binding to α-importin. Although several types of NLSs exist, a typical NLS contains two adjacent residues which must be either arginine or lysine followed by two adjacent residues, one of must be either arginine or lysine while the other must not be acidic (Puntervoll et al., 2003). NLSs are thus highly basic. The α-importin family is conserved from yeast to human and NLSs are thus also highly conserved among eukaryotes (Mason et al., 2009; Sorokin et al., 2007). NLSs are usually verified by observing if the deleted sequence results in the inability of the protein to enter the nucleus.

Nucleolar proteins must have a NLS as well as a nucleolar localization signal (NoLS). Unlike NLSs which always bind α-importin, NoLSs may bind other nucleolar proteins, rDNA, and/or rRNA, and are thus not conserved (Scheer and Weisenberger, 1994; Trimbur and Walsh, 1993). However like NLSs, several NoLSs are often rich in basic residues (Sirri et al., 2008). By definition, a NoLS must localize a molecule/protein to the nucleolus. NoLSs have not yet been identified in Dictyostelium however the region in eIF6 thought to contain a NoLS has been mapped to a subdomain (Balbo and Bozzaro, 2006).
1.7 Hsp32

Hsp32 was the first nucleolar protein identified in *Dictyostelium* and surprisingly is not similar in sequence to Hsp32 from other organisms (De Maria et al., 1997; Moerman and Klein, 1998). It was detected in the nucleolar fraction of cell extract and colocalized with rDNA, verifying that it is a nucleolar protein (Moerman and Klein, 1998). Unsuccessful attempts to knock out the gene suggest that Hsp32 is an essential protein (Moerman and Klein, 1998).

Hsp32 is a nucleolar protein in both stressed and unstressed cells however its subnucleolar distribution changes during heat shock (Moerman and Klein, 1998). In unstressed cells it colocalizes with rDNA appearing as beads on a string around the periphery of the nucleolar patches (Moerman and Klein, 1998; Simon and Olins, 1994). This localization is not dependent on rDNA-binding or rDNA transcription (Moerman and Klein, 1998; Simon and Olins, 1994). Upon heat shock it redistributes throughout the nucleolar patches as well as the nucleoplasm most likely to protect nucleolar proteins from damage, as is the case for other *Dictyostelium* Hsps. (de Maria et al., 1995; De Maria et al., 1997; Moerman and Klein, 1998). This redistribution is not a result of either inhibited rDNA transcription or the five times increase in protein levels that occurs at this time (de Maria et al., 1995; De Maria et al., 1997; Moerman and Klein, 1998). Interestingly, long heat shock (20 h) results in a single-patched nucleolus with more pronounced rDNA beads (Moerman and Klein, 1998; Shaw et al., 1995). This indicates that the structure of the nucleolus in *Dictyostelium* responds to stress, as is true for other organisms (Moerman and Klein, 1998; Shaw et al., 1995).

Hsp32 is 20-25 % identical and 40-50 % similar to the multifunctional nucleophosmin (NPM1) and similar in sequence to the N-terminal of mammalian nucleolin, both highly conserved nucleolar proteins in mammals (Grisendi et al., 2006; Moerman and Klein, 1998). Much of this
similarity results from a highly acidic region rich in aspartic acid (asp) and glutamic acid (glu) residues resulting in a high isoelectric point and a slower gel migration rate (Moerman and Klein, 1998). NPM1 and nucleolin also exhibit slow migration rates, thought to be caused by their highly acidic regions (Lapeyre et al., 1987; Schmidt-Zachmann et al., 1987). These regions are a common feature of nucleolar proteins and are thought to be responsible for binding to basic ribosomal proteins and NLSs of other proteins (Szebeni et al., 1995; Xue and Melese, 1994). Hsp32 also binds DNA, nonselectively but with high affinity, however this binding is not responsible for its localization (Moerman and Klein, 1998).

Hsp32 contains both a monopartite NLS (single stretch of basic residues) and a bipartite NLS (two sets of basic residues separated by eight to ten residues) similar to nucleolar proteins from other organism (Creancier et al., 1993; Dingwall and Laskey, 1991; Moerman and Klein, 1998; Schmidt-Zachmann and Nigg, 1993).

1.8 eIF6

eIF6, also discovered as a 27 kDa β4-integrin in epithelial cells-interacting protein (p27^{BBP}) is, despite its name, not required for the initiation of protein synthesis (Biffo et al., 1997; Si and Maitra, 1999). It is highly conserved in archaebacteria and eukaryotes and required for the biogenesis of the 60S ribosomal subunit in several organisms (Groft et al., 2000; Sanvito et al., 1999; Senger et al., 2001; Si and Maitra, 1999; Wood et al., 1999).

eIF6 was recently identified in Dictyostelium as a nucleolar protein via colocalization with DAPI counterstain (Balbo and Bozzaro, 2006). DAPI has previously been shown to be much less concentrated in nucleoli (Moerman and Klein, 1998). More importantly, eIF6 levels decreased after AM-D treatment, further verifying that it is a nucleolar protein. eIF6 is also present to a lesser degree in the nucleoplasm (Balbo and Bozzaro, 2006). Both the NLS and NoLS have been
mapped to the N-terminal and a second NLS is thought to exist in the C-terminal (Balbo and Bozzaro, 2006). *Dictyostelium* eIF6 shows a high degree of conservation to homologues from other organisms and is thus thought to have a similar function (Balbo and Bozzaro, 2006). Its homologues are partially nucleolar in other organisms as well (Miluzio et al., 2009). As in other organisms, deletion of the *Dictyostelium* eIF6 gene is lethal (Sanvito et al., 1999; Si and Maitra, 1999; Wood et al., 1999).

### 1.9 TRAP1

TRAP1 is a multifunctional protein that shares a high degree of similarity with Hsp90 (Morita et al., 2002). It associates with tumour necrosis factor-receptor which binds tumour necrosis factor, a protein involved in the immune system (Locksley et al., 2001). TRAP1 localizes to mitochondria in several organisms via an N-terminal mitochondria localization sequence but like other mitochondria chaperones (such as Hsp60 and Hsp70) also localizes to non-mitochondrial locations (Cechetto and Gupta, 2000; Felts et al., 2000; Morita et al., 2002; Morita et al., 2001; Soltys and Gupta, 1999; Song et al., 1995). In mammals, TRAP1 is found in nuclei, secretory granules, and at the cell surface (Cechetto and Gupta, 2000).

In *Dictyostelium* the TRAP1 homologue is a nucleolar protein but is also present in other locales (Yamaguchi et al., 2005). It localizes to intra nuclear patches however it is important to note that these patches, although reminiscent of nucleoli, have not been verified as nucleoli in any way. It is thought to play a role in cell cycle progression, cell differentiation, and apoptosis (Morita et al., 2004). TRAP1 is found in the outermost layer of the spore coat where it protects the spores from various physicochemical stresses (Morita et al., 2005; Yamaguchi et al., 2005). Null mutations in the TRAP1 gene are lethal, as is the case for other members of the Hsp90 family.
(Morita et al., 2004). Also, like other Hsps, its expression is induced by heat shock (Morita et al., 2005).

1.10 Putative nucleolar proteins

As detailed in Chapter 2, nucleomorphin isoform NumA1 is a nuclear calmodulin (CaM)-binding protein (CaMBP) in Dictyostelium thought to be involved in the cell cycle (Myre and O’Day, 2002). Isoform NumA2 contains a breast cancer carboxy-terminus (BRCT) domain which in other organisms is present in cell cycle checkpoint proteins (Myre and O’Day, 2004b). Moreover, overexpression of GFP-NumA1 lacking its acidic glu/asp (DEED) domain results in an increased proportion of multinucleate cells (Myre and O’Day, 2002). GFP-NumA1 localizes to discrete arc-like patches adjacent to the inner nuclear envelope (Myre and O’Day, 2002). This pattern is similar to that of eIF6 and TRAP1 suggesting that NumA1 is a nucleolar protein (Balbo and Bozzaro, 2006; Yamaguchi et al., 2005). NumA1 possesses two putative monopartite NLSs ($^{31}$PKSKKKF$^{37}$ and $^{246}$PTKKRSL$^{252}$) and a putative bipartite NLS ($^{48}$KKSYQDPEIIAHSRPRK$^{64}$; Myre and O’Day, 2002). The last four residues of the bipartite NLS ($^{61}$RPRK$^{64}$) may also function as a monopartite NLS. GFP fused to N-terminal residues 1-120 (which contain $^{31}$PKSKKKF$^{37}$ and $^{48}$KKSYQDPEIIAHSRPRK$^{64}$) localizes to the same nuclear patches while GFP-$^{48}$KKSYQDPEIIAHSRPRK$^{64}$ localizes to the nucleus (Myre and O’Day, 2005).

NumA1 interacts with Ca$^{2+}$-binding protein (CBP) 4a in a Ca$^{2+}$-dependent manner and with puromycin-sensitive aminopeptidase (Psa) A (Myre and O’Day, 2004a; Myre, 2005). If NumA1 is a nucleolar protein, CBP4a and PsaA may also reside in the nucleolus. CBP4a is covered in chapter 3 while PsaA is detailed in chapter 4.
NumA1 shares several attributes in common with the multifunctional NPM1 such as multiple putative NLSs, a large glu/asp domain, and links to the cell cycle (Myre and O’Day, 2002; Grisendi et al., 2006). Although NumA1 shares only 14% sequence identity with NPM1, it is similar in other ways to NPM1. This suggests NumA1 may be a functional equivalent of NPM1 in *Dictyostelium*, as this eukaryote does not possess a NPM1 homologue. Moreover, NPM1 is a nucleolar protein in mammals further supporting the notion that NumA1 is nucleolar in *Dictyostelium* (Grisendi et al., 2006). In mammals, NPM1 is a negative regulator of murine double-minute (MDM) 2 (human double-minute 2 in humans) and MDM2 negatively regulates the tumour suppressor p53 (Grisendi et al., 2006; Kurki et al., 2004). However upon DNA damage p53 is activated by checkpoint kinase (Chk) 2 (Rad53 in yeast; Hirao et al., 2000).

NPM1, MDM2, Chk2, and p53 are thus tightly involved in cell cycle events. In order to identify potential nucleolar, cell cycle proteins in *Dictyostelium*, homologues for NPM1, MDM2, and Chk2 were analyzed (no p53 homologue has been identified in *Dictyostelium*).

As mentioned earlier, NumA1 is thought to function as a functional equivalent of NPM1. *Dictyostelium* does not possess a MDM2 homologue however does possess a homologue for switch/sucrose nonfermentable (SWI/SNF) complex member brahma-related gene 1-associated factor (BAF) 60a, Snf12 (dictyBase.org). As detailed in chapter 5, in mammals BAF60a contains a SWIB domain which is homologous and shares a common fold with the MDM2 domain in MDM2 (Bennett-Lovsey et al., 2002). Moreover, like MDM2, BAF60a also interacts with p53 (Lee et al., 2002; Oh et al., 2008). Both MDM2 and BAF60a are also nucleolar (Li et al., 2008; Grisendi et al., 2006). Snf12 thus represents the closest protein in *Dictyostelium* to MDM2 and is thus thought to be nucleolar.
Unlike in other organisms, *Dictyostelium* possesses five homologues for Chk2; forkhead-associated kinase (Fhk) A, FhkB, FhkC, FhkD, and FhkE (dictyBase.org). Since none of these proteins have been characterized FhkA was chosen for this study (chapter 6). Chk2 is a nuclear protein in yeast but given its involvement in cell cycle regulation may also be nucleolar (Smolka et al., 2005).

### 1.11 Aims and objectives

The aim of this study was to identify and characterize novel, nucleolar proteins in order to better understand the nucleolus of *Dictyostelium*. NumA1, CBP4a, PsaA, Snf12, and FhkA were chosen as potential nucleolar proteins to investigate for reasons mentioned above. This goal was divided into the five main objectives listed below:

1. Verify if NumA1, CBP4a, and PsaA are nucleolar proteins.

2. Determine if Snf12 and FhkA are nucleolar proteins.

3. Identify and, where possible, verify NLSs and NoLSs within the nucleolar proteins.

4. Investigate nucleolar protein dynamics during mitosis.

5. Determine if nucleolar subcompartments exist in *Dictyostelium*.

### 1.12 Reproducibility

All images showing cells or nuclei are representative of at least 95% of all cells examined. For all experiments at least three independent replicates were performed and at least 1000 cells or nuclei were examined for each replicate.
1.13 Thesis format

The following chapters, except chapter 9, are structured as individual journal articles which are in preparation for submission.
1.14 References


Cechetto JD and Gupta RS (2000). Immunoelectron microscopy provides evidence that tumor necrosis factor receptor-associated protein 1 (TRAP-1) is a mitochondrial protein which also localizes at specific extramitochondrial sites. Exp Cell Res. 260: 30-39.


Chapter 2
Nucleolar localization and identification of nuclear/nucleolar localization signals of the calmodulin-binding protein nucleomorphin during growth and mitosis in Dictyostelium

This chapter was published as:


I performed the research and writing while my supervisor contributed to experimental design and data analysis and provided editorial input. This chapter was published exactly ‘as is’ except for formatting changes.

2.1 Abstract

The CaMBP nucleomorphin isoform NumA1 is a nuclear number regulator in Dictyostelium that localizes to intra-nuclear patches adjacent to the nuclear envelope and to a lesser extent the nucleoplasm. Earlier studies have shown similar patches to be nucleoli but only three nucleolar proteins have been identified in Dictyostelium. Here, AM-D treatment caused the loss of NumA1 localization, while Ca$^{2+}$ and CaM antagonists had no effect. In keeping with a nucleolar function, NumA1 moved out of the presumptive nucleoli during mitosis redistributing to areas within the nucleus, the spindle fibers, and centrosomal region before re-accumulating in the presumptive nucleoli at telophase. Together, these data verify NumA1 as a true nucleolar protein. Prior to this study, the dynamics of specific nucleolar proteins had not been determined during mitosis in Dictyostelium. Fluorescein isothiocyanate-conjugated peptides equivalent to presumptive NLSs within NumA1 localized to nucleoli indicating that they also act as NoLSs. To our knowledge,
these represent the first precisely defined NoLSs as well as the first NLS/NoLSs identified in *Dictyostelium*. Together, these results reveal that NumA1 is a true nucleolar protein and the only nucleolar CaMBP identified in *Dictyostelium*. The possible use of NLS/NoLS-mediated drug targeting to nucleoli is discussed.
2.2 Introduction

Nucleomorphin isoform NumA1 is a regulator of nuclear number in *Dictyostelium* that interacts with both CaM and CBP4a in a Ca$^{2+}$-dependent manner (Catalano and O’Day, 2008; Myre and O’Day, 2002; 2004a). GFP-NumA1 localizes to discrete intra-nuclear patches adjacent to the nuclear envelope and to a lesser extent the nucleoplasm (Myre and O’Day, 2002). A number of early studies have shown similar intranuclear patches in *Dictyostelium* to be nucleoli which suggest that NumA1 may be a nucleolar protein (Benichou et al., 1983; Kawashima et al., 1979; Maeda and Takeuchi, 1969). Unlike most other organisms, the subnucleolar compartments in *Dictyostelium* overlap and are thus not identifiable as distinct regions. The nucleolus is a multifunctional subnuclear compartment that is involved in ribosome subunit biogenesis and also houses proteins involved in several other processes such as the regulation of cell cycle events (Peterson and Tsai, 2009; Robertson, 2007; Visintin and Amon, 2000). Fittingly, NumA1 is thought to be involved in the cell cycle, since overexpressed GFP-NumA1-ΔDEED results in multinucleate cells and nucleomorphin isoform NumA2 contains a breast cancer carboxy-terminus domain which is found in cell cycle checkpoint proteins in other organisms (Myre and O’Day, 2002; 2004b). The highly acidic DEED domain is a common feature of several nucleolar proteins such as NPM1, nucleoplasmin (NPM2), nucleolin, and Hsp32 (Fig. 2.1; Grisendi et al., 2006; Herve et al., 1999; Moerman and Klein, 1998; Prado et al., 2004). To date, only three nucleolar proteins have been identified in *Dictyostelium*: Hsp32, TRAP1, and eIF6, all of which display a similar pattern of nucleolar localization (Balbo and Bozzaro, 2006; Moerman and Klein, 1998; Yamaguchi et al., 2005). AM-D inhibits rRNA transcription resulting in the dissolution of the nucleolus and is therefore a useful tool in determining if a protein is nucleolar, as done for *Dictyostelium* eIF6 (Balbo and Bozzaro, 2006; Kawashima et al., 1979).
Figure 2.1. Domain structure diagram of NumA1 and other nucleolar proteins. Black represents acidic domains. Number markers represent amino acid position. 

A) Amino acid positions of anti-NumA1 antigen as well as all NLSs. For the sequences of the NLSs see Table 2.2. 

B) Nucleolar proteins that contain acidic domains. NumA1, NPM1, and NPM2 have acidic domains beginning at amino acid position 121/122.

NumA1 contains multiple NLSs, another attribute of many nucleolar proteins. Three are located within N-terminal residues 1-120 (NLS-1, -2, and -4), the region required for the localization of NumA1 to intra-nuclear patches, and the fourth within the C-terminal (NLS-3; Fig. 2.1A; Myre and O’Day, 2002; 2005). A GFP-fused sequence equivalent to NLS-2 has previously been shown to localize to the nucleus (Myre and O’Day, 2005). Although the reason for possessing multiple NLSs is unclear, evidence suggests that they may act cooperatively either to mediate nuclear entry or to modulate the amount of the nuclear-localized protein, as is the case for nuclear human TBX5, nucleolar human ribosomal protein L7a, and transcription factor Nrf2 (Collavoli et al., 2003; Russo et al., 1997; Theodore et al., 2008).
NoLSs, like NLSs, are usually rich in basic residues (Table 2.1). Unlike NLSs however, NoLSs can be viewed as retention signals rather than transport signals since there is no membrane separating the nucleolus from the nucleoplasm (Birbach et al., 2004). Given that nucleolar proteins interact with high affinity binding sites on specific nucleolar target proteins, the sequence of each NoLS will depend on its unique interacting protein (Birbach et al., 2004; Misteli, 2001). Some NoLSs can also act as NLSs and are thus referred to as NLS/NoLSs.

Examples of proteins containing NLS/NoLSs include human NF-κB-inducing kinase, the novel human nucleolar protein phosphatidylinositol 4-kinase and myogenic regulatory factor (Birbach et al., 2004; Kakuk et al., 2008; Ueki et al., 1998; Wang et al., 2005). As the nucleolus gains more attention due to its newly discovered role in cancer, understanding the targeting of nucleolar proteins is of key importance (Maggi Jr. and Weber, 2006; Mijatovic et al., 2008; Montanaro et al., 2008). Since some NoLS peptide analogues may not be capable of nuclear entry on their own, NoLSs that are also capable of nuclear entry (NLS/NoLSs) are most attractive for use in drug delivery to the nucleolus. Although the NoLS in eIF6 has been mapped to a subdomain, no precise NoLS has yet been identified in Dictyostelium (Balbo and Bozzaro, 2006).

In mammals, several nucleolar proteins such as NPM1, nucleolin, and TATA box-binding protein-related factor 2 redistribute from nucleoli to other cellular locales during mitosis due to the dissolution of the nucleolus during this time (Ginisty et al., 1999; Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). If NumA1 is a nucleolar protein, then a similar redistribution during mitosis should be observed. In Dictyostelium, the pattern of microtubule localization is used to determine the stage of mitosis (Cappuccinelli et al., 1979; Kitanishi-Yumura and Fukui, 1987; Roos et al., 1984).
Table 2.1. NoLSs from various proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>NumA1</td>
<td>PKSKKKFK</td>
<td>(Myre and O’Day, 2002)</td>
</tr>
<tr>
<td>NumA1</td>
<td>KKSYPQDEIIAHSPFRK</td>
<td>(Myre and O’Day, 2002)</td>
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<tr>
<td>NumA1</td>
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<td>(Myre and O’Day, 2002)</td>
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<td>NumA1</td>
<td>RPFRK</td>
<td>(Myre and O’Day, 2002)</td>
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<td>RQAARRNRRRWREOR</td>
<td>(Kubota et al., 1989)</td>
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<td>MPKTRRPSSQRKPPTP</td>
<td>(Siomi et al., 1988)</td>
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<td>(Dang and Lee, 1989)</td>
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<td>(Song and Wu, 2005)</td>
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<td>KPRGATLGSQPKRRKS</td>
<td>(Stegh et al., 1998)</td>
</tr>
<tr>
<td>ARF</td>
<td>RGAQLRRPRHSPTRARRCP</td>
<td>(Zhang and Xiong, 1999)</td>
</tr>
<tr>
<td>PTHrP</td>
<td>GKKKGKPGKRRSEQKKRRT</td>
<td>(Henderson et al., 1995)</td>
</tr>
<tr>
<td>MDM2</td>
<td>KKLKKRNNK</td>
<td>(Lohrum et al., 2000)</td>
</tr>
<tr>
<td>Coilin</td>
<td>KKNNKKRNNK</td>
<td>(Hebert and Matera, 2000)</td>
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Basic residues are in bold. HIV; human immunodeficiency virus, HTLV; human T-lymphotropic virus, SVV; survivin, DEDD; death effector domain D, ARF; alternate reading frame, PTHrP; parathyroid hormone-related protein.

Here, we have shown via immunolocalization using polyclonal antibodies against NumA1 together with AM-D treatment that NumA1 localizes to nucleoli and to a lesser extent the nucleoplasm. FITC-peptides were used to show that all NLSs within NumA1 act as NLS/NoLSs and thus represent the only defined NoLSs and NLS/NoLSs in Dictyostelium. Finally, NumA1 demonstrated changes in localization throughout the nucleus and the centrosomal region during mitosis. These results demonstrate that NumA1 is a bona fide nucleolar protein and the only identified nucleolar CaMBP in Dictyostelium. The potential use of NLS/NoLS-mediated nucleolar drug targeting is discussed.
2.3 Materials and methods

2.3.1 Treatment, fixation with ultracold methanol, and immunolocalization

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich®. *Dictyostelium discoideum* AX3 cells were grown in HL-5 at 21 °C shaking at 180 rpm, as previously described (Fey et al., 2007). Cells (150 µL of 2 x 10⁶ cells/mL) were allowed to adhere to 13 x 0.1 mm circular glass coverslips (McCrone™) for 30 min in a humidity chamber. Alternatively, cells were either resuspended in KK₂-phosphate buffer (17.0 mM KH₂PO₄, 7.5 mM K₂HPO₄, pH 6.5, BioShop®) prior to adhering to coverslips for 2 or 4 h (starvation), or treated while adhering to coverslips (in HL-5). Treatment consisted of either 1, 5, or 10 mM CaCl₂ (4 h), 1, 5 or 10 mM EGTA (4 h), 1 or 5 mM 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid (BAPTA; 4 h), 300 µM W-5 (4 h), 50 µM W-7 (4 h), 0.5 mg/mL cycloheximide (1, 2, 4 or 8 h), or 0.05 mg/mL AM-D (4, 8, or 4 h followed by three washes with HL-5 prior to 4 h recovery in HL-5). Cells were fixed with ultracold methanol as previously described (Hagedorn et al., 2006). After blocking [PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄) with 0.2 % gelatin and 0.1 % Triton X100], the cells were incubated with anti-NumA1 for 60 min followed by Alexa Fluor® 555 goat anti-rabbit for 45 min. Anti-NumA1 has previously been verified by western blotting (Myre and O’Day, 2002). The cells were then incubated with anti-α-tubulin (Hybridoma Bank) for 60 min followed by Alexa Fluor® 488 goat anti-mouse. The localization of α-tubulin was used to determine the stage of cell cycle. All secondary antibodies were purchased from Invitrogen™. Five microliters Prolong Antifade (Invitrogen™) containing 4’,6’-diamidino-2-phenylindole (DAPI) was placed on a slide prior to mounting and coverslips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital Sight DS-Ri1 camera and images were analyzed using Nikon
Imaging Software Elements Basic Research 3.0. The level of fluorescence at different cellular locales was measured using Image J and the values analyzed using Student’s $t$-test ($\alpha = 0.05$).

### 2.3.2 Peptide synthesis and localization

The peptides that were synthesized and used in this study are summarized in Table 2.2. IP22-3, a novel motility-enhancing peptide in *Dictyostelium*, was used as a control peptide that does not enter cells (Catalano et al., 2010). All peptides were synthesized via solid phase synthesis, purified to at least 90% via HPLC, N-terminally conjugated to FITC, and verified via mass spectrometry, as previously described (Chan and White, 2000). Cells (1 mL of 1-2 x $10^6$ cells/mL) starved for 0, 0.5, 2, or 4 h were incubated with 200 µM FITC-peptide. FITC-DEED peptides (200 µM) were electroporated (5 µL peptide with 95 µL cells for a final concentration of 200 µM in 1 mm cuvettes, pulsed at 0.85 kV, 25 µF, 1.0 ms, two pulses 5 s apart) and viewed immediately after a 10 min recovery period on ice as previously described (Gaudet et al., 2007). The cells were allowed to incubate with the peptide for up to 10 min while adhering to coverslips. For verification of nucleolar localization, cells were treated with 0.05 mg/mL AM-D for 4 h on coverslips prior to incubation with the peptide. To determine if Ca$^{2+}$ or CaM is required for localization, cells were treated for either 2 or 4 h with either 5 mM EGTA or 5 mM BAPTA or with either 300 µM W-5 or 50 µM W-7 respectively prior to incubation with the peptide. Peptides were also incubated with isolated nuclei. The nuclei were isolated as previously described without using a sucrose gradient (Charlesworth and Parish, 1975). Exponentially growing cells were washed twice with ice-cold Sorensen buffer (14.6 mM KH$_2$PO$_4$, 2.0 mM Na$_2$HPO$_4$, pH 6.1) at 1,500 g for 5 min each spin, then resuspended in 50 mL of buffer 4 (90 mL of buffer 3 and 10 mL Percoll). 2.5 mL of 20% NP40 were added prior to a 5 min spin at 3,000 g. The pellet was resuspended in 20 mL of buffer 3 (5% w/v sucrose, 20 mM KCl, 40 mM MgCl$_2$, 50 mM 4-[2-hydroxyethyl]piperazine-1-ethane sulfonic acid [HEPES]-NaOH, pH 7.5,
Table 2.2. Peptide sequences used in this study.

<table>
<thead>
<tr>
<th>NLS and DEED peptides</th>
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<tr>
<td>NLS-1</td>
<td>$^{31}$PKSKKKF$^{37}$</td>
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<td>NLS-2</td>
<td>$^{48}$KKSYYQDPEIITAHSRPRK$^{64}$</td>
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<tr>
<td>NLS-3</td>
<td>$^{246}$PTKRRSL$^{252}$</td>
</tr>
<tr>
<td>NLS-4</td>
<td>$^{61}$RPRK$^{64}$</td>
</tr>
<tr>
<td>DEED-1</td>
<td>$^{121}$DDEEEDEEDEEDEEDEEDEDE $^{142}$</td>
</tr>
<tr>
<td>NLS-DEED-1</td>
<td>RPRKDDEEEDEEDEEDEEDE $^{142}$</td>
</tr>
<tr>
<td>DEED-2</td>
<td>$^{145}$EEEEEEDYDDEDLNDDEEDEDEDD$^{166}$</td>
</tr>
<tr>
<td>NLS-DEED-2</td>
<td>RPRKDDEEEDEEDEEDEEDEE $^{142}$</td>
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</table>

<table>
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</tr>
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</tr>
<tr>
<td>NLS-1c</td>
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</tr>
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<td>PKSKAASKF</td>
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</tr>
<tr>
<td>NLS-4b</td>
<td>RARK</td>
</tr>
<tr>
<td>NLS-4c</td>
<td>RPAK</td>
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<tr>
<td>NLS-4d</td>
<td>RPRA</td>
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</table>

0.1 % $\beta$-mercaptoethanol [14 mM], 0.038 mg/mL spermidine [0.15 mM], 0.2 mM PMSF) and then spun again for 5 min at 3,000 g. The pellet was then resuspended in 1 mL of buffer 3. After peptide incubation, Hoechst 33258 nuclear stain was added (500 µM) and all cells were slide-mounted and viewed with either a Zeiss LSM 510 confocal microscope using an argon-2 laser (Fig. 2.4C) or a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera (all other peptide-treated cells). Images were captured with either the LSM image capture software or the Nikon Imaging Software Elements Basic Research 3.0 respectively.
2.3.3 Random cell motility, chemotaxis, and development

Agar plates (1 mL) were made containing 0.4 % agar (BioShop®), treatment, and KK₂-phosphate buffer. The treatment for aggregation and migration experiments consisted of 0, 10, 50, 100, 200, or 500 µM of either NLS-1, -2, -3, or -4, either alone or conjugated to FITC. For chemotaxis experiments 10 µM cAMP was added. All peptides and chemicals were added to the KK₂-agar and mixed thoroughly before allowing the agar to cool. Vegetative AX3 cells were concentrated to 1.0 x 10⁸ cells/mL and spotted (0.2 µL) on the cooled KK₂-agar plates. Plates were left at room temperature. Pictures were taken immediately after spotting and 4 h after using a Zeiss Axiovert 100 inverted microscope equipped with a Sony 950 digital camera. The difference in spot diameter was measured using Northern Eclipse image analysis system (Empix Imaging, Mississauga, Ontario) and the distance migrated calculated.

For fruiting body morphogenesis experiments, spore and stalk cells that had developed on peptide-treated agar plates (0, 10, 50, 100, 200, or 500 µM) were incubated with 0.1 % calcofluor, slide-mounted, and viewed with a Zeiss Axiovert 100 inverted epifluorescent microscope.

2.4 Results

2.4.1 Anti-NumA1 detects NumA1 in both nucleoli and in the nucleoplasm

NumA1 was detected by anti-NumA1 in intra-nuclear patches adjacent to the nuclear envelope and to a lesser extent the nucleoplasm, as demonstrated by colocalization with DAPI unstained and stained regions of the nucleus respectively (Fig. 2.2A,B). This pattern was due to the specificity of anti-NumA1, as the cells probed either with the preimmune serum instead of anti-NumA1 or with the secondary antibody only showed low background levels of fluorescence with
Figure 2.2. Effect of AM-D on localization of NumA1. A) Untreated cells (0) or cells treated with 0.05 mg/mL AM-D for either 4 h (4), 8 h (8), or 4 h followed by a 4 h recovery period (R) were fixed in ultracold methanol. Cells on coverslips were probed with anti-NumA1 followed by Alexa Fluor 555® goat anti-rabbit, then with anti-α-tubulin followed by Alexa Fluor 488® goat anti-mouse. Coverslips were slide-mounted with Prolong Antifade containing DAPI. B) An enlarged image of the nucleus shows arrows denoting nucleoli which were diminished after 4 h of treatment and absent after 8 h, but were again visible after recovery. C) Cells probed with either the preimmune serum instead of anti-NumA1 or with only the secondary antibody showed low background levels of fluorescence with no specific localization evident. A Nikon ×100 oil-immersion objective lens with an aperture of 1.30 was used. At least four independent replicates were performed, all showing the same result. Scale bar represents 2 µm.

no specific localization evident (Fig. 2.2C). The level of secondary antibody fluorescence intensity was 0.75 ± 0.02 (mean ± standard error) in these patches, 0.65 ± 0.01 in the nucleoplasm, and 0.43 ± 0.02 in the cytoplasm, while the background level was 0.03 ± 0.00 (with 0 and 1 representing the minimum and maximum detectible levels respectively; Fig. 2.3). All four values are statistically different from each other. The size of the patches decreased dramatically after 4 h of transcription inhibition (0.05 mg/mL AM-D) and were absent after 8 h. After a 4 h recovery period from 4 h treatment, the patches were again visible confirming that
they are nucleoli (Fig. 2.2A,B). The inhibition of transcription did not affect the nucleoplasmic localization of NumA1. No difference in localization was observed when cells were either starved or treated with either 1, 5, or 10 mM CaCl$_2$ (4 h), Ca$^{2+}$ chelators (1, 5, or 10 mM EGTA for 4 h, or 1 or 5 mM BAPTA for 4 h), CaM antagonists (300 µM W-5 or 50 µM W-7 for 4 h), or 0.5 mg/mL of the translation inhibitor cycloheximide (1, 2, 4, or 8 h).

### 2.4.2 FITC peptides localize to nucleoli and nucleoplasm

FITC-NLS peptides equivalent to putative NLSs in NumA1 were used to determine which, if any, are responsible for the localization of NumA1 to nucleoli and therefore act as NoLSs. FITC-NLS-1, -2, -3, and -4 (Table 2.2,) localized predominantly to discrete intra-nuclear patches (Fig. 2.4A,C). Fluorescence was also observed to a lesser extent in the nucleoplasm, as demonstrated by colocalization with Hoechst 33258 nuclear stain, and sometimes diffusely throughout the cytoplasm (Fig. 2.4A,B). This pattern is the same as that observed using GFP-NumA1 (Fig. 2.4E,F; Myre and O’Day, 2002). All peptides displayed the same localization pattern regardless if the cell was starved for 0.5, 2, or 4 h. Treatment with either Ca$^{2+}$ chelators (5 mM EGTA or 5 mM BAPTA) or CaM antagonists (300 µM W-5 or 50 µM W-7) did not affect this localization. Treatment with AM-D (0.05 mg/mL for 4 h) prior to incubation resulted in the diminishment of...
Figure 2.4. Localization of FITC-NLS-1, -2, -3, -4, and all variants. A,C) After incubation with Dictyostelium amoebae for 30 min, all NLS peptides (FITC-NLS-1, -2, -3, -4, and all NLS-1 and -4 variants) localized predominantly to nucleoli, and to a lesser extent the nucleoplasm. A cell incubated with FITC-NLS-1 is shown. The same pattern was observed for each of the FITC-NLS peptides. A) Colocalization of FITC-NLS-1 with (B) Hoechst 33258 nuclear stain. C) Enlarged view of FITC-NLS-1 localization in nucleoli. D) Localization of FITC-NLS-1 in isolated nuclei with arrows pointing to nucleoli. E) GFP-NumA1 (Myre and O'Day, 2002). F) The same GFP-NumA1-expressing cell colocalized with Hoechst 33258 nuclear stain (Myre and O'Day, 2002). G,H) Dictyostelium amoebae were incubated with FITC-NLS-1 (G) or treated with 0.05 mg/mL AM-D (H) for 4 h prior to incubation with FITC-NLS-1. AM-D treatment resulted in smaller intranuclear patches (arrows). Again, all FITC peptides showed the same localization with or without AM-D treatment. I) FITC-DEED-1, -2, -NLS-DEED-1, or -NLS-DEED-2 was electroporated into Dictyostelium amoebae and localization was observed. All peptides colocalized with Hoechst 33258 nuclear stain in the nucleoplasm. FITC-DEED-1 localization is shown; however, the same localization was observed for all DEED peptides. Either a Zeiss x63 oil-immersion objective lens with an aperture of 1.4 (C) or a Nikon x100 oil-immersion objective lens with an aperture of 1.30 (all others) was used. At least four independent replicates were performed, all showing the same result.
these patches, confirming that they are nucleoli (Fig. 2.4G,H). The incubation of isolated nuclei with these peptides resulted in the same pattern of localization (Fig. 2.4D). None of the FITC-DEED peptides entered any of the cells and were therefore electroporated into the cells. The cells were viewed immediately after a 10 min recovery period on ice following electroporation. Electroporated FITC-DEED-1, -2, FITC-NLS-DEED-1, and -2 all localized to the nucleoplasm, but not nucleoli (Fig. 2.4I).

To identify critical residues within the NLSs, variants were synthesized with alanine substitutions at specific positions (Table 2.2). All variants of FITC-NLS-1 and -4 displayed the same localization as the original peptide. In order to rule out the possibility that nucleolar localization was due to a functional effect of the peptide on the cell, the cells were treated with each of the FITC-NLS peptides and subsequently monitored. As expected, there was no effect on migration rate, aggregation size, fruiting body development, or spore viability (data not shown).

2.4.3 FITC-NLS peptides interact with highly acidic motifs (DEED-1 and -2)

Since acidic motifs from other nucleolar proteins have previously been shown to interact with basic NLSs, we investigated the possibility of such an interaction between the peptides in this study. When cells were incubated with both an FITC-NLS peptide and an FITC-DEED peptide, the FITC-NLS peptide did not enter any cells. This was observed for all combinations of either FITC-NLS-1, -2, -3, or -4 with either FITC-DEED-1, -2, FITC-NLS-DEED-1, or -2. This effect was specific for the FITC-DEED peptides as the presence of IP22-3, a peptide that does not enter cells, did not inhibit any of the FITC-NLS peptides from entering cells and localizing to nucleoli (data not shown).
2.4.4 Localization of NumA1 changes during mitosis

Since nucleolar proteins change locations during mitosis, we investigated the localization of NumA1 from prometaphase through telophase. During prometaphase, NumA1 was detected by anti-NumA1 in the centrosomal region as well as in discrete patches adjacent to the inner nuclear membrane however these patches were smaller than nucleoli (Fig. 2.5). During metaphase, these patches were no longer visible but NumA1 was instead localized diffusely at the nuclear envelope region and the spindle fibers, and during anaphase and telophase NumA1 was observed at the nuclear envelope region and the centrosomal region (Fig. 2.5). Only during telophase NumA1 reappeared in nucleoli (Fig. 2.5).

2.5 Discussion

GFP-NumA1 has previously been shown to localize to discrete intra-nuclear patches adjacent to the nuclear envelope and to a lesser extent the nucleoplasm (Myre and O’Day, 2002). This localization is dependent on N-terminal residues 1-120 which contain a bipartite NLS, NLS-2 (Myre and O’Day, 2005). A number of previous studies in Dictyostelium have provided detailed evidence that these patches are in fact nucleoli (Benichou et al., 1983; Kawashima et al., 1979; Maeda and Takeuchi, 1969). Here, we have shown that polyclonal antibodies raised against NumA1 residues 1-120 (anti-NumA1) detect NumA1 in discrete intra-nuclear patches and to a lesser extent the nucleoplasm, the same pattern as observed for GFP-NumA1. These patches were shown to be nucleoli by their diminished size after rRNA transcription inhibition. These findings reveal that NumA1 is a bona fide nucleolar protein which fits well with the proposed role of NumA1 as a regulator of cell cycle since many cell cycle proteins are known to reside in the nucleolus (Myre and O’Day, 2002; Visintin and Amon, 2000). This pattern of localization is a common feature of nucleolar proteins, as NPM1, adenosine deaminases that act on RNA, and
Figure 2.5. Localization of NumA1 in the nucleus throughout mitosis. Immunolocalization using anti-NumA1 and anti-α-tubulin showed the redistribution of NumA1 to the nuclear envelope region and the centrosomal region during prometaphase to telophase. NumA1 was also localized to the spindle fibers during metaphase, anaphase, and telophase, and reappeared in nucleoli only in telophase. Arrows indicate the nuclear envelope region (N), the centrosomal region (C), the spindle fibers (S), and nucleoli (No). The localization of α-tubulin was used to determine the stage of mitosis. A Nikon x100 oil-immersion objective lens with an aperture of 1.30 was used. At least four independent replicates were performed, all showing the same result. Scale bar represents 2 µm.

murine double minute 2 have all been shown to shuttle between the nucleolus and nucleoplasm (Lim and Wang, 2006; Sansam et al., 2003; Tao and Levine, 1999). NumA1 represents the fourth nucleolar protein identified in *Dictyostelium*. The localization of NumA1 is Ca\(^{2+}\)- and CaM-independent, as demonstrated previously by deletion construct analysis as well as in the present study via the use of Ca\(^{2+}\) chelators and CaM antagonists (Myre and O’Day, 2005). Nonetheless, NumA1 represents the only nucleolar CaMBP discovered in *Dictyostelium*. The nucleolar localization of a handful of CaMBPs from other organisms such as calcineurin, CaM kinase II, and myosin light chain kinase suggest a role for CaM in the nucleolus (Guerriero et al., 1981; Ohta et al., 1990; Torgan and Daniels, 2006). Fittingly, CaM has also been shown to bind and localize to mammalian nucleoli (Thorogate and
Torok, 2007; Wong et al., 1991). Taken together, our results further support a potential role for CaM in the nucleolus which may be to regulate the binding of NumA1 to other proteins such as CBP4a (Myre and O’Day, 2004a).

The localization of the FITC-NLS peptides to nucleoli demonstrates that these sequences act as both NLSs and NoLSs and are thus responsible for the nucleolar localization of NumA1. Like NoLSs in other organisms, they are also rich in basic residues (Sirri et al., 2008). Consistent with the NumA1 localization experiments, neither Ca$^{2+}$ nor CaM was required for proper localization of the NLS/NoLS peptides. This appears to be the first precise delineation of both a NoLS and a NLS/NoLS in this eukaryote model and is in keeping with results from studies on mammalian cells where certain NLSs also function as NoLSs (Wang et al., 2005). The nucleoplasmic localization of these NLS/NoLS peptides is thought to be due to nuclear retention via a nuclear binding protein rather than active transport across the nuclear pore complex since isolated nuclei displayed the same localization despite the lack of cytoplasm which would contain nuclear pore complex transporters such as importin. However, the nucleoplasmic localization of NumA1 may also be mediated by the DEED domain, since all FITC-DEED peptides localized to the nucleoplasm after electroporation.

The inability of the FITC-NLS peptides to enter cells in the presence of FITC-DEED peptides suggests an interaction between the two, and NumA1 is thus thought to localize to nucleoli by binding to highly acidic motifs via its NLS/NoLSs. Fittingly, highly acidic motifs are a common feature of nucleolar proteins, a number of which have previously been shown to interact with NLS peptides (Xue and Melese, 1994). The interaction between nucleolar proteins and their targets is specific and of high affinity (Birbach et al., 2004; Misteli, 2001).
Finally, NumA1 has been shown to redistribute to non-nucleolar locations during mitosis such as the nuclear envelope region, the spindle fibers, and the centrosomal region. Several nucleolar proteins in other organisms such as the multifunctional NPM1, nucleolin, and the nucleolar RNA methyltransferase Misu also redistribute during mitosis (Cha et al., 2004; Gas et al., 1985; Hussain et al., 2009; Yun et al., 2008). The loss of nucleolar proteins during nucleolar dissolution at prophase and their reaccumulation during nucleolar reformation at telophase is a common event. This further confirms that NumA1 is a nucleolar protein and suggests a possible role for NumA1 during mitosis. Prior to this study, the nucleolar protein dynamics had not been determined during mitosis in *Dictyostelium*.

Apart from the insight gained into the function and pathways of NumA1, the FITC-NLS/NoLS peptides used in this study may also be of biomedical value. Increasingly, drugs are being conjugated to peptides in order to target their delivery to specific subcellular locales, resulting in an increase in drug efficacy and a decrease in adverse side effects (Arap et al., 1998; Schally and Nagy, 1999). Several nuclear and nucleolar proteins have recently been suggested to be promising targets for anti-cancer drugs (Mijatovic et al., 2008; Yao and Yang, 2005). This is due in part to the role of the nucleolus in cancer as well as the morphological differences between nucleoli in healthy and cancerous cells which act as a visual marker (Maggi and Weber, 2006; Montanaro et al., 2008). However, although successful in *Dictyostelium*, further experiments are needed to determine the capability of these peptides to target drugs to human nucleoli.
2.6 References


Chapter 3
Ca\(^{2+}\)-dependent nucleolar localization of *Dictyostelium* CBP4a during interphase and redistribution throughout mitosis

3.1 Abstract

CBP4a (alternatively CbpD1) was previously shown to be a Ca\(^{2+}\)-dependent binding partner of NumA1, a nuclear and nucleolar CaMBP linked to the cell cycle in *Dictyostelium*. This study shows that CBP4a localizes specifically to the nucleolus in a Ca\(^{2+}\)-dependent manner as shown by treatment with the Ca\(^{2+}\) chelator BAPTA. Addition of the rDNA transcription inhibitor AM-D to cells caused a loss of nucleolar CBP4a with the protein moving to the centrosomal region and discrete cytoplasmic particles rather than the nucleoplasm. CBP4a residues \(^{40}\text{KKCK}^{43}\) represent a functional NLS which targets CBP4a to the nucleus. CBP4a moves outside the nucleolus during prometaphase of mitosis forming islands distributed throughout the nucleoplasm and then also localizes to the metaphase plate during metaphase. These unique protein islands represent novel intranuclear subdomains associated with mitosis that have not been previously identified in *Dictyostelium*. Large islands are present at the nucleoplasmic periphery during telophase reminiscent of the start of nucleolar reformation. Consistent with literature regarding the relationship between the centrosome and nucleolar structure and function, nocodazole treatment caused a loss of nucleolar CBP4a which redistributed to islands at the nucleoplasmic periphery. CBP4a thus represents the fifth nucleolar protein verified to date in *Dictyostelium*. In keeping with NumA1 as a proposed cell cycle protein, CBP4a possesses a putative forkhead-associated domain present in several nuclear proteins involved in the cell cycle. The NLS is suggested to be the NumA1-binding domain the interaction between CBP4a and NumA1 in relation to their possible role in the regulation of cell cycle events is discussed. These results provide unique
insight into nucleolar protein dynamics and their regulation in Dictyostelium suggesting this eukaryote could serve as a model for studying nucleolar structure and function.
3.2 Introduction

Nucleomorphin isoform NumA1 is a nucleolar/nucleoplasmic CaMBP linked to the cell cycle in *Dictyostelium* (Chapter 2; Catalano and O’Day, 2008; Myre and O’Day, 2002; 2004). Deletion of an extensive glu/asp domain, common to many nucleolar proteins, leads to an increase in multinuclearity (Chapter 2; Myre and O’Day, 2002). Yeast two hybrid and coimmunoprecipitation studies, have shown that CBP4a (alternatively CbpD1) binds to NumA1 in a Ca$^{2+}$-dependent manner, an interaction dependent on the NumA1 glu/asp domain (Myre and O’Day, 2004). *Dictyostelium* possesses 13 CBPs including CaM, the major Ca$^{2+}$ effector in all eukaryotes, all of which possess EF-hand Ca$^{2+}$-binding motifs that interact with Ca$^{2+}$ ions (Dharamsi et al., 2000; Dorywalska et al., 2000; Sakamoto et al., 2003; Zhou et al., 2009). CBP4a has been shown to interact with CBP1, a CBP that interacts with the actin cytoskeleton and is involved in cell aggregation (Dharamsi et al., 2000; Dorywalska et al., 2000). With the exception of CaM, calfumirin, and CBP3 the function of the other CBPs is unknown (Catalano and O’Day, 2008; Itoh et al., 1998; Lee et al., 2005).

The nucleolus is a multifunctional structure that is the site of ribosomal subunit biogenesis but also houses cell cycle proteins (Peterson et al., 2009; Robertson, 2007; Visintin et al., 2000). At present, NumA1 is one of only a handful of nucleolar proteins identified in *Dictyostelium* that includes Hsp32, TRAP1, and eIF6 (Chapter 2; Balbo and Bozzaro, 2006; Moerman and Klein, 1998; Yamaguchi et al., 2005). The identification of CBP4a as a nucleolar protein would suggest that it too could function in the regulation of cell cycle events in *Dictyostelium* via nucleolar interactions with NumA1.

Here we show that CBP4a localizes to the nucleoli in a Ca$^{2+}$-dependent manner and redistributes to islands within the nucleoplasm during mitosis. To our knowledge, CBP4a represents the fifth
verified nucleolar protein and only the second to be studied during mitosis. Its unique pattern of localization also indicates there are previously unidentified intranuclear subdomains linked to mitosis in *Dictyostelium.*

### 3.3 Materials and methods

#### 3.3.1 Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich®. 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. All restriction enzymes were purchased from New England BioLabs® Inc. *Dictyostelium* AX3 cells were used for all experiments. Cells were maintained in HL-5 at 21°C shaking at 180 rpm as previously described (Fey et al., 2007).

#### 3.3.2 Polyclonal antibody production

A peptide equivalent to residues $^{20}$KAGDKNGDNQLSKKE$^{34}$ from CBP4a was synthesized using solid phase synthesis (with the addition of an N-terminal cysteine required for subsequent conjugation), purified via high-performance liquid chromatography (HPLC), and verified via mass spectroscopy prior to keyhole limpet hemocyanin (KLH) conjugation via a m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester (MBS) linker (Advanced SynTech Inc.). Conjugation was performed as previously described (Lateef et al., 2007). Polyclonal antibodies were produced in New Zealand White rabbits against this KLH-conjugated peptide according to the standard operating procedures of the University of Toronto. Rabbits were injected with 750 μg of KLH-conjugated peptide in 500 μL PBS mixed with 500 μL Freund’s Complete Adjuvant. Three subsequent boost injections 2-3 weeks apart were performed in the same manner each consisting of 250 μg of KLH-conjugated peptide in 500 μL PBS mixed with 500 μL Freund’s
Incomplete Adjuvant. Western blots performed with serum from blood samples collected prior to exsanguination confirmed the presence of an antibody which detected a protein of the correct molecular weight (O’Day et al., 2009). Exsanguination was performed by the department of Faculty of Medicine at the University of Toronto and the serum was collected. Crude serum was used for immunolocalization experiments and referred to as anti-CBP4a.

3.3.3 Modification of pDM-323 vector

The vectors pDM-317 and pDM-323 (dictyBase.org) were used to generate an N- and C-terminal GFP-fusion protein respectively (Veltman et al., 2009). The cut sites for insertion of a cDNA fragment are BglII and SpeI (for both vectors) however the CBP4a cDNA sequence also contains a SpeI site. To circumvent this problem the vectors were digested with BglII and SpeI and then gel extracted. A small fragment was produced composed of a BglII, SacI, and SpeI site, by annealing two primers, 5’-GATCTGAGCTCA-3’ and 5’-CTAGTGAGCTCA-3’. The primers were dissolved in TE-NaCl (10 mM Tris, pH 7.5, 1 mM ethylenediaminetetraacetic acid [EDTA], 50 mM NaCl) and incubated at 95 °C for 5 min in a water bath, after which the temperature was allowed to slowly decrease to room temperature over the next few hours. The digested vector and annealed primers were then ligated (DNA ligation kit) and competent *Escherichia coli* DH5α cells (200 μL) were transformed with 2 μL of ligation product as previously described (Kimmel et al., 2006). The transformed cells were spread onto LB agar plates supplemented with 100 μg/mL ampicillin overnight at 37 °C. The following day visible colonies were isolated and grown to stationary phase in LB broth supplemented with 100 μg/mL ampicillin and the plasmid was extracted from the culture. An aliquot was digested with SacI and the product analyzed on an agarose gel in order to confirm successful insertion of the SacI site between the BglII and SpeI sites. These modified vectors will now be referred to as either pDM-317-SacI or pDM-323-SacI.
3.3.4 Generation of CBP4a and CBP4a-ΔKKCK cDNA fragment

Total RNA was isolated from vegetative *Dictyostelium AX3* cells as previously described (Pilcher et al., 2007). AccuScript PfuUltra II RT-PCR kit (Stratagene®) was used to synthesize cDNA from the RNA templates using oligo (dT) primers (10 μL total volume, 65 °C for 5 min, then 42 °C for 90 min). PCR primers were then designed to amplify the full length CBP4a cDNA without the stop codon. The forward and reverse primers included either a BglIII (underlined) and kozak site or a SacI (underlined) restriction site respectively as well as two extra bases before the restriction site (forward: 5’-CAAGATCTAAAATGGTTACTAAAAAGGAA-3’; reverse: 5’-CAGAGCTCTTCAGTTGGATAAATTTCATAATA-3’). PCR was performed with the same kit using 1 μL of cDNA product as a template and resulted in the amplification of a ~490 bp fragment (2 min at 95 °C, then 43 cycles of 30 sec at 95 °C, 30 sec at 55 °C, and 30 sec at 68 °C, followed by a final 5 min at 68 °C). The PCR product was gel extracted, digested with both BglIII and SacI, and PCR purified. This fragment was then used for the generation of the full length GFP-fusion proteins.

Alternatively, primers were designed to amplify the regions before and after residues 40KKCK43 generating two fragments that were processed in the same manner (region before: forward: 5’-CAAGATCTAAAATGGTTACTAAAAAGGAA -3’; reverse: 5’-ACCACCGAACAATTCATAAAGTTC -3’, region after: forward: 5’-GGTGGTTACCCAAATTTCCAT -3’; reverse: 5’-CAGAGCTCTTCAGTTGGATAAATTTCATAATA -3’). The bases coding for residues 40KK41 (region before) and 42CK44 (region after) were changed to code for 40GG41 and 42GG43 respectively. After gel extraction, digestion, and PCR purification, these fragments were ligated (blunt end ligation) and the ligation product amplified via PCR using the same primers used to generate the full length CBP4a fragment. The product was again gel extracted, digested, and
PCR purified, and is referred to as CBP4a-ΔKKCK, since $^{40}\text{KKCK}^{43}$ has been changed to $^{40}\text{GGGG}^{43}$.

### 3.3.5 Generation of GFP-fusion protein expressing cell lines

The vectors pDM-317-Sacl and pDM-323-Sacl were digested with BglII and SacI and the products were gel extracted and each ligated with digested CBP4a cDNA product (to produce N-GFP-CBP4a and C-GFP-CBP4a respectively). Alternatively, digested/extracted pDM-323-Sacl was ligated with CBP4a-ΔKKCK (to produce GFP-CBP4a-ΔKKCK which possesses a C-terminal GFP). Competent *E. coli* DH5α cells (200 μL) were transformed with 2 μL of ligation product, colonies were grown to saturation, and the plasmid was extracted (as described above). An aliquot of plasmid was digested with BglII and SacI and the liberation of the CBP4a DNA fragment was observed via analysis using agarose gel. The undigested plasmid was sequenced (Analytical Genetics Technology Center, Toronto) and concentrated to 1 μg/μL. Vegetative *Dictyostelium* AX3 cells were transformed by electroporation as previously described (0.85 kV, 25 μF, 1 ms, 2 pulses 5 sec apart; Gaudet et al., 2007). After 4 days colonies were transferred to HL-5 containing 10 μg/mL G418 within which they were maintained. Alternatively, cells were transformed with pDM-317 which when empty expresses GFP alone.

### 3.3.6 SDS-PAGE and western blotting

Cells (1-2 x 10⁶ cells/mL; either untransformed or expressing a GFP-fusion protein) in HL-5 were resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5 % NP-40, 1 complete protease inhibitor tablet; Roche Diagnostics) and sonicated. Protein levels were quantified using the Bradford assay. Total protein (0.1 μg for GFP alone or 20 μg for all other strains) was separated on a 12 % SDS-PAGE gel. Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Pall corp.). The membrane was blocked with 5 % non-fat milk in
washing buffer (TBS and 0.001 % Tween 20) at 4 °C overnight and subsequently probed with anti-GFP (Santa Cruz®) in 5 % non-fat milk in washing buffer (1:600) for 1 h at room temperature followed by probing with secondary anti-mouse HRP (1:600; Santa Cruz®) in the same conditions. Bands were detected with Amersham ECL Plus™ Western Blotting Detection kit (General Electric) using the STORM Scanner System.

### 3.3.7 FITC peptide localization

A peptide equivalent to residues KKCK from CBP4a was synthesized via solid phase synthesis, purified to 90 % via HPLC, N-terminally conjugated to FITC, and verified via mass spectrometry (Advanced SynTech Inc.). The conjugated peptide (FITC-KKCK) was electroporated (4 µL) with vegetative cells (100 µL of 5.0 x 10^7 cells/mL) as previously described (Chapter 2). After recovering on ice cells were transferred to 10 mL HL-5 in a Petri dish. Cells were resuspended in KK_2 phosphate buffer with 500 µM Hoechst 33258 nuclear stain and slide-mounted. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera and images were analyzed using NIS Elements BR 3.0.

### 3.3.8 Treatment, fixation with ultracold methanol, immunolocalization, and live viewing

GFP-expressing cells were viewed live after adhering to a coverslip for 30 min prior to slide-mounting. For fixation and immunolocalization experiments cells (150 µL of 2 x 10^6 cells/mL) were allowed to adhere to 0.1 mm circular glass coverslips (McCronet™) for 30 min in a humidity chamber. Alternatively, cells were treated while adhering to coverslips (in HL-5) with either 1, 5, or 10 mM CaCl_2 (4 h), 1, 5, or 10 mM EGTA (4 h), 5 mM BAPTA (4 h), 300 µM W-5 (4 h), 50 µM W-7 (4 h), 50 µM nocodazole (6 h), 0.5 mg/mL cycloheximide (1, 2, 4, or 8 h), or 0.05 mg/mL AM-D (4 h, 8 h, or 4 h followed by three washes with HL-5 prior to 4 h recovery in HL-5). Cells were fixed with ultracold methanol as previously described (Hagedorn et al., 2006).
After blocking, cells were incubated with either anti-CBP4a or anti-NumA1 for 60 min followed by Alexa Fluor® 555 goat anti-rabbit (Invitrogen™) for 45 min (Chapter 2). Cells were then incubated with anti-α-tubulin (Hybridoma Bank) for 60 min followed by Alexa Fluor® 488 goat anti-mouse (Invitrogen™) for 45 min. The pattern of microtubules was used to determine cell cycle stage. Prolong Antifade (Invitrogen™) containing DAPI (5 μL) was placed on a slide prior to mounting and coverslips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera and images were analyzed using NIS Elements BR 3.0.

3.4 Results

3.4.1 CBP4a possesses domains characteristic of a nuclear protein

Analysis of CBP4a via the Eukaryotic Linear Motif database revealed the presence of a forkhead-associated (FHA) domain, MVTKKEF, which recognizes phosphorylated threonine residues on the ligand protein (Fig. 3.1A; Puntervoll et al., 2003). CBP4a also contains a putative monopartite NLS, KKCK, and four EF-hand Ca²⁺-binding motifs (EF-hand motif; Fig. 3.1A; dictyBase.org). Of the 13 CBPs in Dictyostelium (including CBP4a) 10 contain at least one putative monopartite NLS, all of which overlap with the end third of an EF-hand motif, and 10 contain at least one putative FHA domain, four of which overlap different areas of an EF-hand motif (Fig. 3.1B; dictyBase.org). A model of CBP4a residues 14-40 was constructed using the SWISS-MODEL Workspace (Fig. 3.1C; Arnold et al., 2006; Guex and Peitsch, 1997; Schwede et al., 2003). The putative NLSs identified always occur on one of the helices (each helix occupies approximately one third of the motif) which flank the central Ca²⁺-binding loop (Zhou et al., 2009).
Figure 3.1. Domain structure diagram of CBP4a and related CBPs. A) CBP4a possesses a putative FHA domain, four EF-hand Ca\(^{2+}\)-binding motifs, and a putative NLS. The antigen site is also indicated. B) All of the 13 CBPs found in Dictyostelium contain EF-hand motifs (dark grey), and 10 contain both putative NLSs (black) and putative FHA domains (white). All putative NLSs overlap with the end third of an EF-hand motif while only some putative FHA domains overlap with different regions of an EF-hand motif. Summary column on right (Sum.) shows NLSs (black) and/or FHA domains (white) overlapping with EF-hand motifs (dark grey). Number markers indicate amino acid position. C) SWISS-MODEL of CBP4a residues 14-40 (three different 3D views). This model represents the structure of an EF-hand motif containing two helical domains flanking a central Ca\(^{2+}\)-binding loop (Zhou et al., 2009). Putative NLSs were always found in the helical domains.

3.4.2 All GFP-fusion proteins were properly expressed except GFP-CBP4a-ΔKKCK

Cell lines were produced expressing either GFP alone, N-GFP-CBP4a, C-GFP-CBP4a, or GFPCBP4a-ΔKKCK (Fig. 3.2A). Western blots of lysates from untransformed cells or cells expressing either GFP, N-GFP-CBP4a, C-GFP-CBP4a, or GFP-CBP4a-ΔKKCK were probed with anti-GFP. GFP alone and N-GFP-CBP4a were detected at the proper size (~29 and 45 kDa respectively) however GFP-CBP4a-ΔKKCK was not detected (Fig. 3.2B). C-GFP-CBP4a was
also detected at the correct size however a higher, stronger band (~49 kDa) and a lower band (~22 kDa) were also detected (Fig. 3.2B).

3.4.3 CBP4a localizes Ca\(^{2+}\)-dependently to nucleoli

To determine if a protein is nucleolar, cells can be treated with AM-D, an rDNA transcription inhibitor that results in the disappearance of nucleoli. In *Dictyostelium* AM-D leads to the disruption of the nucleolus as well as the loss of eIF6 and NumA1 from nucleoli (Chapter 2; Balbo and Bozzaro, 2006; Kawashima et al., 1979). In this organism, nucleoli exist as 2-3 distinct patches on the inner side of the nuclear envelope (Maclean et al., 1984; Sameshima et al., 1991). CBP4a was detected in such discrete intranuclear patches (Fig. 3.3B). In contrast, cells probed with either the secondary antibody alone or with the pre-immune serum in place of anti-CBP4a displayed either no detectable fluorescence or a diffuse pattern of fluorescence throughout the entire cell (with slightly more intensity in the nucleus) respectively (Fig. 3.3A).
Figure 3.3. Effect of AM-D on localization of CBP4a.

A) Cells were probed with either the secondary antibody alone (no primary) or with the preimmune serum followed by the secondary antibody. B) CBP4a localized to intranuclear patches in untreated cells (0 h) that diminished in size and ultimately disappeared after treatment with AM-D for 4h and 8h respectively. Four hour treatment also resulted in the detection of CBP4a in the centrosomal region (C) while 8 h treatment also resulted in the presence of cytoplasmic particles (CP). Treatment for 4 h followed by a 4 h recovery period (R) resulted in only a partial restoration of nucleoplasmic CBP4a. Immunolocalization of α-tubulin was performed to verify cellular integrity and DAPI stain was used to identify the nucleus. Nucleoli (No) and cytoplasmic particles (CP) are indicated with arrows. Merged image shows overlay of CBP4a (red), α-tubulin (green), and DAPI (blue). An enlarged view of the nucleus (bottom row) shows arrows denoting nucleoli, which were diminished after 4 h and absent after 8 h of treatment, or the centrosomal region (C), which contained CBP4a after 4 h of treatment. At least four independent replicates were performed, all of which showed the same result. Scale bar represents 2 μm.

After 4 h of treatment with AM-D (0.05 mg/mL) the size of these patches decreased dramatically completely disappearing after 8 h treatment, confirming that they are nucleoli (Fig. 3.3B).

During AM-D treatments, CBP4a did not concentrate in the nucleus after leaving the nucleolus but instead accumulated at the nuclear periphery and centrosomal region after 4 h and then in cytoplasmic particles after 8 h (Fig. 3.3B). Even after 4 h of recovery from 4 h of treatment,
CBP4a had not entirely moved back into nucleoli suggesting there may be some restriction of their movement after localizing to these particles.

Treatment with 5 mM BAPTA (4 h) also resulted in the disappearance of nucleolar CBP4a however it did not accumulate in the centrosomal region or in cytoplasmic particles (Fig. 3.4A,B). In contrast, BAPTA treatment did not affect the localization of NumA1, as documented previously (Fig. 3.4B; Chapter 2). No effect on the nucleolar localization of CBP4a was observed when cells were treated with either 1, 5, or 10 mM CaCl$_2$ (4 h) or the Ca$^{2+}$ chelator EGTA (1, 5, or 10 mM for 4 h).

Since NumA1 is a CaMBP it was possible that CaM could indirectly affect CBP4a localization through interactions with NumA1. However CaM antagonists (300 μM W-5 or 50 μM W-7 for 4 h) had no effect on localization. As well, 0.5 mg/mL of the translation inhibitor cycloheximide (1, 2, 4, or 8 h) also had no effect (data not shown). In contrast, nocodazole (50 μM), which disrupts the microtubular cytoskeleton, also lead to a loss of nucleolar CBP4a but did not result in centrosomal or cytoplasmic localization (Fig. 3.4C). Instead CBP4a was present in islands around the periphery of the nucleus. These islands were much smaller than nucleoli.

### 3.4.4 GFP-CBP4a localizes to nucleoplasm and nucleoli

In living cells N-GFP-CBP4a localized throughout the entire cell. However when cells were fixed it was observed more in the nucleoplasm than the cytoplasm, while even less was detected in nucleoli (Fig. 3.5A). C-GFP-CBP4a localized predominantly in the nucleoplasm but also throughout the cytoplasm. When cells were fixed, C-GFP-CBP4a localized predominantly in nucleoli with a lesser amount detected in the nucleoplasm (Fig. 3.5A). GFP alone was detected throughout the entire cell in both live and fixed cells.
3.4.5 Residues KKCK represent a true NLS

GFP-CBP4a-ΔKKCK was not detected in either live or fixed cells, in accordance with western blot experiments that also did not detect the protein (Fig. 3.5A). Electroporated FITC-KKCK peptide localized predominantly in the nucleus but was also present throughout the entire cell (Fig. 3.5B).

3.4.6 CBP4a redistributes during mitosis

Nucleolar proteins redistribute to non-nucleolar locales during mitosis due to the nucleolar dissolution that occurs during this time (Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). During prometaphase CBP4a was not localized to nucleoli, but instead present in islands...
within the nucleoplasm (Fig. 3.6). During metaphase the islands were also present as well as at least two larger islands at the metaphase plate region. During anaphase only the nucleoplasmic islands remained. Finally, during telophase large islands were present along the inner nuclear membrane reminiscent of reforming nucleoli (Fig. 3.6). A diagrammatic representation of the localization of both CBP4a and NumA1 during nuclear division (karyokinesis) is presented in Figure 3.7.

### 3.5 Discussion

CBP4a is one of 13 CBPs in *Dictyostelium* and has been shown to bind NumA1, a protein linked to the cell cycle, in a Ca\(^{2+}\)-dependent manner (Myre and O’Day, 2002; 2004; Sakamoto et al.,
The recent finding that NumA1 is a nucleolar protein suggests that the same may be true for CBP4a (Chapter 2). This study has shown via immunolocalization that CBP4a localizes to discrete intra-nuclear patches adjacent to the nuclear membrane. This localization was also confirmed with N-GFP-CBP4a. Treatment with AM-D resulted in the disappearance of these patches confirming that they are nucleoli. Although EGTA had no effect, treatment with BAPTA (which chelates Ca\(^{2+}\) with more specificity than EGTA) also resulted in the disappearance of these patches demonstrating that this localization is Ca\(^{2+}\)-dependent. In contrast, BAPTA had no effect on the localization of NumA1, in accordance with previous results (Chapter 2). The unchanged localization observed when cells were treated with CaM antagonists demonstrates that the nucleolar localization of CBP4a is not CaM-dependent. The unaltered localization when
Figure 3.7. Patterns of CBP4a and its binding partner NumA1 during karyokinesis in Dictyostelium. During interphase CBP4 (red) and NumA1 (blue) colocalize in the nucleolus (red and blue stripes) while NumA1 also shows nucleoplasmic localization (light blue) to a lesser extent. During prometaphase, CBP4a reorganizes as nucleoplasmic islands distributed throughout the nucleoplasm while NumA1 remains at the nuclear periphery but in smaller patches. During metaphase the CBP4a islands localize around the periphery of the nucleoplasm as well as the metaphase plate while NumA1 localizes at the nuclear envelope region and along spindle fibers. This pattern remains constant during anaphase with the exception of the disappearance of the CBP4a islands at the metaphase plate region. During telophase the CBP4a islands begin to enlarge while NumA1 reappears in nucleoli and the nucleoplasm but is still present along the nuclear envelope region. It is not clear if the large CBP4a islands during telophase colocalize with NumA1 in reforming nucleoli (Chapter 2).

cells were treated with the translation inhibitor cycloheximide suggests that CBP4a is a relatively stable protein with a low turnover rate. Together, these results show that CBP4a is a Ca^{2+}-dependent nucleolar protein. They also suggest that CBP4a and NumA1 interact in the nucleolus not only in a Ca^{2+}-dependent manner as suggested by Myre and O’Day (Myre and O’Day, 2004) but also in a CaM-independent manner.
CBP4a and 4b evolved on a separate lineage from the majority of the other CBPs in *Dictyostelium* but previous data is insufficient to determine any potential differences in function or localization between them (Sakamoto et al., 2003). Although the region in CBP4b most similar to the antigen site in CBP4a is the same except for one residue (CBP4b contains A instead of G at position 26) analysis of mRNA expression reveals that in vegetative cells CBP4a mRNA is approximately 8.3-times more abundant that that of CBP4b meaning any detection of CBP4b by anti-CBP4a would be minimal (Parikh et al., 2010; Rot et al., 2009).

The GFP-fusion proteins showed different localization patterns depending on which end GFP was fused suggesting that the presence of GFP interferes with CBP4a localization. GFP is almost twice the size of CBP4a and therefore may sterically hinder interactions between CBP4a and any target proteins. The difference in N-GFP-CBP4a localization between live and fixed cells may be due to the over-expression of this fusion protein. In live cells once the nucleolus becomes saturated the remaining fusion protein may disperse throughout the nucleoplasm and to a lesser extent the cytoplasm. Upon methanol precipitation (fixation) any soluble N-GFP-CBP4a would be washed away, leaving only the nucleolar fraction to be detected. The larger and smaller bands detected by western blotting of C-GFP-CBP4a may be due to post-translational modification and breakdown products respectively suggesting that N-GFP-CBP4a is the more stable fusion protein.

It is interesting to note that several CBPs in *Dictyostelium* possess both putative NLSs and FHA domains suggesting that several of these proteins may function in the nucleus. Accordingly, NLS and FHA domains were found to be either both present or both absent. In all of these proteins the NLS overlaps with the end third of an EF-hand motif, a helical region not directly involved in Ca$^{2+}$-binding but which changes conformation upon Ca$^{2+}$-binding to the adjacent loop region.
(Zhou et al., 2009). This suggests that Ca$^{2+}$-binding may be involved in the regulation of NLSs. Because these motifs remain unverified and given the little information available on CBPs in *Dictyostelium* further studies are needed to verify this relationship.

Residues KKCK have been verified as a true NLS since FITC-KKCK localized preferentially to the nucleus. This NLS does not appear to localize CBP4a to the nucleolus. It also appears to be crucial for the stability of GFP-CBP4a since its deletion most likely results in the degradation of the fusion protein. A similar situation was encountered previously with the site-directed mutation of NumA1 residues $^{48}$KK$^{49}$ to $^{48}$EF$^{49}$ (Myre and O’Day, 2005). This change abolished the stability of the GFP-fusion protein but the mRNA was still detected (Myre and O’Day, 2005). Perhaps CBP4a residues $^{40}$KKCK$^{43}$ are critical for the proper folding of the protein, possibly interacting with acidic residues $^{133}$EDVDSDDD$^{140}$, as basic NLSs have previously been shown to interact with highly acidic sequences (Xue and Melese, 1994). In fact the functional NLS in NumA1 is unable to enter cells and localize to the nucleolus when in the presence of highly acidic peptides suggesting an interaction between the two (Chapter 2). Alternatively, cysteine residue 42 may be required to form a disulfide bond with one of two other cysteines in the protein, a post-translational modification necessary for the stability of the protein.

CBP4a and NumA1 represent, to our knowledge, the only nucleolar proteins for which protein dynamics during mitosis have been examined in *Dictyostelium* (Chapter 2). During mitosis, NumA1 distributes diffusely in the nucleoplasm as well as at the nuclear periphery, in the centrosomal region, and at the spindle fibers region. In contrast, its binding partner CBP4a redistributes to islands within the nucleoplasm during mitosis and at the metaphase plate region during metaphase. In other species, the redistribution from the nucleolus to non-nucleolar locales during mitosis is a common feature of nucleolar proteins (Kieffer-Kwon et al., 2004; Lam et al.,
2005; Ochs et al., 1983). The fact that NumA1 and CBP4a show identical nucleolar localization in interphase cells but discrete and different localizations during mitosis suggests that the Ca^{2+}-dependent binding is a nucleolar event. However both proteins only localize to the centrosomal region during nucleolar disruption from either mitosis (NumA1) or treatment with AM-D (CBP4a). The interaction between NumA1 and CBP4a requires the presence of the NumA1 acidic glu/asp domain which when deleted results in a dramatic increase in multinuclearity (Myre and O’Day, 2002; 2004). Similar acidic sequences from other nucleolar proteins have been shown to interact with NLSs suggesting that CBP4a may interact with the glu/asp domain in NumA1 via the putative NLS identified here (Durocher and Jackson, 2002).

NumA1 is thought to be involved in the regulation of cell cycle events suggesting that CBP4a may also have a similar function (Chapter 2; Myre and O’Day, 2002). Fittingly, CBP4a possesses a putative FHA domain which is usually found in proteins involved in the regulation of transcription, DNA damage repair, and cell cycle checkpoints (Durocher and Jackson, 2002). Moreover, the FHA domain is most prevalent among nuclear proteins (Durocher and Jackson, 2002). Another relevant result here was the disruption of nucleoli by nocodazole treatment. In Dictyostelium there is a relationship between the position of the nucleolus and the centrosome (Sameshima et al., 1991). During mitosis, NumA1 leaves the nucleolus to localize more with microtubules and the centrosome (Chapter 2). A diagrammatic representation of the nuclear and nucleolar dynamics of NumA1 and CBP4a during karyokinesis is presented in Figure 3.7. The distinct localization patterns of these proteins indicate there are intranuclear subdomains linked to mitosis that have not been previously identified in Dictyostelium. Further investigation into the relationship between CBP4a and NumA1 should yield further insight into the function of these two nucleolar proteins and their relationship to the aforementioned cell cycle events. Based on the unique pattern of CBP4a during mitosis, it appears that there are many subtle events
occurring that remain to be investigated such as the possibility of nucleolar subcompartments in *Dictyostelium*. Thus more can be learned about nucleolar structure and function in *Dictyostelium* by studying the behaviour of the currently known nucleolar proteins after pharmacological treatments and in various available mutant cell lines.
3.6 References


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Chapter 4

*Dictyostelium* PsaA is a nucleoplasmic NumA1-binding protein that relocates to the cytoplasm during mitosis

Yekaterina Poloz performed the colocalization of PsaA and NumA1 (Fig. 4.2F).

4.1 Abstract

NumA1 is a nucleolar/nucleoplasmic protein linked to cell cycle in *Dictyostelium*. It interacts with PsaA which in other organisms is a Zn\(^{2+}\)-metallopeptidase thought to be involved in cell cycle progression and is involved in several human diseases. To better understand the relationship between NumA1 and the cell cycle and to shed light on these biomedical issues *Dictyostelium* PsaA was investigated. This study has shown that *Dictyostelium* PsaA contains domains characteristic of the M1 family of Zn\(^{2+}\)-metallopeptidases: a GAMEN motif and a Zn\(^{2+}\)-binding domain. PsaA colocalizes with NumA1 in the nucleoplasm and is also present to a lesser extent in the cytoplasm. During mitosis PsaA redistributes mainly throughout the cytoplasm. It possesses a functional NLS necessary for nuclear entry. To our knowledge this is the first NLS identified in a Psa from any organism. Treatment with Ca\(^{2+}\) chelators or CaM antagonists indicate that neither Ca\(^{2+}\) nor CaM are involved in PsaA localization. Treatment with the aminopeptidase inhibitor Bestatin Methyl Ester (BME) resulted in decreased cell density after 48 h which was not due to cell death. BME-treated cultures contained fewer multinucleate cells however cell size was unaffected. These results show that BME-treated cells grow more slowly and undergo cell division less frequently. Together, these results support a role for PsaA in regulating the cell cycle. *Dictyostelium* PsaA demonstrates all of the attributes common to
eukaryotic Psa and displays the same localization suggesting it may serve as a model for studying Psa function in other organisms.
4.2 Introduction

NumA1 is a nucleolar/nucleoplasmic CaM-binding protein in *Dictyostelium*, one of only a handful of identified nucleolar proteins in this organism (Chapter 2; Catalano and O’Day, 2008; Myre and O’Day, 2002). Overexpression of GFP-NumA1 lacking its acidic glu/asp (DEED) domain leads to an increase in multinuclearity suggesting that NumA1 is involved in the regulation of mitosis (Myre and O’Day, 2002). Moreover, isoform NumA2 contains a BRCT domain which is found predominantly in proteins involved in the regulation of cell cycle checkpoints (Myre and O’Day, 2004b). Together this data suggests that NumA1 is involved in the regulation of cell cycle events. In order to better understand the involvement of NumA1 in the cell cycle its binding partners must be examined. Recently NumA1 has been shown to interact with CBP4a in a Ca$^{2+}$-dependent manner presumably in the nucleolus where the two proteins colocalize (Chapter 2; Myre and O’Day, 2004a). NumA1 has also been shown to interact with PsaA via yeast-two hybrid analysis (Myre, 2005). Although the relationship between NumA1 and CBP4a has recently been examined PsaA remains an uncharacterized protein in *Dictyostelium* (Chapter 2a).

In other organisms Psa is a well studied Zn$^{2+}$-metallopeptidase that is characterized by the presence of an exopeptidase GAMEN motif and a Zn$^{2+}$-binding domain (Constam et al., 1995). It localizes to both the nucleus and cytoplasm while associating with the spindle fibers during mitosis and is inhibited by both Bestatin Methyl Ester (BME) and puromycin, both of which are often used in assessing its presence in tissues (Constam et al., 1995; Hui et al., 1993; Taylor, 1993a). Psa is involved in proteolytic events that mediate processes such as embryogenesis, reproduction, proliferation, meiosis, and establishment of polarity (Brooks et al., 2003; Lee and Kim, 2009; Lyczak et al., 2006; Sanchez-Moran et al., 2004). It is also required for proper
spermatogenesis, copulatory behavior, and fertility in mice and is thought to regulate the cell cycle via cyclin degradation (Constam et al., 1995; Osada et al., 2001; Takahashi et al., 1989). Interestingly, other Zn$^{2+}$-metallopeptidases have also been shown to inhibit cell proliferation suggesting that these peptidases are involved in the regulation of cell cycle events (Takahashi et al., 1987; Takahashi et al., 1989).

Psa is also involved in several human diseases. For example it inhibits Tau-induced neurodegeneration and is thus involved in Alzheimer’s disease (Karsten et al., 2006). It also shows an unusual ability to bind glutamine (Q) peptides for poly-Q digestion and is therefore also involved in several poly-Q neurodegenerative diseases such as Huntington’s disease (Bhutani et al., 2007). In fact Psa is the major peptidase responsible for digesting poly-Q peptides released by proteasomes after protein degradation (Bhutani et al., 2007). For reasons unknown *Dictyostelium* tolerates surprisingly long poly-Q tracts which in humans would lead to disease (Loomis, 2006). Determining how this organism tolerates such tracts may be the key to solving this problem in humans. *Dictyostelium* is used for the study of several biomedical diseases such as bacterial and viral pathogens as well as several diseases affecting the central nervous system (Annesley and Fisher, 2009; Myre et al., 2011). The study of Psa in *Dictyostelium* would thus not only help us better understand cell cycle events and the relationship to NumA1 but may also shed light on these aforementioned biomedical issues.

This study has shown that PsaA colocalizes with NumA1 in the nucleoplasm and is also present to a lesser degree in the cytoplasm in growing cells. During mitosis it redistributes throughout the cytoplasm. PsaA possesses several conserved domains common to Psa from other species including a GAMEN motif, Zn$^{2+}$-binding domain, putative nuclear export signal (NESs), and at least one functional NLS that is necessary for nuclear entry. To our knowledge this is the first
NLS that has been verified in a Psa from any organism. BME treatment resulted in a decreased rate of both growth and frequency of cell division suggesting that PsaA (and perhaps PsaB) may be involved in these processes. The relationship between PsaA and NumA1 with respect to cell cycle events is discussed.

4.3 Materials and methods

4.3.1 Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich®. 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. All restriction enzymes were purchased from New England BioLabs® Inc. Dictyostelium AX3 cells were used for all experiments. Cells were maintained in HL-5 at 21 °C shaking at 180 rpm as previously described (Fey et al., 2007).

4.3.2 Generation of GFP vectors and cell lines

Vectors pDM-317 and pDM-323 (dictyBase.org) were used to generate constructs that would express GFP alone, Dictyostelium PsaA with an N-terminally-fused GFP (N-GFP-PsaA), or Dictyostelium PsaA with a C-terminally-fused GFP (C-GFP-PsaA; Veltman et al., 2009).

Fragments inserted into these vectors must be done using BglII and SpeI cut sites, however since PsaA contains multiple BglII restriction sites a SacI site was inserted into both vectors between the BglII and SpeI sites as previously described (Chapter 3). Full length PsaA was then amplified using primers CAGAGCTCAAAATGTGTAATATAATGATC (forward), and CAACTAGTTTTTTAATCCAATTTAATAATC (reverse) which incorporated a kozak site as well as the appropriate restriction sites (underlined) as previously described using an elongation cycle of 90 sec (Chapter 3). This fragment was incorporated into both pDM-317 and
pDM-323 vectors (with SacI and SpeI cut sites) and the resulting constructs were transformed in *Dictyostelium* as previously described (Chapter 3). Empty pDM-317 was also transformed in order to express GFP alone. After 4 days of selection colonies were transferred to HL-5 containing 10 µg/mL G418 within which they were maintained.

The deletion constructs (GFP-PsaA-ΔNLS-1 and -2) were generated in a similar manner, however only the regions before and after the deletion were amplified using primers

CAGAGCTCAAAATGTGTAATATAATGATC (forward) and
CAGGATCTTGGGTCAATGAG (reverse) for the region before NLS-1 (Frag-1),
CAGGATCTCAGATGGTCAAG (forward) and
CAACTAGTTTTTTTAATCCAAATTAAATG (reverse) for the region after NLS-1 (Frag-2), CAGAGCTCAAAATGTGTAATATAATGATC (forward) and
CAAGGCTTAGCCTCAGGCA (reverse) for the region before NLS-2 (Frag-3), and
CAAGGCTGACATCCAGAAGTC (forward) and
CAACTAGTTTTTTTAATCCAAATTAAATG (reverse) for the region after NLS-2 (Frag-4). Frag-1 was then ligated with Frag-2 and Frag-3 with -4 and the ligation products amplified with PCR using the same primers used to amplify full length PsaA. PCR products were extracted, digested, and PCR purified prior to incorporation into pDM-323 (with SacI and SpeI cut sites) as previously described (Chapter 3). GFP-PsaA-ΔNLS-1 and -2 thus possess a BamHI and HindIII site in place of NLS-1 and -2 respectively.

GFP-NLS-1 and -2 were made from sets of primers equivalent to NLS-1 and -2 with sticky-end SacI and SpeI restriction sites (CAAAATGAAGAAATTCAGA; NLS-1 forward,
CTAGTTCTGAATTCTTCTTTTGAGCT; NLS-1 reverse,
CAAAATGAGAAAGAGATTTA; NLS-2 forward,
CTAGTAAATCTCTTTCTCATTTTGAGCT; NLS-2 reverse). These primers were annealed (NLS-1 forward with NLS-1 reverse and NLS-2 forward with NLS-2 reverse) and incorporated into pDM-323 (with SacI and SpeI cut sites). These fusion proteins thus possess a C-terminal GFP.

4.3.3 Antibody production

A peptide equivalent to a unique C-terminal sequence from PsaA (816QQVEDFFKQ825; Fig. 4.1A) was synthesized using solid phase synthesis (with the addition of an N-terminal cysteine required for subsequent conjugation), purified via HPLC, and verified via mass spectroscopy prior to KLH conjugation via an MBS linker (Advanced Syntech Inc., Markham, Ontario, Canada). Conjugation was performed via the method described by Lateef et al., 2007. Polyclonal antibodies against this KLH-conjugated peptide were produced in New Zealand White rabbits at the University of Toronto at Mississauga Animal Care Facility according to University of Toronto protocols for antibody production and animal care as previously described (Chapter 3; O’Day et al., 2009). The preimmune serum (rabbit serum prior to any injections) was kept and used as a control for immunolocalization experiments. The specificity of the crude serum was verified as anti-PsaA and used for all western blots and immunolocalization experiments.

4.3.4 SDS-PAGE and western blotting

Cells (1-2 x 10⁶ cells/mL; either untransformed or expressing a GFP-fusion protein) were resuspended in lysis buffer and sonicated as previously described (Chapter 3). 20 mg (or 0.1 mg for cells expressing GFP alone) total protein was separated on a 12 % SDS-PAGE gel, transferred to a PVDF membrane, blocked, and probed with either anti-PsaA (1:200) or anti-GFP (1:600; Santa Cruz®) followed by either anti-rabbit HRP (1:800; Santa Cruz®; for detection of anti-PsaA) or anti-mouse HRP (1:800; Santa Cruz®; for detection of anti-GFP) as previously
described (Chapter 3). Alternatively, anti-PsaA was incubated overnight at 4 °C with the antigen peptide prior to probing (blocking experiments). Bands were detected with Amersham ECL Plus™ Western Blotting Detection kit (General Electric) using the STORM Scanner System.

4.3.5 Live cell viewing, treatment, fixation, and immunolocalization

GFP-expressing cells were viewed live after adhering to a coverslip for 30 min prior to slide-mounting. Just before mounting Hoechst 33258 nuclear stain was added to some cells for colocalization experiments. For fixation and immunolocalization experiments cells (150 μL of 2 x 10^6 cells/mL) were allowed to adhere to 13 x 0.1 mm circular glass coverslips (McCrone™) for 30 min in a humidity chamber. Alternatively, cells were treated while adhering to coverslips (in HL-5) with either 1, 5, or 10 mM CaCl₂ (4 h), 1, 5, or 10 mM EGTA (4 h), 1 or 5 mM BAPTA (4 h), 300 μM W-5 (4 h), 50 μM W-7 (4 h), 0.5 mg/mL cycloheximide (1, 2, 4, or 8 h), or 0.05 mg/mL AM-D (4 h, 8 h, or 4 h followed by three washes with HL-5 prior to 4 h recovery in HL-5). Cells were fixed with ultracold methanol as previously described (Hagedorn et al., 2006).

After blocking (PBS with 0.2 % gelatin and 0.1 % Triton X100), cells were incubated (60 min) with anti-PsaA (1:20) or anti-GFP (1:500; GFP-PsaA-ΔNLS-1, -2, NLS-1, or -2; Santa Cruz®) followed by either Alexa Fluor® 555 goat anti-rabbit (1:40; Invitrogen™) or Alexa Fluor® 488 goat-anti mouse (1:100; Invitrogen™) respectively for 45 min in blocking buffer. Untransformed cells were then incubated (60 min) with anti-α-tubulin (1:100; Hybridoma Bank) followed by Alexa Fluor® 488 goat anti-mouse (1:100; 45 min). C-GFP-PsaA-expressing cells (mitosis experiments) were probed only with anti-α-tubulin followed by Alexa Fluor® 555 goat anti-mouse. For detection of GFP-fusion protein by anti-Psa, cells expressing either GFP, N-, or C-GFP-PsaA were probed with anti-PsaA followed by Alexa Fluor® 555 goat anti-rabbit. For colocalization of PsaA and NumA1 C-GFP-PsaA-expressing cells were probed with anti-NumA1 (1:40) followed by Alexa Fluor® 555 goat anti-rabbit (1:40). Anti-NumA1 has been used
previously (Chapter 2; O’Day et al., 2009). The localization of α-tubulin was used to determine the stage of cell cycle and cell integrity. 5 μL Prolong Antifade (Invitrogen™) containing DAPI (5 μL) was placed on a slide prior to mounting and coverslips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera and images were analyzed (ex. intensity measurements) using Nikon Imaging Software Elements Basic Research 3.0. The Student’s t-test (α = 0.05) was used to calculate significance for all graphs. Significant differences from the control are indicated with an asterisk.

4.3.6 Effects of BME on growth and development

Growing, vegetative cells (1-2 x 10^6 cells/mL) were diluted to 1.0 x 10^3 cells/mL and transferred (3 mL) into a well in a 12-well multi-well plate (2.5 cm diameter/well). Cells were treated with either 0, 10, 50, 100, 300, or 600 μM BME (Calbiochem®) and allowed to grow at 21 °C shaking at 180 rpm for 48 h. A haemocytometer was used to measure cell density after 0, 24, and 48 h. Each culture was measured three times at each time point and the average cell density per culture was calculated and considered a single replicate. At least three replicates were performed for each concentration. BME-treated cells were also stained with both fluorescein diacetate (FDA) and propidium iodide (PI), or fixed and probed as described above with either anti-α-tubulin (to determine if BME-treated cells underwent mitosis), anti-PsaA and anti-α-tubulin (in order to determine the effect of BME on PsA localization), or anti-NumA1 (to determine the effect of BME on nucleolar size), prior to viewing. As well, C-GFP-PsaA-expressing cells that were treated with BME were fixed and probed with anti-α-tubulin prior to viewing. Nikon Imaging Software Elements Basic Research 3.0 was used to measure the area of either the entire cell, the nucleus, or the nucleoli of BME-treated cells. The Student’s t-test (α = 0.05) was used to
calculate significance for all graphs. Significant differences from the control are indicated with an asterisk. Standard deviation bars are shown on all graphs.

4.4 Results

4.4.1 Protein domains of PsaA

A BLAST search revealed Dictyostelium PsaA (dictyBase.org) is highly similar to Drosophila, mouse, and human Psa with an E-value of $2e^{-178}$, $e^{-165}$, and $2e^{-163}$ respectively and a ClustalW2 alignment shows the N-terminal to be most highly conserved. Within this region lies the highly conserved GAMEN motif, characteristic of the M1 family of metallopeptidases. Dictyostelium PsaA possesses this motif ($^{77}$GAMEN$^{81}$) as do the other Psas (Fig. 4.1A,B). Of the known Zn$^{2+}$-metallopeptidases, about half also contain the motif HExxHx$_{18}$E, where x represents any amino acid, which forms part of the Zn$^{2+}$-binding domain as determined through crystallographic studies (Constam et al., 1995; Rawlings and Barrett, 1995). This motif is relatively common, but can be more stringently defined for Zn$^{2+}$-metallopeptidases as abxHEbbHbc, where 'a' is most often valine or threonine, 'b' is an uncharged residue, and 'c' a hydrophobic residue (Rawlings and Barrett, 1995). A sequence comparison of this Zn$^{2+}$-binding domain between Dictyostelium PsaA and Psa from Drosophila, mouse, and human is shown in figure 4.1B. Dictyostelium PsaA contains the sequence $^{309}$VIGHELAHQW(x$_{16}$)E$^{335}$ which conforms to both the HExxHx$_{18}$E and the more stringent abXHEbbHbc motif.

PsaA also contains a WW domain ($^{332}$WLNEGFMGYLVTYDLYPKW$^{353}$) which potentially could interact with NumA1 via a complementary WW-interacting motif (WWIM).

Scanning of the NumA1 sequence revealed three complementary class IV WWIMs ($^{27}$EGTTPK$^{32}$, $^{242}$IPLSPT$^{247}$, and $^{292}$ILESPS$^{297}$) two of which exist within residues 161-340, the region required for PsaA-interaction (Myre, 2005).
Figure 4.1. Domain structure diagram and alignment of PsaA with Psa from other organisms. 

A) Domain structure diagram and alignment of PsaA with Psa from Drosophila, mouse, and human. Amino acid length is indicated (aa) as well as GAMEN motifs, Zn$^{2+}$-binding domains, NLSs, NESs, the antigen site for PsaA, and the WW domain (dictyBase.org). Sequences have been shifted and gaps inserted (grey line within PsaA and Psa from Drosophila) in order to align the Zn$^{2+}$-binding domain and corresponding NLSs in the other proteins respectively. The N-terminal is the most conserved region as indicated.

B) Alignment of the GAMEN motif, Zn$^{2+}$-binding domain, NES, and NLS from these different organisms.

C) Diagram of all GFP-fusion proteins used in this study.
Like Psa from other organisms, *Dictyostelium* PsaA possesses multiple putative NLSs, 481KKFR484 (NLS-1), and 680RKRF683 (NLS-2; Fig. 4.1A,B). Human, mouse, and *Drosophila* Psa possess the sequence 726RRRF729, 727RRRF730, or 682RFRR685 respectively at a similar position to that of NLS-2 (Fig. 4.1A,B). *Dictyostelium* PsaA also possesses two putative NESs, 10LVEKVDRLVL19 and 555LVPVIESLEL564, as do Psa from *Drosophila*, mouse, and human (Fig. 4.1A). A sequence comparison of *Dictyostelium* PsaA NLS-2 and NES-2 to corresponding NLS and NES in *Drosophila*, mouse, and human is presented in figure 4.1B.

### 4.4.2 Validation of anti-PsaA specificity and GFP-fusion protein expression

A diagram of all GFP-fusion proteins used in this study is presented in figure 4.1C. Western blotting with anti-PsaA revealed a band slightly greater than 95 kDa, corresponding to the molecular weight of PsaA (predicted 99 kDa; Fig. 4.2A). This band was not detected when anti-PsaA was preincubated (blocked) with a peptide equivalent to the antigen sequence prior to probing (Fig. 4.2A). Anti-PsaA also detected endogenous PsaA and C-GFP-PsaA in the lysate of cells expressing C-GFP-PsaA however only endogenous PsaA was detected in cells expressing GFP alone (Fig. 4.2B). The endogenous PsaA band in these blots appeared as a doublet due to the high amount of protein separation needed for these experiments however it is unlikely that the second band represents PsaB since PsaB does not contain the PsaA antigen sequence. Anti-GFP detected GFP and C-GFP-PsaA in the lysate of cells transformed with the plasmid coding for either GFP or C-GFP-PsaA respectively (Fig. 4.2B). GFP was not detected in the lysate of untransformed cells. These results confirm that anti-PsaA specifically detects PsaA (both endogenous and GFP-fused), and that GFP, N-GFP-PsaA, and C-GFP-PsaA were properly expressed.
Figure 4.2. Antibody specificity, GFP-fusion protein expression, and colocalization of PsaA with NumA1. 

A) Western blot of lysate from untreated cells probed with anti-PsaA. The antibody was used directly (Reg) or incubated with the antigen peptide overnight (Block) prior to probing. PsaA was detected slightly higher than 95 kDa.

B) Western blot of lysate from untransformed cells (AX3) or cells expressing either GFP alone (GFP) or C-GFP-PsaA (G-P) probed with either anti-PsaA or anti-GFP. A band slightly higher than 95 kDa was detected by anti-PsaA in untransformed cells and in cells transformed with C-GFP-PsaA (arrow). Anti-PsaA also detected a band at ~125 kDa, corresponding to C-GFP-PsaA, which was dramatically darker indicating that this GFP-fusion protein was overexpressed. Anti-GFP also detected C-GFP-PsaA as well as GFP alone.

C) Western blot of lysate from cells expressing either GFP (GFP), N-GFP-PsaA (N), C-GFP-PsaA (C), GFP-PsaA-ΔNLS-1 (Δ1), Δ2, Δ-GFP-NLS-1 (1), or Δ2 (2). All GFP-fusion proteins were detected at their predicted molecular weight however for GFP alone a larger band was also detected at ~32 kDa. Other bands were sometimes detected at ~21 kDa. All numbers represent molecular weight (kDa).

D) Live cells expressing either GFP alone, N-GFP-PsaA, or C-GFP-PsaA were stained with Hoechst 33258 prior to mounting. GFP alone dispersed throughout the cell whereas N-GFP-PsaA and C-GFP-PsaA localized predominantly to the nucleoplasm and to a lesser extent the cytoplasm. N-GFP-PsaA and C-GFP-PsaA did not localize to nucleoli (arrows) which were identified by the Hoechst 33258 unstained nuclear regions. Merged image shows overlay of GFP fluorescence (green) with Hoechst 33258 stain (blue).

E) Anti-PsaA detected N-GFP-PsaA and C-GFP-PsaA (only C-GFP-PsaA is shown) in the nucleoplasm but only endogenous PsaA in cells expressing GFP alone. The nucleoplasmic fluorescence intensity of secondary antibody (detecting anti-PsaA) is much greater in cells expressing C-GFP-PsaA which further attests to the specificity of anti-PsaA. Merged image shows overlay of GFP fluorescence (GFP fluor.; green), anti-PsaA (red), and DAPI (blue).

F) PsaA colocalizes with NumA1 in the nucleoplasm but not in nucleoli (arrows), where NumA1 is most abundant. Merged image shows overlay of C-GFP-PsaA (green), NumA1 (red), and DAPI (blue). Scale bar represents 2 µm.
GFP alone was detected around the predicted molecular weight of 27 kDa however a higher band of ~32 kDa was also detected, most likely representing post-translationally modified GFP (Fig. 4.2C). N-GFP-PsaA, C-GFP-PsaA, GFP-PsaA-ΔNLS-1, and -2 were all detected around their predicted molecular weight of ~125 kDa (Fig. 4.2C). GFP-NLS-1 and -2 were also detected around their predicted molecular weights of ~27 (Fig. 4.2C). Lower bands most likely representing break-down products were also detected in the lanes of GFP-PsaA-ΔNLS-1, -2, GFP-NLS-1, and -2 (Fig. 4.2C).

4.4.3 Anti-PsaA detects PsaA in the nucleoplasm, centrosomal region, microtubules, and to a lesser extent the cytoplasm

Immunolocalization was performed in order to determine the cellular localization of PsaA. Cells probed with only the secondary antibody or with the preimmune serum in place of anti-PsaA displayed either no visible fluorescence or diffuse fluorescence throughout the entire cell respectively (Fig. 4.3A). In contrast, PsaA was detected with anti-PsaA in the nucleoplasm, centrosomal region, and microtubules, as demonstrated by colocalization with DAPI nuclear stain and α-tubulin, and to a lesser extent the cytoplasm (Fig. 4.3B). Treatment with 50 μM nocodazole for 6 h resulted in the disappearance of both microtubules and the microtubular localization of PsaA, however PsaA was still detected in the nucleoplasm (Fig. 4.3B).

Since NumA1 is a CaMBP we were interested to see if Ca^{2+} or CaM might affect PsaA localization through NumA1. No change in localization was observed when cells were treated with either 1, 5, or 10 mM CaCl_2 (4 h), Ca^{2+} chelators (1, 5, or 10 mM EGTA for 4 h, or 1 or 5 mM BAPTA for 4 h), or CaM antagonists (300 μM W-5 or 50 μM W-7 for 4 h). As well, 0.5 mg/mL of the translation inhibitor cycloheximide (1, 2, 4, or 8 h) also did not affect localization (data not shown).
4.4.4 No effect of transcription inhibition on PsaA localization

Nucleolar disruption after AM-D treatment has been shown to result in the disappearance of NumA1 from nucleoli (Chapter 2). In order to determine if the localization of PsaA is dependent on either proper NumA1 localization or on transcription, cells were treated with AM-D prior to immunolocalization with anti-PsaA. The localization of PsaA was unaffected after either 4 or 8 h of AM-D treatment (Fig. 4.3C).
4.4.5 N-GFP-PsaA and C-GFP-PsaA localize to the nucleoplasm and to a lesser extent the cytoplasm but not the centrosomal region or microtubules

Both N-GFP-PsaA and C-GFP-PsaA localized primarily to the nucleoplasm but were not detected in the nucleolus of live cells as demonstrated by colocalization with either Hoechst 33258 stained or unstained nuclear regions respectively (Fig. 4.2D). Both fusion proteins were also detected in the cytoplasm, albeit to a lesser extent. The same pattern was observed when cells were fixed (using DAPI as a nuclear marker instead of Hoechst 33258; Fig. 4.2E). In contrast, in cells expressing GFP alone fluorescence was not observed at any specific cellular locale, but rather was detected throughout the cell in live cells and was not detected in fixed cells (Fig. 4.2D,E). Neither N-GFP-PsaA nor C-GFP-PsaA localized to the centrosomal region or microtubules. As well, anti-PsaA detected N-GFP-PsaA and C-GFP-PsaA but not GFP alone in fixed cells as demonstrated by the difference in fluorescence intensity (Fig. 4.2E).

4.4.6 PsaA only colocalizes with NumA1 in the nucleoplasm

PsaA colocalized with NumA1 in the nucleoplasm but not in nucleoli (Fig. 4.2F).

Overexpression of N-GFP-PsaA or C-GFP-PsaA did not affect the localization of NumA1 (Fig. 4.2F). These results also confirm that PsaA does not localize to nucleoli, since there is no overlap of nucleolar NumA1 and PsaA.

4.4.7 No effect of overexpression of C-GFP-PsaA

Western blotting revealed a dramatic difference in band intensity between endogenous PsaA and C-GFP-PsaA in the lysates of cells expressing this fusion protein (Fig. 4.2B). Similar results were obtained from immunolocalization of C-GFP-PsaA using anti-PsaA, as the fluorescence was much brighter than that observed when probing for endogenous PsaA in cells expressing GFP alone (Fig. 4.2E). These results confirm that C-GFP-PsaA was overexpressed.
Overexpression of C-GFP-PsaA had no effect on the overall morphology of the cell or on the rate of cell culture growth (data not shown). There was also no effect on the localization of the PsaA-binding protein NumA1 (Fig. 4.2F).

4.4.8 PsaA redistributes from nucleoplasm to cytoplasm during mitosis
The redistribution of PsaA from the nucleoplasm to the cytoplasm during mitosis was observed via immunolocalization as well as with the localization of both N-GFP-PsaA and C-GFP-PsaA (Fig. 4.4A, 5). During interphase the level of nucleoplasmic fluorescence of endogenous PsaA detected by anti-PsaA was 1.83-times the level of cytoplasmic fluorescence (Fig. 4.4B). However during prometaphase the level of nucleoplasmic PsaA decreased 0.83-times from 1.83 ± 0.11 (mean ± standard deviation) to 1.52 ± 0.10 while the cytoplasmic levels increased 1.46-times from 1.00 ± 0.09 to 1.46 ± 0.11 (Fig. 4.4B). A similar distribution was observed during metaphase, anaphase, and telophase, however during anaphase and telophase cytoplasmic fluorescence levels of endogenous PsaA were 0.82 and 0.79-times lower than during prometaphase respectively (Fig. 4.4B).

4.4.9 NLS-2 is required for nucleoplasmic localization of PsaA
GFP-PsaA-ΔNLS-1 showed relatively low levels of fluorescence but still localized throughout the cell, predominantly within the nucleus of fixed cells (Fig. 4.6A). The level of GFP-PsaA-ΔNLS-2 fluorescence was 0.6-times less in the nucleus and 1.7-times greater in the cytoplasm compared to full length C-GFP-PsaA (Fig. 4.6A,C). However the ratio of intensity of nuclear to cytoplasmic fluorescence of GFP-NLS-2 was no different than that of GFP-NLS-1 or GFP alone (Fig. 4.6B).
Figure 4.4. Immunolocalization of PsaA during mitosis. A) During mitosis (from prometaphase through telophase) PsaA left the nucleus and dispersed throughout the entire cell. It was also detected at the spindle fibers and microtubules by anti-PsaA. Merged image shows overlay of PsaA (red), α-tubulin (green), and DAPI (blue). Scale bar represents 2 µm. B) Relative intensity level of fluorescence during mitosis. Between interphase and prometaphase the level of nuclear fluorescence (black diamonds) decreased while the level of cytoplasmic fluorescence (white squares) increased. Throughout the rest of mitosis both nuclear and cytoplasmic levels decreased slightly. Asterisks (top; nucleus, bottom; cytoplasm) represent significant differences from interphase levels. Scale bar represents 2 µm.
4.4.10 BME treatment results in slower progression through the cell cycle by decreasing both the rate of cell growth and frequency of cell division

PsA was not detected by anti-PsA in the nucleoplasm of BME-treated (600 µM) cells suggesting that either PsA is absent from the nucleoplasm in these cells or that BME-binding blocks anti-PsA from detecting PsA (Fig. 4.7A). The presence of C-GFP-PsA in the nucleoplasm of BME-treated (600 µM) cells confirms that the latter is true and strongly implies an interaction between BME and PsA (Fig. 4.7B).
In order to examine the effects of BME on growth, cells were treated with a range of concentrations and cell density was measured. In untreated cultures, cell density increased 18.46-times in 48 h however increasing BME concentration resulted in a smaller increase in cell density, with 600 µM resulting in only a 3.25-times increase after 48 h (Fig. 4.8A). This represents an 82.3 % inhibition by BME. In order to determine if BME was cytotoxic, BME-
Figure 4.7. Effect of BME on localization of PsaA. A) Untreated cells or cells treated with 600 µM BME (bestatin) were fixed and probed with anti-PsaA. Nuclear PsaA was not detected in cells treated with BME (arrow). Merged image shows overlay of PsaA (red), α-tubulin (green), and DAPI (blue). B) The same experiment preformed with cells expressing C-GFP-PsaA in which case the protein was detected. Merged image shows overlay of C-GFP-PsaA (green), α-tubulin (red), and DAPI (blue). Scale bar represents 2 µm. C) Average cell doubling time increased with increasing BME concentration. Asterisks represent significant differences from untreated cells. D) BME treatment did not affect cell size. Cell area divided by nuclear number was calculated and was the same regardless of BME concentration.

treated cells were stained with both FDA (which enters only live cells, is then cleaved and fluoresces green) and PI (which enters all cells and fluoresces red; Fig. 4.8B). No dead cells were detected in untreated cultures or cultures treated with either 10, 50, or 100 µM BME. Cultures treated with either 300 µM or 600 µM BME contained 99.14 ± 1.22 % or 95.78 ± 3.25 % live cells respectively (Fig. 4.8C). As well, cells were observed in all stages of the cell cycle in cultures treated with either 0, 10, 50, 100, 300, or 600 µM BME (Fig. 4.8D). These results show that the BME-induced inhibition of cell culture growth rates was due to a decreased frequency of
Figure 4.8. Effect of BME on growth and cell viability. A) Increase in cell density over time for cells either untreated (black diamonds) or treated with either 10 (cross), 50 (white diamonds), 100 (white squares), 300 (white triangles), or 600 µM (black squares) BME (bestatin). Asterisks represent significant differences from untreated cells at 48 h. B) Sample pictures of a cell stained with either FDA or PI. Scale bar represents 2 µm. C) Percent live cells in BME-treated cultures after 24 h. These values were the same after 48 h (data not shown). D) BME-treated cells (all concentrations) were observed at all stages of the cell cycle. Cells were fixed, stained with DAPI (blue), and probed with anti-α-tubulin (green) in order to determine cell cycle stage. Sample cell from a BME-treated (600 µM) culture is shown. Scale bar represents 2 µm. E) Untreated cells or cells treated with 600 µM BME were fixed and probed with anti-NumA1 in order to measure nucleolar size. Nucleoli (arrows) of BME-treated cells were smaller than those of untreated cells. Merged image shows overlay of NumA1 (red) and DAPI (blue). Scale bar represents 2 µm. F) Quantification of nucleolar area in untreated and BME-treated (600 µM) cells. The asterisk represents significant difference. G) Proportion of mononucleate cells (dark grey), binucleate cells (light grey), or cells with more than two nuclei (white) within untreated cultures (0) or cultures treated with different concentrations of BME. Asterisks represent significant differences in number of mononucleate cells relative to untreated cells. H) Cell area divided by nuclear number for mononucleate (Mono) and binucleate (Bi) cells. Cell area is proportional to nuclear number. I) Cell concentration (dark grey) and nuclear concentration (light grey) within cultures of untreated cells (0) or cells treated with different concentrations of BME. The inherent multinuclearity of Dictyostelium hides the true effect of BME. Asterisks represent significant differences relative to untreated cells.
cell division, resulting in an increased doubling time, as opposed to cell death. To clarify this, the doubling time of BME-treated cells in log phase was calculated from the data obtained between 24 and 48 h. Doubling time increased with increasing BME concentration to a maximum of 3.70-times, from $8.22 \pm 0.96$ h at 0 µM to $30.41 \pm 3.07$ h at 600 µM (Fig. 4.7C). A decreased frequency of cell division would result in a longer interphase and if the growth rate was unaffected, cells would thus be larger. However, BME-treated cells were the same size as untreated cells meaning their growth rate must also have been slower than untreated cells (Fig. 4.7D). Fittingly, BME-treated cells (600 µM) possessed nucleoli 0.23-times smaller ($4.69 \pm 0.93\%$ of nuclear area) than untreated cells ($20.07 \pm 2.93\%$), a phenomenon associated with slow growth in human cells (Fig. 4.8E,F; Matsumoto et al., 1991). Together, these results show that BME treatment results in a slower progression through the cell cycle resulting from both a slower rate of growth and a decreased frequency of cell division.

4.4.11 Multinuclearity in *Dictyostelium* diminishes the apparent effect of BME

Upon examining the effects of BME on cell morphology it appeared as though BME-treated cells were smaller, however the difference was due to the decreased number of multinucleate cells. Multinuclearity in *Dictyostelium* axenic cultures is generally thought to be a result of impaired cytokinesis due to the lack of substratum. Fittingly, the cell area in multinucleate cells was found to be proportional to the number of nuclei (Fig. 4.8H). The proportion of mononucleate cells in a BME-treated culture increased with increasing concentration to a maximum of 2.23-times (from $38.89 \pm 4.81\%$ at 0 µM to $86.67 \pm 6.54\%$ at 600 µM; Fig. 4.8G). This difference in proportion of multinucleate cells thus masks the true effect of BME on cell growth. Ignoring aberrant cytokinesis and measuring the concentration of nuclei in a culture instead of the concentration of cells shows that after 48 h the concentration of nuclei in a BME-treated (600 µM) culture is 0.11-
times (89 % inhibition) that of the untreated culture, as opposed to 0.18-times (82 % inhibition), as would be calculated by measuring cell concentration (Fig. 4.8I).

4.5 Discussion

In order to better understand the role of NumA1 in the cell cycle in *Dictyostelium* we examined its binding proteins. NumA1 interacts with CBP4a in a Ca\(^{2+}\)-dependent manner presumably in the nucleolus where the two colocalize (Chapter 3; Myre and O’Day, 2004a). NumA1 also interacts with PsaA (Myre, 2005). In other organisms Psa is a Zn\(^{2+}\)-metallopeptidase thought to be involved in the regulation of cell cycle progression and several diseases such as Huntington’s disease and Alzheimer’s disease (Bhutani et al., 2007; Karsten et al., 2006; Takahashi et al., 1987; 1989). This study has shown that *Dictyostelium* PsaA is similar to Psa from *Drosophila*, mouse, and human. All contain a GAMEN motif and Zn\(^{2+}\)-binding domain, characteristics of the M1 family of Zn\(^{2+}\)-metallopeptidases (Constam et al, 1995; Rawlings and Barrett, 1995). The involvement of Zn\(^{2+}\)-metallopeptidases in the progression of the cell cycle suggests that PsaA may also be involved in cell cycle events (Peer, 2011).

As shown by both immunolocalization and N- and C-terminal GFP fusion proteins, PsaA colocalizes with NumA1 in the nucleoplasm but not the nucleolus of interphase cells, suggesting this is where they interact, perhaps via the WWIM within PsaA. PsaA also localizes to a lesser extent in the cytoplasm. This is consistent with previous work, as immunohistochemical analysis of Psa from a murine neuroblastoma cell line revealed both a cytoplasmic and nuclear distribution in COS-7 cells and 3T3 fibroblasts (Constam et al., 1995). This localization was not altered when either transcription or translation was inhibited, suggesting that it is a relatively stable protein. There was also no effect of Ca\(^{2+}\) chelators or CaM antagonists indicating that neither Ca\(^{2+}\) nor CaM affect its localization. Although Psa from mouse and rat have previously
been shown to localize to microtubules the immunolocalization of *Dictyostelium* PsaA in the centrosomal region and at microtubules was not supported by the GFP data (Constam et al., 1995; Yamamoto et al., 2002). Furthermore, proteomic analysis of the centrosome did not detect the presence of PsaA (Reinders et al., 2006). Thus the centrosomal localization of PsaA remains in question.

Although GFP-PsaA-ΔNLS-1 did not fluoresce as intensely as the full length fusion protein, it localized mainly to the nucleus, demonstrating that NLS-1 is not necessary for proper nuclear entry. GFP-PsaA-ΔNLS-2 however demonstrated a decreased ability to enter the nucleus. Proper nuclear localization of PsaA is therefore at least partially dependent on NLS-2 and probably requires the presence of other unidentified domains since GFP-NLS-2 did not preferentially localize to the nucleus. Although functional NLSs have not been identified in Psa from other organisms, this study has identified two putative NLSs in *Drosophila*, mouse, and human Psa. In each protein the second NLS is similar in sequence and situated in a similar position within the protein as NLS-2 from *Dictyostelium* PsaA. The previously observed nuclear localization of Psa in COS-7 cells and 3T3 fibroblasts may be attributed to these unverified NLSs (Constam et al., 1995). The two putative NESs found in *Dictyostelium* PsaA as well as in Psa from *Drosophila*, mouse, and human may be involved in the nuclear export observed during mitosis, however this remains to be verified.

BME inhibits aminopeptidases by binding to the Zn$^{2+}$-binding domain within the substrate-binding domain (Taylor, 1993a). Of the nine aminopeptidases present in *Dictyostelium*, only PsaA and PsaB possess a Zn$^{2+}$-binding domain that conforms to the consensus sequence HExxHx$_{18}$E. In fact both of these motifs also conform to the more stringent sequence abXHEbbHbc. The decreased rate of cell growth and decreased frequency of cell division are
therefore most likely a result of either PsaA or PsaB inhibition, or perhaps both. The fact that anti-PsaA was unable to detect PsaA when cells were treated with BME but the fluorescence from C-GFP-PsaA was still visible suggests that BME does not decrease the amount of nuclear PsaA but rather interacts with PsaA, blocking antibody binding. The involvement of PsaA in the regulation of growth and cell division fits well with the rest of the data presented in this study as well as with previous work on PsaA from other organisms. Although the mechanism of action is unknown, the inhibition of growth by BME is directly proportional to the increase in cell division frequency. Since these cells remain the same size despite the much longer doubling time, it suggests that a single factor is causing both events. The reason BME caused a decrease in multinuclearity is unclear but one possibility is that PsaA negatively regulates cytokinesis. If so, then the inhibition of PsaA would result in increased cytokinesis.

In summary, *Dictyostelium* PsaA demonstrates all of the attributes common to eukaryotic Psa and localizes to the same areas within the cell. The redistribution of PsaA during mitosis and the effects of BME on the rate of growth and division together with previous work implicating Psa in the regulation of the cell cycle suggest that *Dictyostelium* PsaA may also function in cell cycle events as it does in other species. Given the accumulating evidence placing NumA1 in a cell-cycle role through the identification of a BRCT-domain containing isoform, and the finding that overexpression of dominant negative mutants lacking the highly acidic glu/asp (DEED) domain induces highly multinucleate cells, the identification of a specific interaction with PsaA might suggest that PsaA regulation by NumA1 may be required for proper progression through the cell cycle.
4.6 References


Chapter 5

BAF60a homologue Snf12 is a nucleolar and nucleoplasmic protein involved in stress response in *Dictyostelium*

5.1 Abstract

The SWI/SNF nucleosome remodeling complex remodels nucleosomes to regulate gene transcription. It has many ties to cancer and acts as a tumour suppressor by regulating p53-mediated transcription of cell cycle genes. Complex member BAF60a (Snf12 in yeast) mediates the interaction with p53. *Dictyostelium* is a model for several fundamental cellular processes and human diseases. However comparatively little is known about its cell cycle. Given the involvement of BAF60a in cell cycle regulation and tumour suppression in humans its *Dictyostelium* homologue Snf12 was investigated. This study shows via immunolocalization as well as GFP-fused Snf12 that Snf12 is a predominately nucleoplasmic protein which also localizes to nucleoli in ~20% of cells. It possesses a NLS (\(^{372}\text{KRKR}^{375}\)) that is both necessary and sufficient for both nuclear/nucleolar localization. It represents only the second NoLS and NLS/NoLS identified in *Dictyostelium*. Snf12 possesses conserved SWIB and COG domains which are not involved in its localization. Cycloheximide treatment resulted in the absence of Snf12 suggesting that it has a relatively high turnover rate. Both heat shock (2 h, 30 °C) and AM-D treatment (0.05 mg/mL for 4 h) resulted in an increase in nucleolar Snf12 indicating that Snf12 is involved in stress response. AM-D treatment also resulted in the bulging of nucleolar localized Sn12 from the nucleus after 4 h treatment. These bulges were eventually detected as Snf12 cytoplasmic circles after 8 h. During prometaphase Snf12 was detected less in the nucleus and more in the cytoplasm. During metaphase and anaphase it was detected throughout the entire
cell before reaccumulating in the nucleus during telophase. The potential role of *Dictyostelium* Snf12 in the regulation of cell cycle events is discussed.
5.2 Introduction

The SWI/SNF is a nucleosome remodeling complex composed of 9-12 proteins called BAFs (Bennett-Lovsey et al., 2002; Reisman et al., 2009; Wang et al., 1996a; 1996b). These proteins are highly conserved and found in organisms ranging from yeast to humans (Reisman et al., 2009). The SWI/SNF complex remodels nucleosomes in order to expose/conceal genes thereby regulating their transcriptional activity (Kwon et al., 1994; Sudarsanam and Winston, 2000; Wang et al., 1996b). The complex is involved in several processes such as cell proliferation, differentiation, and DNA repair (Reisman et al., 2009). It also acts as a tumour suppressor by regulating p53-mediated transcription, which is just one example of the many links between the SWI/SNF complex and cancer (Lee et al., 2002; Wang et al., 2007; Reisman et al., 2009; Weissman and Knudsen, 2009).

Each member of the SWI/SNF complex has a different function. For example Brahma and Brahma-related gene 1 are catalytic subunits, BAF155 is a scaffold protein that controls stability, while SNF5 and BAF57 mediate the recruitment of transcription factors (Sohn et al., 2007; Cheng et al., 1999; Link et al., 2005; Belandia et al., 2002). BAF60a (Snf12 in yeast) is one of the most versatile, interacting with several forms of transcription factors including p53 (Hsiao et al., 2003; Ito et al., 2001; Lee et al., 2002; Oh et al., 2008; Assmann et al., 2006; Chien et al., 2003; Wang et al., 1996b). It possesses a SWIB domain which lies within a larger conserved domain called COG5531 (COG), both of which the function is unknown (Marchler-Bauer et al., 2009; Oh et al., 2008). The SWIB domain is homologous and shares a common fold with the MDM2 domain from MDM2, the master regulator of p53 (Bennett-Lovsey et al., 2002). MDM2 interacts with p53 via its MDM2 domain however BAF60a interacts with p53 via its N-terminal, not its SWIB domain (Bennett-Lovsey et al., 2002; Lee et al., 2002; Oh et al., 2008).
Dictyostelium is a single-celled amoeba that has proved an excellent model organism for the study of a multitude of cellular events. It is used for the study of several fundamental cellular processes as well as several biomedical diseases such as those caused by bacterial and viral pathogens as well as several diseases affecting the central nervous system (Annesley and Fisher, 2009; Williams, 2010). Despite the extensive study of this organism relatively little is known about its cell cycle. Interestingly, Dictyostelium possess several homologues of key cell cycle regulators however no p53 homologue has yet been identified (Eichinger et al., 2005; Mayanagi et al., 2005). Understanding cell cycle events in Dictyostelium is crucial since it would almost certainly lead to a better understanding of cell cycle regulation in human cells. Given the involvement of BAF60a in the regulation of cell cycle events and cancer in higher organisms its Dictyostelium homologue Snf12 was investigated in order to better understand cell cycle regulation in this model eukaryote. Moreover, given the interaction of BAF60a with p53 in human cells studying Snf12 in Dictyostelium may lead to the discovery of a functional equivalent of p53.

This study shows that Dictyostelium Snf12 is a nucleoplasmic protein that also localizes to nucleoli to a lesser extent. Its nucleolar localization increased when cells were either heat shocked or treated with the transcription inhibitor AM-D indicating that it is involved in the stress response. Snf12 possesses a NLS that is both necessary and sufficient for proper localization. This NLS also acts as a NoLS and is thus a NLS/NoLS. During mitosis Snf12 redistributed throughout the cytoplasm. The potential role of Snf12 in the regulation of cell cycle events in Dictyostelium is discussed.
5.3 Materials and methods

5.3.1 Materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich®. 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.

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 (Fey et al., 2007).

5.3.2 Generation of GFP vectors and cell lines

Vectors pDM-317 (for N-terminal GFP) and pDM-323 (for C-terminal GFP) were used to generate all GFP-fusion protein constructs (Veltman et al., 2009; dictyBase.org). A SacI site was inserted into both vectors between the BglII and SpeI sites as previously described (Chapter 3).

Full length Snf12 was then amplified using primers CAGAGCTCAAAATGTCTAAAAATGGTAC (forward), and CAACTAGTAGGAGTTGAGGAGTTGAA (reverse) which incorporated a kozak site as well as the appropriate restriction sites (underlined) as previously described using an elongation cycle of 90 sec (Chapter 3, 4). This fragment was incorporated into both pDM-317 and pDM-323 vectors (with SacI and SpeI cut sites) and the resulting constructs were transformed in Dictyostelium as previously described (Chapter 3, 4). These transformed cells expressed N-GFP-Snf12 and C-GFP-Snf12 respectively. Empty pDM-317 was also transformed in order to express GFP alone. After 4 days of selection colonies were transferred to HL-5 containing 10 µg/mL G418 within which they were maintained.
All deletion constructs (GFP-Snf12-ΔSWIB, -ΔCOG, -ΔNLS-1, -ΔNLS-2, and -ΔNLS-3) were generated in a similar manner, however only the regions before and after the deletion were amplified (see Table 5.1 for a list of primers used). However due to the small length of the region before NLS-1 this fragment was generated by annealing two complementary primers equivalent to this region (forward; CAAAATGTCTAAAAATGGTACAACTGCT, reverse; GATCCACCAGTTGTACCATTATTAGACATTGAGCT) which incorporated a sticky-end SacI and BamHI site at the 5’ and 3’-end respectively. For each deletion construct the region before was ligated to the region after the deletion and the ligation product was amplified with PCR using the same primers used to amplify full length Snf12. PCR products were extracted, digested, and PCR purified prior to incorporation into pDM-323 (with SacI and SpeI cut sites) as previously described (Chapter 3, 4). Both GFP-Snf12-ΔSWIB and -ΔCOG therefore possessed a BamHI restriction site in place of either the SWIB domain or the COG domain while GFP-Snf12-ΔNLS-1, -2, and -3 possessed a BamHI, HindIII, and PstI site in place of NLS-1, -2, and -3 respectively.

GFP-SWIB, -COG, -COG-NLS, -COG-1, and -COG-2 were constructed via PCR of these domains (see Table 5.2 for a list of primers). GFP-NLS-1, -NLS-2, and -NLS-3 were made from sets of primers equivalent to these domains with incorporated sticky-end SacI and SpeI restriction sites (see Table 5.2 for a list of primers used). The forward and reverse primers were annealed to produce the fragment. Fragments were incorporated into either GFP-Snf12-NLS-3 (GFP-COG-1, -2; with BglII and SacI cut sites) or pDM-323 (all others; with SacI and SpeI cut sites) as previously described (Chapter 3, 4). GFP-COG-1 and -2 thus possess NLS-3 before the C-terminal GFP. All of these fusion proteins possess a C-terminal GFP.
Table 5.1. List of primers used to generate GFP-fusion deletion constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Region before/after deletion</th>
<th>For./Rev.</th>
<th>Primers</th>
<th>Restr. site</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-Snf12-ΔSWIB</td>
<td>Before</td>
<td>F</td>
<td>CAGAGCTCAAAATGTCTAAAAATGGTAC</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAGGATCTACGTGATCTAAAATACATTAAA</td>
<td>BamHI</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>F</td>
<td>CAGGATCCCCCTGATCCATTAGAA</td>
<td>BamHI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAACTAGTGGAGTTTAGAGGAGTTGAA</td>
<td>SpeI</td>
</tr>
<tr>
<td>GFP-Snf12-ΔCON</td>
<td>Before</td>
<td>F</td>
<td>CAGAGCTCAAAATGTCTAAAAATGGTAC</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAGGATCCATCCCCATCCACAGTA</td>
<td>BamHI</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>F</td>
<td>CAGGATCCATTTTGAGGAGGAGTTGAA</td>
<td>SpeI</td>
</tr>
<tr>
<td>GFP-Snf12-ΔNLS-1</td>
<td>Before</td>
<td>F</td>
<td>(See materials and methods)</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>(See materials and methods)</td>
<td>BamHI</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>F</td>
<td>CAGGATCTCAATGCTCTAAAATGGTAC</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAACTAGTGGAGTTTAGAGGAGTTGAA</td>
<td>SpeI</td>
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<tr>
<td>GFP-Snf12-ΔNLS-2</td>
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<td>CAGAGCTCAAAATGTCTAAAAATGGTAC</td>
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<td></td>
<td></td>
<td>R</td>
<td>CAACTAGTGGAGTTTAGAGGAGTTGAA</td>
<td>SpeI</td>
</tr>
<tr>
<td>GFP-Snf12-ΔNLS-3</td>
<td>Before</td>
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<td>CAGAGCTCAAAATGTCTAAAAATGGTAC</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAACTAGTGGAGTTTAGAGGAGTTGAA</td>
<td>SpeI</td>
</tr>
</tbody>
</table>

Restriction sites are underlined. For a diagrammatic representation of each GFP-fusion protein see figure 5.1B.

5.3.3 Antibody production

A peptide equivalent to a unique C-terminal sequence from Snf12

\(^{362}\text{NHHIQKVWQHKRKFMEK}^{380}\); Fig. 5.1A) was synthesized using solid phase synthesis
(with the addition of an N-terminal cysteine required for subsequent conjugation), purified via
HPLC, and verified via mass spectroscopy prior to KLH conjugation via an MBS linker
(Advanced Syntech Inc., Markham, ON). Conjugation was performed as previously described
(Lateef et al., 2007). Polyclonal antibodies against the KLH-conjugated peptide were produced
in New Zealand White rabbits at the University of Toronto at Mississauga Animal Care Facility
Table 5.2. List of primers used to generate GFP-fusion domain constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>For./ Rev.</th>
<th>Primers</th>
<th>Restr. site</th>
</tr>
</thead>
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<td>GFP-SWIB</td>
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<td>CAACTAGTTGATGATAATAGTCTCTTAAATAATTG</td>
<td>SpeI</td>
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<tr>
<td>GFP-CON</td>
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<td>SacI</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAACTAGTTGAAATCTCTTTTCTTATTG</td>
<td>SpeI</td>
</tr>
<tr>
<td>GFP-CON-NLS-3</td>
<td>F</td>
<td>CAGAGCTCAAAATGCTCAACACGAA</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CAACTAGTTTTCATGAATTCTAC</td>
<td>SpeI</td>
</tr>
<tr>
<td>GFP-CON-1</td>
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<tr>
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<td>R</td>
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<td>SacI</td>
</tr>
<tr>
<td>GFP-CON-2</td>
<td>F</td>
<td>CAAGATCTAAAAATGGACTGATCCATTAGAA</td>
<td>BglII</td>
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<td></td>
<td>R</td>
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<td>SacI</td>
</tr>
<tr>
<td>GFP-NLS-1</td>
<td>F</td>
<td>AAAATGAAATAGGAAAGAAAAACAGAAATTAA</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>CTAGTTAATTTCTCTTTTTATCTTGGAGCT</td>
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<tr>
<td>GFP-NLS-2</td>
<td>F</td>
<td>AAAATGAAAGAGAAAACGTGAA</td>
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<td>GFP-NLS-3</td>
<td>F</td>
<td>AAAATGAAAGAGAAACGTGAA</td>
<td>SacI</td>
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<tr>
<td></td>
<td>R</td>
<td>CTACTACGTCTCTCTTTGAGCT</td>
<td>SpeI</td>
</tr>
</tbody>
</table>

Restriction sites (or portions of restriction sites) are underlined. For a diagrammatic representation of each GFP-fusion protein see figure 5.1B.

according to University of Toronto protocols for antibody production and animal care as previously described (Chapter 3, 4). The preimmune serum (rabbit serum prior to any injections) was kept and used as a control for immunolocalization experiments. Exsanguination was performed by the department of Faculty of Medicine at the University of Toronto. The specificity of the crude serum was verified as anti-Snf12 and used for all western blots and immunolocalization experiments.

5.3.4 **SDS-PAGE and western blotting**

Cells (1-2 x 10^6 cells/mL) were resuspended in lysis buffer (20 mM Tris-HCl pH 6.8; 1 % SDS; 1 mM EDTA; 1 mM PMSF; 1 complete protease inhibitor tablet) and sonicated. The Bradford
Figure 5.1. Domain structure diagram of Snf12 in *Dictyostelium*, yeast, and human and of GFP-fusion proteins used in this study. A) Snf12 in *Dictyostelium*, yeast, and human (BAF60a) all possess a conserved COG domain however only in *Dictyostelium* and human does the protein also possess a SWIB domain within the COG domain. Putative motifs are indicated. Numbers represent amino acid position. B) Several GFP-fusion proteins were made with either a N- or C-terminally-fused GFP. All possess a sequence equivalent to NLS-3 except for GFP-SWIB, -COG, and -Snf12-ΔNLS-3. All GFP-fusion proteins were detected by anti-GFP via western blotting (right of construct). All GFP-fusion proteins were also detected by anti-Snf12 except for those lacking the antigen site (GFP alone, GFP-SWIB, -COG, -COG-1, -COG-2, -NLS-1, -NLS-2, and -NLS-3). GFP-Snf12-ΔNLS-3 was detected by anti-Snf12 as a faint band.
assay was used to quantify protein levels. 20 mg total protein (0.1 mg for cells expressing GFP alone) was separated on a 12 % SDS-PAGE gel and transferred onto a PVDF membrane (Pall corp.). The membrane was blocked with 5 % non-fat milk in washing buffer (TBS and 0.001 % Tween 20) at 4 °C overnight and then probed with either anti-GFP (1:600; Santa Cruz®) or anti-Snf12 (1:200) in 5 % non-fat milk in washing buffer for 1 h at room temperature followed by probing with either secondary anti-mouse HRP (1:800; Santa Cruz®) or anti-rabbit HRP (1:800; Santa Cruz®) respectively in the same conditions. Bands were detected with Amersham ECL Plus™ Western Blotting kit (General Electric) using the STORM Scanner System.

5.3.5 Live cell viewing, treatment, fixation, and immunolocalization

GFP-fusion protein-expressing cells were viewed live after adhering to a coverslip for 30 min prior to slide-mounting. Hoechst 33258 nuclear stain was added to some cells for colocalization experiments. Fixation in ultracold methanol and immunolocalization was performed as previously described (Hagedorn et al., 2006). Cells were either untreated or treated while adhering to coverslips (in HL-5) with either 1, 5, or 10 mM CaCl₂ (4 h), 1, 5, or 10 mM EGTA (4 h), 1 or 5 mM BAPTA (4 h), 300 µM W-5 (4 h), 50 µM W-7 (4 h), 0.5 mg/mL cycloheximide (1, 2, 4, or 8 h), or 0.05 mg/mL AM-D (4 h, 8 h, or 4 h followed by three washes with HL-5 prior to 4 h recovery in HL-5). Alternatively, cells were heat shocked (30 °C, 2 h) prior to fixation. After blocking, cells were incubated with either anti-NumA1 (1:40; Chapter 2; O’Day et al., 2009), anti-Snf12 (1:40), or anti-GFP (1:500; Santa Cruz®) for 60 min followed by either Alexa Fluor® 555 (1:40; to detect anti-NumA1 or anti-Snf12) or 488 (1:100; to detect anti-GFP) goat anti-rabbit for 45 min. For some experiments cells were then probed with anti-α-tubulin (1:100; Hybridoma Bank) for 60 min followed by either Alexa Fluor® 488 or 555 goat anti-mouse (1:100) for 45 min. The localization of α-tubulin was used to determine the stage of cell cycle as well as cellular integrity. All secondary antibodies were purchased from Invitrogen™. 5 µL
ProlongAntifade (Invitrogen™) containing DAPI (5 µL) was placed on the slide prior to mounting and coverslips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera and images were analyzed (e.g. intensity measurements) using Nikon Imaging Software Elements Basic Research 3.0. The Student’s t-test ($\alpha = 0.05$) was used to calculate significance for all graphs. Significant differences from the control are indicated with an asterisk.

5.3.6 Development

Cells expressing either GFP alone, N-GFP-Snf12, or C-GFP-Snf12 were resuspended in KK$_2$ phosphate buffer and plated on SM-agar plates containing 100 µg/mL G418. Cells were allowed to develop into fruiting bodies which were slide-mounted with 0.001 % calcofluor (UVITEX). Pictures were taken with the same microscope as described above.

5.4 Results

5.4.1 *Dictyostelium* Snf12 is similar to yeast Snf12 and human BAF60a

*Dictyostelium* possesses homologues for five of the seven SWI/SNF complex members in yeast (Table 5.3; dictyBase.org). *Dictyostelium* Snf12 is similar in sequence to Snf12 from yeast and BAF60a from humans (Fig. 5.1A). The gene for Snf12 is present in two copies within the *Dictyostelium* genome however it is not known whether both are actively transcribed (dictyBase.org). *Dictyostelium* Snf12 possesses a SWIB domain (residues 236-310) and a conserved COG domain (residues 204-355; Fig. 5.1A; Marchler-Bauer et al., 2009). Human BAF60a possesses both a SWIB domain and a COG domain however yeast Snf12 possesses only the COG domain (Fig. 5.1A).
Table 5.3. *Dictyostelium* homologues of SWI/SNF proteins (dictyBase.org).

<table>
<thead>
<tr>
<th>Yeast protein</th>
<th><em>Dictyostelium</em> homologue</th>
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<tbody>
<tr>
<td>SWI1</td>
<td>(none)</td>
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<tr>
<td>SNF2</td>
<td>DDB_G0285205 or DDB_G0271052</td>
</tr>
<tr>
<td>SWI3</td>
<td>DDB_G0277033</td>
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<td>SNF5</td>
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<td>SNF12</td>
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<tr>
<td>ARP4</td>
<td>Arp4</td>
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<tr>
<td>RSC1</td>
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*Dictyostelium* Snf12 possesses three putative NLSs, NLS-1 (NKKKKTKKL₁⁶), NLS-2 (KRKF₁⁸³), and NLS-3 (KRKR₃⁷⁵; Fig. 5.1A). Similarly, human BAF60a possesses five putative NLSs while yeast Snf12 possesses only one (Fig. 5.1A). *Dictyostelium* Snf12 also possesses a putative NES, NES-1 (IRTLRLSI₁²⁰) as does yeast Snf12 (VDFDGLDI²⁸³) as well as two putative calmodulin (CaM)-binding domains (CaMBDs), FSSFFKKVF₁⁹² (CaMBD-1) and ILALWHYIKSNTLL²⁷³ (CaMBD-2; Fig. 5.1A; la Cour et al., 2004; Yap et al., 2000).

5.4.2 Validation of anti-Snf12 specificity and GFP-fusion protein expression

Several GFP-fusion protein-expressing cell lines were produced in order to study the various domains within Snf12. A diagram of each fusion protein is presented in figure 5.1B. Western blotting of lysate (from cells expressing each GFP-fusion protein) with anti-Snf12 revealed a band of ~55 kDa which corresponds to the molecular weight of Snf12 (predicted 52 kDa; Fig. 5.1B; dictyBase.org). All GFP-fusion proteins were detected with anti-GFP at the correct molecular weight (Fig. 5.1B). All GFP-fusion proteins were also detected with anti-Snf12 at the predicted molecular weight except for GFP-SWIB, -COG, -COG-1, -2, -NLS-1, -2, and -3,
which did not contain the antigen sequence for anti-Snf12 (Fig. 5.1B). GFP-Snf12-ΔNLS-3 was detected as a faint band most likely since it lacks a portion of the antigen sequence (\textsuperscript{372}KRKR\textsuperscript{375}).

### 5.4.3 Snf12 localizes to the nucleoplasm and nucleoli

N-GFP-Snf12 and C-GFP-Snf12 both localized throughout the nucleoplasm of live cells as demonstrated by colocalization with Hoechst 33258 nuclear stain (Fig. 5.2C). In contrast, GFP alone dispersed throughout the entire cell. N-GFP-Snf12 and C-GFP-Snf12 also localized to nucleoli in ~20 % of cells (Fig. 5.2D). Immunolocalization experiments showed the same result, as anti-Snf12 resulted in nucleoplasmic staining whereas cells probed with either the secondary antibody alone or preimmune serum in place of anti-Snf12 displayed little to no fluorescence or diffuse fluorescence throughout the entire cell respectively (Fig. 5.2A). Moreover, anti-Snf12 detected N-GFP-Snf12 and C-GFP-Snf12 in cells expressing these constructs but only detected endogenous Snf12 in cells expressing GFP alone (Fig. 5.2B). N-GFP-Snf12 and C-GFP-Snf12 were also present in the nucleus of spores but not in stalk cells (Supplementary figure 5.1).

Due to the presence of putative CaMBDs the possible role of Ca\textsuperscript{2+} or CaM in the localization of Snf12 was examined. No change in localization was observed when cells were treated with either 1, 5, or 10 mM CaCl\textsubscript{2} (4 h treatment), Ca\textsuperscript{2+} chelators (1, 5, or 10 mM EGTA for 4 h, or 1 or 5 mM BAPTA for 4 h), or CaM antagonists (300 μM W-5 or 50 μM W-7 for 4 h; data not shown).

### 5.4.4 Effect of SWIB and COG domains on Snf12 localization

GFP-Snf12-ΔSWIB and GFP-COG-NLS localized to the nucleus as demonstrated by colocalization with Hoechst 33258 nuclear stain while GFP-SWIB, -COG, and -COG-1 localized throughout the entire cell (Fig. 5.3). GFP-COG-2 localized predominately to the nucleus but was
Figure 5.2. N-GFP-Snf12, C-GFP-Snf12, and immunolocalization of Snf12. A) Cells were fixed and probed with the secondary antibody alone (No primary), preimmune serum and secondary antibody (Preimmune), or anti-Snf12 and secondary antibody (Snf12). Cells were then probed with anti-α-tubulin. Only cells probed with anti-Snf12 displayed nuclear fluorescence as demonstrated by colocalization with DAPI. Merged image shows overlay of secondary antibody fluorescence (red), α-tubulin (green), and DAPI (blue). Scale bar represents 2 µm. B) Cells expressing either GFP alone, N-GFP-Snf12 (not shown), or C-GFP-Snf12 were probed with anti-Snf12. Anti-Snf12 detected only endogenous Snf12 in cells expressing GFP alone but detected both endogenous Snf12 and N/C-GFP-Snf12 in cells expressing that fusion protein, as demonstrated by the difference in fluorescence intensity. Merged image shows overlay of anti-Snf12 (red), GFP fluorescence (GFP fluor.; green), and DAPI (blue). Scale bar represents 2 µm. C) Images of GFP-fusion protein fluorescence (GFP fluor.) for GFP alone, N-GFP-Snf12, or C-GFP-Snf12. N-GFP-Snf12 and C-GFP-Snf12 were detected in the nucleus as demonstrated by colocalization with Hoechst 33258 whereas GFP alone was detected throughout the entire cell. Merged image shows overlay of GFP fluorescence (green) and Hoechst 33258 (blue). Scale bar represents 2 µm. D) In ~20 % of cells N-GFP-Snf12, C-GFP-Snf12, and GFP-NLS-3 were detected in the nucleoplasm as well as in nucleoli (arrows) as demonstrated by colocalization with Hoechst 33258 stained and unstained nuclear regions respectively (only C-GFP-Snf12 is shown). Merged image shows overlay of C-GFP-Snf12 (green) and Hoechst 33258 (blue). Scale bar represents 2 µm.
Figure 5.3. Localization of GFP-fused conserved domains and deleted domains in live cells. GFP-ΔSWIB and GFP-COG-NLS were detected in the nucleus as demonstrated by colocalization with Hoechst 33258 whereas GFP-ΔCOG was not detected. GFP-SWIB, -COG, and -COG-1 were detected throughout the entire cell while GFP-COG-2 was detected predominately in the nucleus but also in the cytoplasm. Merged image shows overlay of GFP fluorescence (GFP fluor.; green) and Hoechst 33258 (blue). Scale bar represents 2 µm.
also present throughout the cytoplasm and GFP-Snf12-ΔCOG was not detected anywhere in the cell (Fig. 5.3).

### 5.4.5 NLS-3 is responsible for both nucleoplasmic and nucleolar localization of Snf12

In order to determine which potential NLS was responsible for the nucleoplasmic and nucleolar localization of Snf12, GFP deletion constructs were made lacking each of the three NLSs (Fig. 5.1B). Each NLS was also expressed on its own, fused to GFP. GFP-Snf12-ΔNLS-1 and -2 were detected in the nucleoplasm and fluoresced as intensely as the full length C-GFP-Snf12 (Fig. 5.4A,C). GFP-Snf12-ΔNLS-3 fluoresced less intensely in the nucleus and more intensely in the cytoplasm (Fig. 5.4A,C). Both live and fixed cells displayed the same pattern of fluorescence. Accordingly, GFP-NLS-1 and -2 were detected throughout the entire cell displaying a similar pattern of fluorescence as GFP alone (Fig. 5.4B,C). GFP-NLS-3 fluoresced more in the nucleus than in the cytoplasm (Fig. 5.4B,C). Together these results show that NLS-3 is both necessary and sufficient for proper nucleoplasmic localization of C-GFP-Snf12. As was observed for C-GFP-Snf12, GFP-NLS-3 localized to nucleoli as well as the nucleoplasm in ~20% of cells and thus also acts as a NoLS (Fig. 5.2D).

Interestingly, NLS-3 is similar in sequence to the reverse sequence of the functional NLS/NoLS from NumA1 (Chapter 2). Only one of the first two residues in the NLS/NoLS from NumA1 is basic, contrary to the consensus sequence for monopartite NLSs, which require the first two to be basic (Puntervoll et al., 2003). Reversing the order however complies with this consensus.
Figure 5.4. NLS-3 is necessary and sufficient for nuclear localization. **A** and **B**. Live (left column) or fixed cells (next three columns) expressing GFP-fusion proteins. **A**. C-GFP-Snf12, GFP-Snf12-ΔNLS-1 (ΔNLS-1), -2 (ΔNLS-2), -3 (ΔNLS-3). **B**. GFP alone (GFP), GFP-NLS-1 (NLS-1), -2 (NLS-2), or -3 (NLS-3). Merged image shows overlay of anti-GFP (green) and DAPI (blue). Scale bars represent 2 µm. **C**. The level of nuclear fluorescence (black bars) of GFP-Snf12-ΔNLS-3 (ΔN3) was less than that of C-GFP-Snf12 (Snf12) while cytoplasmic levels (grey bars) were greater. Both nucleoplasmic and cytoplasmic fluorescence levels of GFP-Snf12-ΔNLS-1 (ΔN1) and -2 (ΔN2) were the same as that of C-GFP-Snf12. Nucleoplasmic fluorescence intensity of GFP-NLS-3 (N3) was greater than that of GFP alone (GFP) while cytoplasmic levels were less. Both nucleoplasmic and cytoplasmic fluorescence levels of GFP-NLS-1 (N1) and -2 (N2) were the same as GFP alone. At least 100 cells were counted per replicate, and at least four replicates were performed for each. Asterisks represent significant differences from C-GFP-Snf12. **D**. Sequence comparison of all putative (unbolded) and verified (bold) monopartite NLSs from Snf12, NumA1, and CBP4a (Chapter 2, 3). NLS-3 from Snf12 is similar to the NLS in CBP4a and to the reverse NLS-4 from NumA1. These three NLSs conform to a common consensus, KRxR, where ‘x’ represents any amino acid.
5.4.6 Effect of cycloheximide on localization of Snf12

Snf12 was not detected in cells treated with the translation inhibitor cycloheximide in contrast to NumA1, a nucleoplasmic/nucleolar protein that shows a constant pattern of localization after cycloheximide treatment (Chapter 2; Fig. 5.5A).

5.4.7 Effect of heat shock or AM-D on localization of Snf12

In order to determine the effect of cellular stress on the localization of Snf12, cells were heat shocked for 2 h. Heat shock resulted in a higher fluorescence intensity level of C-GFP-Snf12 in nucleoli (Fig. 5.5B). Furthermore, C-GFP-Snf12 was nucleolar in all cells that underwent heat shock and was also detected in the nucleoplasm, albeit to a lesser extent (Fig. 5.5B).

AM-D treatment has previously been shown to result in the disappearance of nucleoli and nucleolar proteins by inhibiting rDNA transcription (Chapter 2, 3; Kawashima et al., 1979; Balbo and Bozzaro, 2006). Given the nucleolar C-GFP-Snf12 observed in ~20% of untreated cells as well as in all cells that underwent heat shock, cells were treated with AM-D in order to observe the effect on the localization of Snf12. After 4 h of AM-D treatment Snf12 was detected by anti-Snf12 in nucleoli, which were bulging out from the nucleus on the opposite side from the centrosome (Fig. 5.5C,D,E). After 8 h it was detected in cytoplasmic islands near the nucleus (Fig. 5.5C,D,E). After recovery from AM-D treatment these islands remained in the cytoplasm. Together, these results show that Snf12 redistributes to the nucleolus in response to cell stress.

5.4.8 Snf12 redistributes to the cytoplasm during mitosis

In mammals several nucleolar proteins have been shown to change location during mitosis (Ginisty et al., 1999; Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). Recently, *Dictyostelium* nucleolar proteins NumA1 and CBP4a have also been shown to redistribute
Figure 5.5. Effect of cycloheximide, heat shock, and AM-D on Snf12 localization. A) Untreated or cycloheximide-treated cells were fixed and probed with anti-Snf12 followed by anti-NumA1. Snf12 was not detected in cycloheximide-treated cells. In accordance with previous results, NumA1 was detected (Chapter 2). Merged image shows overlay of Snf12 (green), NumA1 (red), and DAPI (blue). Scale bar represents 2 µm. B) Localization of C-GFP-Snf12 in untreated or heat shocked cells. Heat shock resulted in an increase in nucleolar fluorescence intensity. Asterisk represents significant difference. Scale bar represents 2 µm. C) Untreated cells (0 h) or cells treated with AM-D for either 4 h, 8 h, or 4 h followed by 4 h recovery (4 h recover) were fixed and probed with anti-Snf12 followed by anti-α-tubulin. After 4 h treatment Snf12 was detected in nucleoli (arrow) that were bulging from the nucleus, always on the opposite side of the nucleus as the centrosome. After 8 h Snf12 was detected in cytoplasmic circles (arrow) adjacent to the nucleus. These circles remained (arrow) despite recovery from treatment. Merged image shows overlay of Snf12 (red), α-tubulin (green), and DAPI (blue). Scale bar represents 2 µm. D) Enlarged view of the nucleus from the merged image in (A). Scale bar represents 2 µm. E) Diagrammatic representation of the localization of Snf12 (black) during AM-D treatment.
throughout the nucleus during this time (Chapter 2, 3). We therefore examined the localization of Snf12 throughout the various stages of mitosis. During prometaphase Snf12 was detected by anti-Snf12 in the nucleoplasm however to a lesser extent than during interphase (Fig. 5.6A). The cytoplasmic levels of Snf12 also increased at this time. During metaphase and anaphase the cytoplasmic levels increased further overwhelming any nucleoplasmic fluorescence that otherwise might be visible. During telophase cytoplasmic levels decreased to that observed during prometaphase and nucleoplasmic fluorescence was again visible (Fig. 5.6A). The distribution of C-GFP-Snf12, N-GFP-Snf12, and GFP-COG-NLS throughout the cell during mitosis was similar to that observed using anti-Snf12 (Fig. 5.6B).

5.5 Discussion

Snf12 is a Dictyostelium homologue of the highly conserved BAF60a (Snf12 in yeast), a member of the SWI/SNF nucleosome remodeling complex (Lee et al., 2002; Oh et al., 2008). The involvement of BAF60a in the transcriptional regulation of several cell cycle genes in other organisms prompted the investigation of the role of Snf12 in Dictyostelium in order to better understand the cell cycle in this model eukaryote (Reisman et al., 2009; Weissman and Knudsen, 2009). This study has shown via immunolocalization as well as with GFP-fusion proteins that Snf12 localizes predominately to the nucleoplasm of Dictyostelium cells. A similar localization has been observed in mice (Li et al., 2008). In ~20 % of cells Snf12 was also detected in nucleoli. It is unclear why Snf12 localizes to nucleoli in only a portion of cells however it may be related to their stage in the cell cycle. A similar situation exists in yeast where SWI/SNF complex member Snf2 localizes predominantly to nuclei with a small percentage localizing to nucleoli (Dror and Winston, 2004).
Figure 5.6. Snf12 redistributes to the cytoplasm during mitosis. A) Cells were fixed and probed with anti-Snf12 followed by anti-α-tubulin. Snf12 was detected less in the nucleus and more in the cytoplasm of prometaphase and telophase cells. During metaphase and anaphase Snf12 was detected throughout the entire cell. Merged image shows overlay of Snf12 (red), α-tubulin (green), and DAPI (blue). Scale bar represents 2 µm. B) Cells expressing either N-GFP-Snf12, C-GFP-Snf12, or GFP-COG-NLS were fixed and probed with anti-GFP followed by anti-α-tubulin (Only GFP-COG-NLS is shown). During prometaphase these proteins were detected less in the nucleus whereas during metaphase, anaphase, and telophase they were detected throughout the entire cell. Merged image shows overlay of anti-GFP (green), α-tubulin (red), and DAPI (blue). Scale bar represents 2 µm.

Of the three putative NLSs only NLS-3 (\textsuperscript{372}KRKR\textsuperscript{375}) was both necessary and sufficient for nucleoplasmic and nucleolar localization. It therefore represents a NLS/NoLS. This represents only the second NoLS and NLS/NoLS identified in Dictyostelium. It is similar in sequence to both the NLS/NoLS identified in NumA1 and the verified NLS in CBP4a which may or may not be responsible for localizing CBP4a, however is capable of concentrating FITC to the nucleus (Chapter 2, 3). NumA1 and CBP4a are two nucleolar proteins in Dictyostelium also linked to the regulation of cell cycle events (Chapter 2, 3). The Snf12 and NumA1 NLS/NoLSs are similar to
NoLSs in other organisms in that they contain a high proportion of basic residues (Musinova et al., 2010; Wang et al., 2010; Zhou et al., 2010). In fact six consecutive basic residues (either arginine or lysine) have been shown to be sufficient for nucleolar localization in HeLa cells (Musinova et al., 2010; Wang et al., 2010). However the situation is not this simple since residues KRKR (Snf12 NLS/NoLS), RPRK (NumA1 NLS/NoLS), and KKCK (CBP4a NLS) all contain a high proportion of basic residues but show different localization patterns in *Dictyostelium* (Chapter 2, 3). Furthermore, although both NLSs and NoLSs contain a high proportion of basic residues, NoLSs do not necessarily also function as NLSs, and thus it is important to distinguish between NLS, NoLS, and NLS/NoLS (Scott et al., 2010).

Snf12 redistributed to nucleoli when cells were either heat shocked or treated with AM-D indicating that Snf12 is involved in the stress response in *Dictyostelium*. *Dictyostelium* Hsp32 has also been shown to redistribute from the nucleolar periphery to throughout the nucleolus during heat shock (Moerman and Klein, 1998). This is not surprising given that the nucleolus is the central hub for coordinating the response to cell stress (Boulon et al., 2010). Nucleolar composition is known to change in response to stress (Boulon et al., 2010). In fact heat shock and AM-D treatment have both been shown to result in the nucleolar accumulation of several proteins (Kodiha et al., 2005; Mark et al., 2001). Although AM-D treatment usually leads to nucleolar proteins leaving the nucleolus, the fact that some increase in concentration instead reinforces the fact that the nucleolus is not just a ribosome factory (Andersen et al., 2005). Perhaps when *Dictyostelium* cells are stressed Snf12 is sequestered in nucleoli in order to inhibit the SWI/SNF complex-mediated transcription of cell cycle genes, leading to a temporary halt in the cell cycle. This would be similar to the tumour suppressor ARF which binds to and sequesters the p53 negative regulator MDM2 in the nucleolus thereby activating p53, a negative
regulator of growth and division (Iwakuma and Lozano, 2003; Tao and Levine, 1999; Levine, 1997).

The redistribution of Snf12 to nucleoli is followed by the appearance of Snf12 cytoplasmic circles, adjacent to the nucleus. These circles are thought to leave the nucleus via the budding-off of nucleoli. The significance of these morphological changes in nucleolar structure is unknown however a similar response has been observed for CBP4a (Chapter 3). This could represent a stress-induced nucleolar aggregate, similar to the nucleolar aggregates observed after proteasome inhibition (Latonen, 2011; Latonen et al., 2011). These ‘aggresomes’ are thought to represent nucleoplasmic proteins targeted for degradation via the proteasome pathway (Latonen, 2011; Latonen et al., 2011). It is possible that AM-D induces a similar response in *Dictyostelium* however further study is needed to determine whether these cytoplasmic circles are eventually degraded.

In order to gain insight into the function of the SWIB and COG domains GFP-fusion and deletion constructs were tested. GFP-SWIB and -COG localized throughout the entire cell probably because these fusion proteins lacked NLS-3. Accordingly, GFP-COG-NLS and -COG-2, which possessed NLS-3, localized predominately to the nucleus. Although GFP-COG-1 possessed NLS-3 it did not preferentially localize to the nucleus, possibly due to the tertiary structure of this fusion protein which may have prevented exposure of NLS-3. It is not known why GFP-Snf12-ΔCOG was not detected in cells since it was detected at the proper molecular weight on a western blot. GFP-Snf12-ΔSWIB localized to the nucleoplasm indicating that the SWIB domain is not involved in the localization of Snf12. Together, these results confirm that NLS-3 is the only functional NLS/NoLS in Snf12 while the SWIB and COG domains have
functions other than localization. Further study is needed in order to better understand the role of these domains within Snf12.

A common feature of nucleolar proteins is their redistribution to non-nucleolar locales during mitosis (Cha et al., 2004; Gas et al., 1985; Hussain et al., 2009; Yun et al., 2008). To date the only nucleolar proteins studied during mitosis are NumA1 and CBP4a, both of which redistribute to non-nucleolar locales during mitosis (Chapter 2, 3). Fittingly, Snf12 redistributed throughout the cytoplasm during mitosis. This redistribution was similar to that of the NumA1-binding protein PsaA during mitosis (Chapter 3). Interestingly, GFP-COG-NLS also redistributed throughout the cytoplasm during mitosis suggesting that the putative NES is not responsible for this nuclear export. Snf12 may be needed in the cytosol at this time or perhaps its presence in the nucleus during mitosis would be detrimental.

Given the function of BAF60a in other organisms, *Dictyostelium* Snf12 is thought to be a member of the SWI/SNF complex responsible for the transcriptional regulation of cell cycle genes in the nucleoplasm. It may either sequester other cell cycle proteins or is itself sequestered in the nucleolus as a result of cell stress. Although no p53 equivalent has been identified in *Dictyostelium* the presence of Snf12, a p53-binding protein in other organisms, suggests that a *Dictyostelium* functional equivalent to p53 may exist.
5.6 References


Sudarsanam P and Winston F (2000). Recent advances in understanding transcriptional control by the Snf/Swi family of nucleosomes remodeling complexes. Trends Genet. 16: 345-351.


5.7 Supplementary figures

Supplementary figure 5.1. N-GFP-Snf12 and C-GFP-Snf12 localize to spore nuclei. Cells expressing either GFP alone, N-GFP-Snf12 (not shown), or C-GFP-Snf12 were allowed to develop into fruiting bodies. Both N-GFP-Snf12 and C-GFP-Snf12 were detected in the nucleus of spore cells whereas GFP alone was detected throughout the entire spore, as demonstrated by colocalization with calcofluor. No fluorescence was detected in stalk cells. Merged image shows overlay of GFP fluorescence (GFP fluor.; green) and calcofluor (blue). Scale bar represents 2 µm.
Chapter 6
Rad53 homologue FhkA is a nucleolar protein that redistributes to the cytoplasm during mitosis in *Dictyostelium*

6.1 Abstract

*Dictyostelium* is a model eukaryote used for the study of several cellular processes and human diseases however relatively little is known about its cell cycle. It possesses homologues for most cell cycle proteins, few of which have been characterized. Several recently identified nucleolar proteins in *Dictyostelium* are thought to be involved in cell cycle events. Fittingly, the nucleolus is known to house several cell cycle proteins. Yeast Rad53 (Chk2 in humans) is a well characterized nuclear cell cycle protein involved in DNA damage response. The *Dictyostelium* homologue, FhkA, was thus examined in order to determine if this cell cycle related protein resides in the nucleolus. This study shows that FhkA is a nucleolar protein. It was detected via immunolocalization at the periphery of the nucleolar patches being more concentrated on the side adjacent to the nuclear envelope. AM-D treatment (4 h) resulted in the budding of nucleolar FhkA from the nucleus. After 8 h FhkA was detected in cytoplasmic circles adjacent to the nuclear envelope, opposite the side of the centrosome. Like other nucleolar proteins in *Dictyostelium*, FhkA redistributed throughout the entire cell during mitosis but was also detected at the nuclear envelope region and spindle fibers region from prometaphase through telophase.
6.2 Introduction

*Dictyostelium* is a model eukaryote used to study a multitude of fundamental cellular processes including growth, motility, and development (Williams, 2010). It is also an excellent model for the study of several human diseases such as bacterial and viral infections as well as several diseases affecting the central nervous system (Annesley and Fisher, 2009). Despite this, relatively little is known about its cell cycle. *Dictyostelium* possesses homologues for most cell cycle proteins however few have been characterized to any significant degree (Eichinger et al., 2005). Recently, several nucleolar proteins have been identified in *Dictyostelium* some of which are thought to be involved in the cell cycle (NumA1, its binding partner CBP4a, and Snf12; Chapter 2, 4, 5; Myre and O’Day, 2002; 2004a; 2004b). This is not surprising since the nucleolus is known to house several proteins involved in the regulation of cell cycle events (Peterson and Tsai 2009; Robertson 2007; Visintin and Amon 2000). The nucleolus and nucleolar proteins of *Dictyostelium* have received relatively little attention in recent years however investigating nucleolar protein in *Dictyostelium* may help us better understand the cell cycle.

Rad53 (Chk2 in humans) is a tumour suppressor involved in DNA damage response (Stracker et al., 2009; Bartek et al., 2001). It functions downstream of the well studied ATM and ATR, two central proteins in the DNA damage response signaling cascade (Bartek et al., 2001). Rad53 is a nuclear protein that possesses a C-terminal bipartite NLS required for nuclear entry (Smolka et al., 2005). It also possesses a kinase domain and two FHA domains (Hammet et al., 2003; Pellicoli and Foiani, 2005). It is phosphorylated at several serine and threonine sites (Smolka et al., 2005; Sweeney et al., 2005). Cell cycle kinase CDK1 phosphorylates Rad53 in metaphase suggesting that CDK1 may regulate Rad53 (Diani et al., 2009). The intimate relationship between Rad53 and the cell cycle led to investigation of the *Dictyostelium* Rad53 homologue,
FhkA, in order to better understand these events in *Dictyostelium*. Unlike in other organisms, *Dictyostelium* possesses five Rad53 family members, FhkA, B, C, D, and E (Goldberg et al., 2006). The significance of this expanded family is unknown. Given the relationship between the nucleolus and the cell cycle it was also of interest to determine if FhkA is a nucleolar protein.

This study shows that *Dictyostelium* FhkA is a nucleolar protein localizing to the nucleolar periphery in interphase cells. As is the case for other nucleolar proteins, FhkA leaves the nucleolus after treatment with the rDNA transcription inhibitor AM-D and during mitosis it redistributes throughout the entire cell.

6.3 Materials and methods

6.3.1 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 (Fey et al., 2007).

6.3.2 Antibody production

A peptide equivalent to a unique C-terminal sequence from FhkA (\textsuperscript{554}NQLQLNEGELLKKRTFLSN\textsuperscript{573}; Fig. 6.1A) was synthesized using solid phase synthesis (with the addition of an N-terminal cysteine required for subsequent conjugation), purified via HPLC, and verified via mass spectroscopy prior to KLH conjugation via an MBS linker (Advanced Syntech Inc., Markham, ON). Conjugation was performed via the method described
Figure 6.1. Domain structure diagram of FhkA/Rad53/Chk2. A) FhkA (Rad53 in yeast, Chk2 in humans) possesses several putative FHA domains (asterisk indicates verified domains; Hammett et al., 2003; Pellicioli and Foiani, 2005). NLSs and the antigen site for anti-FhkA are indicated. Numbers represent amino acid position. B) FhkA was detected by anti-FhkA at ~70 kDa.

by Lateef et al., 2007. Polyclonal antibodies against this KLH-conjugated peptide were produced in New Zealand White rabbits at the University of Toronto at Mississauga Animal Care Facility according to University of Toronto protocols for antibody production and animal care as previously described (Chapter 3; O’Day et al., 2009). The preimmune serum (rabbit serum prior to any injections) was kept and used as a control for immunolocalization experiments. The specificity of the crude serum was verified as anti-FhkA and used for all western blots and immunolocalization experiments.

6.3.3 SDS-PAGE and western blotting

Cells (1-2 x 10^6 cells/mL) were resuspended in lysis buffer and sonicated. The Bradford assay was used to quantify protein levels. 20 mg total protein was separated on a 12 % SDS-PAGE gel and transferred onto a PVDF membrane (Pall corp.). The membrane was blocked with 5 % non-fat milk in washing buffer (TBS and 0.001 % Tween 20) at 4 °C overnight and then probed with anti-FhkA (1:200) in 5 % non-fat milk in washing buffer for 1 h at room temperature followed by probing with secondary anti-rabbit HRP (1:800; Santa Cruz®) in the same conditions. Bands
were detected with Amersham ECL Plus™ Western Blotting kit (General Electric) using the
STORM Scanner System.

6.3.4 Treatment, fixation, and immunolocalization

Fixation in ultracold methanol and immunolocalization was performed as previously described
(Chapter 2; Hagedorn et al., 2006). Cells were either untreated or treated while adhering to
coverslips (in HL-5) with either 0.5 mg/mL cycloheximide (1, 2, 4, or 8 h) or 0.05 mg/mL AM-D
(4 h, 8 h, or 4 h followed by three washes with HL-5 prior to 4 h recovery in HL-5). After
blocking, cells were incubated with anti-FhkA for 60 min followed by Alexa Fluor® 555 goat
anti-rabbit (Invitrogen™) for 45 min. Cells were then incubated with anti-α-tubulin (Hybridoma
Bank) for 60 min followed by Alexa Fluor® 488 goat anti-mouse (Invitrogen™) for 45 min. The
localization of α-tubulin was used to determine the stage of cell cycle. 5 µL ProlongAntifade
(Invitrogen™) containing DAPI (5 µL) was placed on a slide prior to mounting and coverslips
were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent
microscope equipped with a Nikon Digital-Sight DS-Ri1 camera and images were analyzed (e.g.
intensity measurements) using Nikon Imaging Software Elements Basic Research 3.0.

6.4 Results

6.4.1 Dictyostelium FhkA is similar to yeast Rad53 and human Chk2
A ClustalW2 alignment showed that the amino acid sequence of Dictyostelium FhkA is 28.8 %
identical and 53.6 % conserved to yeast Rad53 and 27.8 % identical and 45.7 % conserved to
human Chk2. It possesses several putative FHA domains common to nuclear proteins (Fig. 6.1A;
Durocher and Jackson, 2002; Puntervoll et al., 2003). Rad53 and Chk2 also possess multiple
FHA domains some of which have been verified (Hammet et al., 2003; Pellicioli and Foiani,
FhkA possesses a putative monopartite NLS in the C-terminal similar to the C-terminal bipartite NLS within Rad53 and Chk2.

6.4.2 FhkA localizes to the periphery of nucleoli

Probing of western blots with anti-FhkA revealed a band at ~70 kDa corresponding to the predicted molecular weight of FhkA (66 kDa) demonstrating the specificity of anti-FhkA (Fig. 6.1B). FhkA was detected via immunolocalization using anti-FhkA at the periphery of the nucleolar patches, as demonstrated by colocalization with DAPI unstained nuclear regions (Fig. 6.2A,B). It was detected predominantly at the nucleolar periphery adjacent to the nuclear envelope. Alternatively cells were probed with either the secondary antibody alone or the preimmune serum in place of anti-FhkA. These cells displayed either no fluorescence or diffuse fluorescence throughout the cell respectively (Fig. 6.2A).

6.4.3 AM-D treatment leads to redistribution of FhkA from nucleoli to cytoplasmic circles

AM-D is an rDNA transcription inhibitor. Accordingly, AM-D treatment has previously been shown to result in the disappearance of nucleolar proteins as well as the entire nucleolus (Chapter 2, 3, 5; Balbo and Bozzaro, 2006; Kawashima et al., 1979). Here, 4 h of AM-D treatment resulted in the bulging of FhkA from the nucleus (Fig. 6.3A,B). FhkA was most prevalent at the periphery of the bulge and less prevalent in the region joining the bulge to the nucleus. After 8 h of treatment these bulges appeared as circles outside of but adjacent to the nucleus (Fig. 6.3A,B). FhkA was detected around the periphery of each circle but was less prevalent in the region of the circle adjacent to the nucleus. After recovery from AM-D treatment FhkA was not detected in the nucleus or nucleolus but was still present in cytoplasmic circles that were not adjacent to the nucleus (Fig. 6.3A,B). The bulges and nuclei-associated circles were always located away from the centrosomal area (Fig. 6.3A,B).
6.4.4 FhkA redistributes to the cytoplasm during mitosis

A common feature of mammalian nucleolar proteins is their redistribution to non-nucleolar locales during mitosis (Ginisty et al., 1999; Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). Recently, Dictyostelium nucleolar proteins have also been shown to redistribute during mitosis (Chapter 2, 3, 5). FhkA was therefore examined to determine if it undergoes a similar pattern of redistribution. During prometaphase and metaphase FhkA was detected by anti-FhkA throughout the entire cell as well as at the nuclear envelope region (Fig. 6.4). During anaphase it was detected throughout the entire cell but not predominately at the nuclear envelope region. During telophase however, it was also detected throughout the entire cell, at the nuclear envelope region, and at the spindle fibers region (Fig. 6.4).
Figure 6.3. Effect of AM-D on FhkA localization. A) AM-D treatment (4 h) resulted in the budding (B) of nucleolar-localized FhkA from the nucleus. After 8 h FhkA was detected as cytoplasmic circles adjacent to the nucleus, always on the opposite side as the centrosome. After recovery (4 h) from AM-D treatment FhkA was still detected in cytoplasmic circles which were not adjacent to the nucleus. Merged image shows overlay of FhkA (red), α-tubulin (green), and DAPI (blue). Scale bar represents 2 µm. B) Enlarged view of FhkA localization after AM-D treatment (enlarged from merged images in (A)). FhkA budding (B), circles (C), and centrosomes (Ct) are indicated. C) Diagrammatic representation of FhkA localization after AM-D treatment. Black represents FhkA.
Figure 6.4. FhkA redistributes throughout the cell during mitosis. During prometaphase through telophase FhkA was detected throughout the entire cell. It was also detected at the nuclear envelope region (N) and spindle fibers region (S). Although multinucleate cells are sometimes shown, the pattern was the same in mononucleate cells. Merged image shows overlay of FhkA (red), α-tubulin (green), and DAPI (blue). Scale bar represents 2 µm.

6.5 Discussion

*Dictyostelium* is a model for the study of several fundamental cellular processes and biomedical diseases however little is known about its cell cycle (Annesley and Fisher, 2009; Williams, 2010). Given the relationship between the cell cycle and the nucleolus this study investigated the possibility that the *Dictyostelium* Rad53 homologue, FhkA, was a nucleolar protein. This study has shown via immunolocalization that *Dictyostelium* FhkA is a nucleolar protein which localizes to the periphery of nucleolar patches in interphase cells. At present, seven nucleolar proteins have been identified in *Dictyostelium* including FhkA.

The localization of FhkA to the nucleolar periphery is similar to that observed for Hsp32 (Moerman and Klein, 1998). Hsp32 was detected in the shape of a beaded string around the
periphery of each nucleolar patch, a pattern corresponding to the rDNA in Dictyostelium (Moerman and Klein, 1998; Simon and Olins, 1994). Hsp32 has been shown to interact with rDNA however this interaction is not responsible for its localization (Moerman and Klein, 1998). Although FhkA and Hsp32 may colocalize around the nucleolar periphery, FhkA displays a slightly different pattern of localization: it was detected more on the side adjacent to the nuclear envelope and it did not appear as a beaded string.

AM-D treatment has previously been shown to result in nucleolar dissolution accompanied with the disappearance of nucleolar proteins in Dictyostelium (Chapter 2, 3, 5; Balbo and Bozzaro, 2006; Kawashima et al., 1979). Fittingly, FhkA also left the nucleolus after treatment with AM-D. Nucleolar FhkA budded off from the nucleus in a manner similar to that previously observed for CBP4a and Snf12. Also, as previously observed for CBP4a and Snf12, these buds were on the opposite side of the nucleus as the centrosome (Chapter 3, 5).

Nucleolar proteins redistribute to non-nucleolar locales during mitosis (Ginisty et al., 1999; Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). This is also the case in Dictyostelium (Chapter 2, 3, 5). Fittingly, FhkA also redistributed throughout the entire cell during this time. It was also detected at the nuclear envelope region from prometaphase through telophase. It was not possible to determine if FhkA was adjacent to the inner nuclear envelope, outer nuclear envelope, or colocalized with the nuclear envelope, however the pattern was similar to that observed for NumA1. Moreover, like NumA1, FhkA was detected at the spindle fibers region as well. NumA1, CBP4a, Snf12, and FhkA represent the first nucleolar proteins for which their dynamics during mitosis has been studied.
6.6 References


Stracker TH, Usui T, and Petrini JHJ (2009). Taking the time to make important decisions: The checkpoint effector kinases Chk1 and Chk2 and the DNA damage response. DNA Repair. 8: 1047-1054.


Chapter 7
Identification of nucleolar subcompartments in Dictyostelium

7.1 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 those caused by viral infections and cancer. Dictyostelium is a model eukaryote for the study of fundamental biological processes as well as several human diseases however 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 AM-D. Different proteins leave 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. This study shows that Dictyostelium nucleolar proteins NumA1, eIF6, and Bud31 localize throughout the entire nucleolus while CBP4a localizes to only a portion, representing nucleolar subcompartment (NoSC) 1. SWI/SNF complex member Snf12 localizes to a smaller area within NoSC1 representing a second nucleolar subcompartment, NoSC2. The NLS/NoLS, KRKR, from Snf12 localized GFP to NoSC2, and thus also appears to function as a nucleolar subcompartment localization signal. 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.
Introduction

The nucleolus is the site of ribosomal subunit biogenesis however it also houses several proteins involved in a multitude of cellular processes such as DNA replication, DNA repair, regulation of stress response, and the regulation of cell cycle events (Boisvert et al., 2007; Pederson and Tsai, 2009; Visintin and Amon, 2000). It is linked to several human diseases including viral infections and cancer (Andersen et al., 2005; Boisvert et al., 2007; Shiue et al., 2010). The human nucleolar proteome contains more than 700 proteins of which 70% are involved in non-ribosomal processes (Andersen et al., 2005). Nucleolar proteins are also highly conserved as 90% have clear homologues in yeast (Andersen et al., 2005; Boisvert et al., 2007). Eukaryotes typically have either a tripartite nucleolus comprised of three subcompartments, the FC (site of untranscribed rDNA), DFC (site of rRNA transcription and modification), and GC (site of ribosomal subunit assembly) or a bipartite nucleolus comprised of regions equivalent to the FC and GC (Boisvert et al., 2007; Thirty and Lafontaine, 2005).

Dictyostelium is a model eukaryote for the study of several fundamental cellular processes however little is known about its nucleolus. Its nucleolus consists of 2-4 patches adjacent to the inner nuclear envelope (Maclean et al., 1984; Sameshima et al., 1991). Unlike in other organisms no subcompartments are visible and it is therefore neither bipartite nor tripartite. Rather, granular and fibrillar material are interspersed throughout (Maeda and Takeuchi, 1969). The rDNA, which is not visible by electron microscopy, exists not in the center but around the periphery of each patch (Moerman and Klein, 1998; Simon and Olins, 1994; Maeda and Takeuchi, 1969). Polysphondylium possess a nucleolus which is morphologically similar to that of Dictyostelium however it has not been examined to any level of detail and the location of the rDNA has not
been determined (Hohl et al., 1970). Dictyostelium thus represents the only organisms for which this type of nucleolus has been studied.

Transcription of rDNA leads to nucleolar formation (Karpen et al., 1988; Tschochner and Hurt, 2003). Accordingly, treatment with the transcription inhibitor AM-D results in the loss of nucleoli and nucleolar proteins in Dictyostelium (Chapter 2, 3, 5, 6; Balbo and Bozzaro, 2006; Kawashima et al., 1979). Snf12 is unique in that it first accumulates in the nucleolus after AM-D treatment before eventually leaving (Chapter 5). In other organisms the AM-D-induced nucleolar dissolution results from the segregation of the different subcompartments. The recent findings that different proteins leave the nucleolus in different ways after AM-D treatment in Dictyostelium therefore suggests that nucleolar subcompartments may exist. The identification of nucleolar subcompartments in Dictyostelium would allow for a better understanding of the structure/function relationship of the Dictyostelium nucleolus.

Until recently only three nucleolar proteins had been identified in Dictyostelium: Hsp32, eIF6, and TRAP (Moerman and Klein, 1998; Balbo and Bozzaro, 2006; Yamaguchi et al., 2005). The recent discovery of four additional nucleolar proteins (NumA1, CBP4a, Snf12, and FhkA) as well as Bud31 identified here have allowed the investigation of the existence of functional subcompartments in the nucleolus of Dictyostelium via colocalization experiments (Chapter 2, 3, 5, 6).

This study has identified two novel nucleolar subcompartments which were called nucleolar subcompartment (NoSC) 1 (containing CBP4a) and NoSC2 (containing Snf12). NoSC2 is contained within NoSC1. This study has also identified a NoSC2 localization signal. Given these new findings, this study has found similarities between the redistribution of nucleolar proteins in
response to AM-D treatment and during mitosis. A model for the AM-D-induced redistribution is presented.

7.3 Materials and methods

7.3.1 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 (Fey et al., 2007). 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.

7.3.2 Antibodies and GFP-fusion protein-expressing cell lines

Anti-NumA1, anti-CBP4a, and anti-Snf12 were produced and verified as previously described (Chapter 2, 3, 5; O’Day et al., 2009). Cell lines expressing either C-GFP-Snf12 or GFP-NLS-3 (GFP-KRK) from Snf12 were made and verified as previously described (Chapter 5). 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 (Chapter 3). Primers used to amplify eIF6 were CAGAGCTCAAAATGGCTACAAGATTAC (forward) and CAACTAGTTACATTATCAACAATTGAATTTC (reverse). Primers used to amplify Bud31 were CAGAGCTCAAAATGCCAAAAATAAAA (forward) and CAACTAGTACTTTCCATTGCCTTCA (reverse). Restriction sites (underlined) and kozak site were incorporated. Full length eIF6 and Bud31 were ligated into pDM-323 (for expression of C-terminally-fused GFP) as previously described (Chapter 3). Constructs for GFP-eIF6, GFP-Bud31, and pDM-317 (for expression of GFP alone) were transformed into Dictyostelium as previously described (Chapter 3). Colonies were selected and maintained in 10 µg/mL G418.
7.3.3 SDS-PAGE and western blotting

Cells (1-2 x 10^6 cells/mL) were resuspended in lysis buffer and sonicated. The Bradford assay was used to quantify protein levels. 20 mg total protein (0.1 mg for cells expressing GFP alone) was separated on a 12 % SDS-PAGE gel and transferred onto a PVDF membrane (Pall corp.). The membrane was blocked with 5 % non-fat milk in washing buffer (TBS and 0.001 % Tween 20) at 4 °C overnight and then probed with anti-GFP (1:600; Santa Cruz®) in 5 % non-fat milk in washing buffer for 1 h at room temperature followed by probing with secondary anti-mouse HRP (1:800; Santa Cruz®) in the same conditions. Bands were detected with Amersham ECL Plus™ Western Blotting kit (General Electric) using the STORM Scanner System.

7.3.4 Live cell viewing, treatment, fixation, and immunolocalization

GFP-fusion protein-expressing cells were viewed live after adhering to a coverslip for 30 min prior to slide-mounting. Fixation in ultracold methanol and immunolocalization was performed as previously described (Hagedorn et al., 2006). Cells were allowed to adhere to coverslips (in HL-5). After blocking, cells were incubated with either anti-NumA1 (1:40) or anti-Snf12 (1:40) 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™. ProlongAntifade (Invitrogen™) containing DAPI (5 µL) was placed on the slide prior to mounting and coverslips 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.
7.4 Results

7.4.1 Snf12 localizes to a portion of the nucleolus

C-GFP-Snf12 was recently shown to localize predominately to the nucleoplasm and to a lesser extent the nucleolus (Chapter 5). In order to determine if Snf12 localizes throughout the entire nucleolus C-GFP-Snf12 was colocalized with the nucleolar marker NumA1 (Chapter 2). The edge of the nucleolar area occupied by NumA1 colocalized with the edge of the DAPI-stained nucleoplasm (Fig. 7.1A). This demonstrates that NumA1 occupies the entire nucleolus, since DAPI does not stain nucleoli (Moerman and Klein, 1998). C-GFP-Snf12 was detected in a smaller region within the NumA1-occupied area (Fig. 7.1A). Anti-Snf12 detected C-GFP-Snf12 throughout the nucleoplasm but only in a portion of the nucleolus (Fig. 7.1B).

7.4.2 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 unstained nuclear regions (Fig. 7.2A). 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. 7.2B). Its shape varied from globular to linear (Fig. 7.2B).
Figure 7.1. 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 DAPI unstained nuclear regions (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 unstained nuclear regions. Entire cell (Cell) and enlarged image of nucleus (Nuc.) are shown. Only C-GFP-Snf12 is shown. Scale bar represents 2 µm.

7.4.3 GFP-eIF6 and GFP-Bud31 colocalize with NumA1 throughout the entire nucleolus

Dictyostelium Bud31 is a putative RNA splicing factor and DNA transcription factor homologous to Bud31 from yeast and humans (Masciardri et al., 2004; dictyBase.org). This suggests that it may be a nucleolar protein in Dictyostelium. This was confirmed as GFP-Bud31 localized throughout the nucleus but predominately to nucleoli of both live and fixed cells (Fig. 7.3A,B,C). eIF6 has previously been shown to be a nucleolar protein in Dictyostelium (Balbo and Bozzaro, 2006). Here, a GFP-eIF6-expressing cell line was made using the vector pDM-323 instead of pDEX-GFPC, which was used by Balbo and Bozzaro (2006). As expected, GFP-eIF6 was detected predominately in nucleoli of both live and fixed cells, displaying the same pattern of localization previously observed by Balbo and Bozzaro (2006; Fig. 7.3A,B,C). Colocalization of either GFP-Bud31 or GFP-eIF6 with NumA1 (via immunolocalization)
revealed that both GFP-fusion proteins colocalize with NumA1 throughout the entire nucleolus (Fig. 7.3B,C). Western blots confirmed that both GFP-Bud31 and GFP-eIF6 were properly expressed (data not shown).
7.4.4 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 (Chapter 3; Myre and O’Day, 2004).

Colocalization of either GFP-eIF6 or GFP-Bud31 with CBP4a (via immunolocalization) revealed...
that CBP4a occupies only a portion of the nucleolar area occupied by GFP-eIF6 and GFP-Bud31 (Fig. 7.3C). Colocalization of C-GFP-Snf12 with CBP4a (via immunolocalization) revealed that C-GFP-Snf12 occupies a smaller area within the area occupied by CBP4a (Fig. 7.3D). Thus C-GFP-Snf12 colocalizes with only a portion of CBP4a and CBP4a occupies only a portion of the nucleolus.

7.4.5 GFP-KRKR localizes Snf12 to a region of the nucleolus

Residues $^{372}$KRKR$^{375}$ are both necessary and sufficient for the nucleoplasmic and nucleolar localization of C-GFP-Snf12 (Chapter 5). This study has shown that C-GFP-Snf12 localizes to only a portion of the nucleolus suggesting that the NLS/NoLS KKRK also localizes to this same region. As expected, GFP-KKCK localized to a portion of the nucleolus (Fig. 7.1B). The variations in the size and shape of this region were the same as that observed with C-GFP-Snf12.

7.5 Discussion

Nucleolar organization and function are conserved in eukaryotes, from yeast to humans, with most organisms possessing either a bipartite or tripartite nucleolus (Kressler et al., 2010; Thirty and Lafontaine, 2005). The structure of the Dictyostelium nucleolus is unique in that it is neither bipartite nor tripartite. Furthermore, nucleolar subcompartments have not been previously identified. AM-D treatment in higher eukaryotes leads to the condensation and subsequent separation of the FC and GC nucleolar regions (Shav-Tal et al., 2005). Accordingly, in Dictyostelium different proteins leave the nucleolus in different ways after AM-D treatment suggesting that nucleolar subcompartments may exist (Chapter 2, 3, 5, 6).

This study has found that nucleolar proteins NumA1, eIF6, and Bud31 localize throughout the entire nucleolus while CBP4a localizes to a smaller region within. This region must therefore be different in some way from the rest of the nucleolus, otherwise CBP4a would be distributed
evenly throughout. The region thus represents a nucleolar subcompartment which was called NoSC1. C-GFP-Snf12 localizes to a region within NoSC1 which thus represents a second subcompartment which was called NoSC2. The NLS/NoLS from Snf12 (KRKR) also localized to NoSC2 and thus represents the first nucleolar subcompartment localization signal (NoSCLS) identified in Dictyostelium.

C-GFP-Snf12 localized differently in different cells. The localization pattern varied in size, shape, and position. This variation is most likely not due to a variation in C-GFP-Snf12 expression level since variation in the level of nucleoplasmic C-GFP-Snf12 did not correlate with the variability of size, shape, or position of nucleolar-localized C-GFP-Snf12 (data not shown). Accordingly, 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 showed the same variation (Chapter 5; data not shown). Even GFP-KKCK showed the same pattern of variability as the full length C-GFP-Snf12. Since the size and shape of the entire nucleolus changes with varying conditions and developmental stage it is not unreasonable to suggest that a nucleolar subcompartment may also undergo morphological changes (Matsumoto et al., 1991; Sameshima et al., 1991). Together, this evidence strongly suggests that either the size, shape, and position of NoSC2 are variable or NoSC2 represents several different subcompartments.

Eight nucleolar proteins have been identified in Dictyostelium which localize to one of five main patterns: nucleolus and nucleoplasm (NumA1, eIF6, and Bud31), nucleolus only (TRAP1), NoSC1 only (CBP4a), NoSC2 and nucleoplasm (Snf12), or the nucleolar periphery (Hsp32 and FhkA; Fig. 7.4). Hsp32 and FhkA represent a third possible nucleolar subcompartment, NoSC3. Since this is where the rDNA is located NoSC3 may be the Dictyostelium equivalent of the FC
region (Moerman and Klein, 1998; Simon and Olins, 1994). It is important to note that Hsp32 localizes around the nucleolar periphery in a beaded string pattern whereas FhkA localizes more on the side adjacent to the nuclear envelope and does not appear as a beaded string. However if NoSC3 is equivalent to the FC region, NoSC1 and 2 would be located interior to the rDNA. This is interesting since in other organisms the GC and DFC subcompartments lie exterior to the rDNA.

The diversity in localization patterns strongly suggests diversity in function. Accordingly, most of these eight proteins are each thought to function differently; Hsp32 functions in heat shock response, TRAP1 in the formation of the spore coat, NumA1 and CBP4a have ties to cell cycle regulation, and Snf12 is involved in stress response (Chapter 2, 3, 5; Moerman and Klein, 1998; Balbo and Bozzaro, 2006; Yamaguchi et al., 2005; Myre and O’Day, 2002). As more nucleolar proteins are identified it will be interesting to see if each nucleolar subcompartment houses proteins with similar functions and if there are additional, as yet unidentified, subcompartments.

AM-D treatment has previously been shown to result in the disappearance of the nucleolus as well as nucleolar proteins in *Dictyostelium* (Chapter 2, 3, 5, 6; Kawashima et al., 1979). These proteins leave the nucleolus in different ways: the NumA1-occupied area decreases in size whereas CBP4a, Snf12, and FhkA appear to bud off from the nucleus (Chapter 2, 3, 5, 6). This budding fits with recent work demonstrating that nucleoli behave like liquid-like droplets rather than solids (Brangwynne et al., 2011). The identification of NoSC1 and NoSC2 has allowed the building of 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. 7.4). Given the relationship between the centrosome, microtubules, and the nucleolus in *Dictyostelium* the nucleolar budding may be directed by microtubules, since
Figure 7.4. 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, possibly representing NoSC3. Black represents protein localization and grey represents the nuclear envelope. B) After AM-D treatment NumA1 leaves 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 grey. Given the relationship between centrosome position, microtubules, and the nucleolus, microtubules may help guide the budding nucleolus in the proper direction. (Chapter 2, 3, 5, 6; Balbo and Bozzaro, 2006; Moerman and Klein, 1998; Yamaguchi et al., 2005).

budding always occurs on the opposite side of the nucleus as the centrosome. In fact, in developing cells the microtubules that run along side the nucleus are attached to the nucleus via cross bridges (Sameshima et al., 1991). These microtubules are responsible for determining
nucleolar position as well as the presence and direction of the nucleolar protruding nozzle that exists at this time (Sameshima et al., 1991).

The nucleolus normally breaks down during mitosis with nucleolar proteins redistributing to various non-nucleolar locales (Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983; Ginisty et al., 1999). Accordingly, NumA1, CBP4a, Snf12, and FhkA all display a unique pattern of redistribution during mitosis (Chapter 2, 3, 5, 6). Interestingly, NumA1, which is not detected in the cytoplasm after AM-D treatment, is not detected in the cytoplasm during mitosis. As well, other nucleolar proteins that are detected in the cytoplasm after AM-D treatment are also detected there during mitosis. This further supports the idea that each nucleolar subcompartment houses proteins with similar functions. Although this relationship is certainly noteworthy the details remain to be elucidated since CBP4a is cytosolic after AM-D treatment yet nuclear during mitosis.
7.6 References


Chapter 8
Nuclear export during mitosis in *Dictyostelium*: Signal to leave or signal to stay?

8.1 Abstract

Several lower eukaryotes undergo closed mitosis during which the nuclear envelope remains intact but becomes permeable. *Dictyostelium* is a model eukaryote that undergoes closed mitosis however it is unknown whether the nuclear envelope becomes permeable. Recently nucleolar FhkA, Snf12, and PsaA have been shown to redistribute throughout the cytoplasm during mitosis. Snf12 and PsaA possess putative NESs yet it is unclear whether these NESs are functional, the proteins are actively exported from the nucleus or the nuclear envelope becomes permeable during mitosis. This study shows that GFP-Snf12-ΔNES, GFP-PsaA-ΔNES-1, and -2 redistribute to the cytoplasm during mitosis demonstrating that these putative NESs are not responsible for this export. GFP-KRKR (the NLS/NoLS from Snf12) also redistributes to the cytoplasm during mitosis suggesting that the nuclear envelope becomes permeable during this time. GFP-KRKR is 14.6% more concentrated in the cytoplasm than in the nucleus during anaphase suggesting it is actively exported from the nucleus. This study also shows that during mitosis some nuclear proteins remain in the nucleus, some diffuse into the cytoplasm, and some are more concentrated in the cytoplasm.
8.2 Introduction

Mitosis is the process of cell division. It involves karyokinesis (nuclear division) followed by cytokinesis (cell division). Higher eukaryotes usually undergo open mitosis during which the nuclear envelope breaks down, allowing the mitotic spindle fibers access to the chromosomes, and the nucleolus breaks into its component parts. Lower eukaryotes usually undergo closed mitosis during which the nucleus remains intact. In closed mitosis tubulin must enter the nucleus in order to form the mitotic spindle fibers. The nuclear envelope is a barrier which keeps nucleoplasmic and nucleolar proteins separate from the rest of the cell however during open mitosis the nucleoplasm and cytoplasm are free to mix. The situation is less understood in closed mitosis. However in some organisms the nuclear envelope has been shown to become semi-permeable during this time, allowing nucleocytoplasmic diffusion to occur (De Souza and Osmani, 2007). This is accomplished differently depending on the organism. For example in Saccharomyces cerevisiae the nuclear pore complex (NPC) is modified, in Aspergillus nidulans it is partially disassembled, and in Caenorhabditis elegans the nuclear envelope undergoes localized breakdown near the centrosome (De Souza and Osmani, 2007).

Nucleolar proteins redistribute to non-nucleolar locales during mitosis (Cha et al., 2004; Gas et al., 1985; Hussain et al., 2009; Yun et al., 2008). Studying this redistribution has provided insight into the function of several nucleolar proteins. Determining the mechanism by which nucleolar proteins redistribute to cytoplasmic areas during closed mitosis would allow for a better understanding of these proteins in lower eukaryotes. Given the tight relationship between the nucleolus and several human diseases including cancer and viral infections, the study of nucleolar protein dynamics in different organisms is key (Andersen et al., 2005; Boisvert et al., 2007; Shiue et al., 2010).
*Dictyostelium* is a eukaryotic microbe used for the study of several fundamental cellular processes such as cell growth, chemotaxis, development, and cytokinesis (Williams, 2010). It undergoes closed mitosis. The regulation of cell cycle events in this organism is largely unknown mainly due to the high motility level of most strains which prevents identification of cell cycle stage during live cell imaging (Muramoto and Chubb, 2008). However it is possible to identify the mitotic stages in fixed cells via α-tubulin localization (Cappuccinelli et al. 1979; Kitanishi-Yumura and Fukui 1987; Roos et al. 1984).

*Dictyostelium* nucleolar and nucleoplasmic proteins have previously been shown to distribute throughout the cytoplasm during mitosis. The recently identified Snf12, homologue of SWI/SNF complex member BAF60a, is a nucleolar and nucleoplasmic protein in *Dictyostelium* that redistributes throughout the entire cell from prometaphase through telophase (Chapter 5, 6). A second nucleolar protein, Rad53/Chk2 *Dictyostelium* homologue FhkA, displays a similar pattern of redistribution (Chapter 6). PsaA, a nucleoplasmic protein in *Dictyostelium*, is more concentrated in the cytoplasm than in the nucleus during mitosis, suggesting it is actively exported (Chapter 4). However other nucleolar proteins such as nucleomorphin isoform NumA1 and CBP4a remain predominately intra-nuclear during this time (Chapter 2, 3).

In order to better understand nuclear protein dynamics in *Dictyostelium* during mitosis we examined nucleolar Snf12 and nucleoplasmic PsaA in more detail. Both Snf12 and PsaA possess at least one putative NES (Chapter 4, 5). NESs are ~10 residue leucine-rich sequences required for nuclear export (Dong et al., 2009). NES deletion has been shown to result in nuclear retention for several proteins (Renault et al., 2011; Kazgan et al., 2010). If the NESs in Snf12 and PsaA are responsible for nuclear export during mitosis, their deletion should prevent redistribution of these proteins to the cytoplasm. Snf12 also possesses a NLS/NoLS, $^{372}$KRKR$^{375}$, that is both
necessary and sufficient for the nuclear/nucleolar localization of this protein (Chapter 5). If the nuclear envelope in *Dictyostelium* becomes permeable during mitosis, GFP-KRKR should redistribute throughout the cell during this time.

This study provides evidence suggesting that the nuclear envelope in *Dictyostelium* becomes at least semi-permeable during mitosis. Whereas some proteins appear to diffuse throughout the cell, some appear to be actively exported while others remain in the nucleus. It is therefore likely that some nuclear proteins possess a nuclear retention signal, different from the classical NLS, while others may have a novel type of NES.

### 8.3 Materials and methods

#### 8.3.1 Materials

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich®. 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. 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 (Fey et al., 2007).

#### 8.3.2 Generation of GFP-fusion protein-expressing cell lines

Both PsaA and Snf12 possess putative NESs (Fig. 8.1; Chapter 4, 5). All GFP-fusion protein constructs and deletion constructs were made as previously described with a C-terminally fused GFP (Fig. 8.1; Chapter 4, 5).
Figure 8.1. Domain structure diagram of GFP-fusion proteins. Cell lines were produced expressing GFP-PsaA-ΔNES-1, -2, GFP-PsaA-NES-1, -2, GFP-Snf12-ΔNES, and GFP-Snf12-NES. Western blots (right) show each GFP-fusion protein was detected by anti-GFP. G-P represents GFP-PsaA and G-S represents GFP-Snf12. Numbers represent amino acid position. PsaA and Snf12 NES sequences are also shown.

For GFP-PsaA-ΔNES-1 the region before the deletion was made from annealing two primers (CAAAATGTGTAAATATAATGATCATAAAATATG; forward, and GATCCATATTTATGATCATTATATTTACACATTTT; reverse) resulting in a fragment with SacI and BamHI sticky ends as previously described (Chapter 3, 4). Restriction sites are underlined. The region after was amplified using primers CAGGATCCCAGAAAATGTTGTGAC (forward) and CAACTAGTTTTTTTATCCAATTTAATAATC (reverse). The primers used to generate GFP-PsaA-ΔNES-2 and GFP-Snf12-ΔNES are as follows:

CAGAGCTCAAAAATGTGTAATATAATGATC (forward) and CAAAGCTTTGTGGATTATAC (reverse; GFP-PsaA-ΔNES-2; region before),

CAGAGCTTCCAGCTCAAGATCG (forward) and CAACTAGTTTTTTTATCCAATTTAATAATC (reverse; GFP-PsaA-ΔNES-2; region after),

CAGAGCTCAAAAATGTCTAAAAATGAGTAC (forward) and
CAGGATCCGTATTTTAATACTGGTCTTC (reverse; GFP-Snf12-ΔNES; region before), and
CAGGATCCCTATAACACATACTCCAACC (forward) and
CAACTAGTAGGAGTTGAGAGTTGAG (reverse; GFP-Snf12-ΔNES; region after). Thus
GFP-PsaA-ΔNES-1 and -2 possessed a BamHI and HindIII restriction site in place of NES-1 and
-2 respectively and GFP-Snf12-ΔNES possessed a BamHI restriction site in place of NES-1.

GFP-PsaA-NES-1, -2, and GFP-Snf12-NES were made by annealing sets of primers:

CAAATGGTTAGGAAAAGGTAGATGATTGCTATTAA (GFP-PsaA-NES-1; forward) and
CTAGTTAATACCAATCTACGTTCTCATAGTATTTTTGAGCT (GFP-PsaA-
NES-1; reverse), CAAAATGGTACCTGTAATTGAGTCATTGGAACCTTA (GFP-PsaA-
NES-2; forward) and
CTAGTGATTCCATGACTCAATTACAGGTACCAACATTTTGAGCT (GFP-PsaA-NES-
2; reverse) and CAAAATGATTCCATGACTCAATTACAGGTACCAACATTTTGAGCT (GFP-Snf12-NES; forward) and
CTAGTGATTCCATGACTCAATTACAGGTACCAACATTTTGAGCT (GFP-Snf12-
NES; reverse).

GFP-PsaA and GFP-KRKR were used in previous studies (Chapter 4, 5). GFP-KRKR was
previously referred to as GFP-NLS-3 and the expression of this GFP-fusion protein has been
verified via western blotting (Chapter 5). GFP-PsaA was previously referred to as C-GFP-PsaA
(Chapter 4).

8.3.3 SDS-PAGE and western blotting

Cells (1-2 x 10^6 cells/mL) were resuspended in lysis buffer and sonicated. Protein levels were
quantified using the Bradford assay. 0.1 µg (GFP alone) or 20 µg (all other strains) total protein
were separated on a 12 % SDS-PAGE gel and transferred onto a PVDF membrane (Pall corp.).
The membrane was blocked with 5 % non-fat milk in washing buffer (TBS and 0.001 % Tween
20) at 4 °C overnight and probed with anti-GFP (Santa Cruz®) in 5 % non-fat milk in washing buffer (1:600) for 1 h at room temperature followed by probing with secondary anti-mouse HRP (1:600; Santa Cruz®) in the same conditions. The membrane was then probed with anti-α-tubulin (Hybridoma Bank) followed by secondary anti-mouse HRP in the same conditions. Bands were detected with Amersham ECL Plus™ Western Blotting Detection kit (General Electric) using the STORM Scanner System.

8.3.4 Fixation with ultracold methanol, immunolocalization, and live viewing

GFP-expressing cells were viewed live after adhering to a coverslip for 30 min prior to slide-mounting. For fixation and immunolocalization experiments cells (150 μL of 2 x 10⁶ cells/mL) were allowed to adhere to 0.1 mm circular glass coverslips (McCrone™) for 30 min in a humidity chamber. Cells were fixed with ultracold methanol as previously described (Hagedorn et al., 2006). After blocking, cells were either slide mounted immediately or incubated with either anti-GFP for 60 min followed by Alexa Fluor® 488 goat anti-rabbit (Invitrogen™) for 45 min. Cells were then incubated with anti-α-tubulin (Hybridoma Bank) for 60 min followed by Alexa Fluor® 555 goat anti-mouse (Invitrogen™) for 45 min. The pattern of microtubules was used to determine cell cycle stage. ProlongAntifade (Invitrogen™) containing DAPI (5 μL) was placed on a slide prior to mounting and coverslips were then sealed using nail polish. Cells were viewed with a Nikon 50i epifluorescent microscope equipped with a Nikon Digital-Sight DS-Ri1 camera and images were analyzed using NIS Elements BR 3.0.
8.4 Results

8.4.1 GFP-fusion proteins were properly expressed

PsaA and Snf12 both contain putative NESs (Chapter 4, 5). To determine if putative NESs are responsible for the nuclear exit of either PsaA or Snf12 several GFP-fusion protein-expressing cell lines were made (Fig. 8.1). Western blots probed with anti-GFP verified that all GFP-fusion proteins were expressed properly (Fig. 8.1).

8.4.2 Localization of GFP-PsaA-ΔNES-1, -2, GFP-Psa-NES-1, and -2 during interphase and mitosis

To determine if the putative NESs within PsaA are involved in nuclear export during mitosis cell lines were made expressing a GFP-fusion protein either lacking a putative NES or expressing GFP-NES alone (Fig. 8.1). In both interphase and mitotic cells GFP-PsaA-ΔNES-1 and -2 were expected to localize in a similar pattern as full length GFP-PsaA while GFP-NES-1 and -2 were expected to localize throughout the cytoplasm but not in the nucleus. In interphase cells GFP-PsaA-ΔNES-1 was detected in the nucleoplasm but to a lesser extent compared with C-GFP-PsaA with more detected in the cytoplasm (Fig. 8.2A). In contrast, GFP-PsaA-ΔNES-2 was not concentrated in the nucleus at all but was detected diffusely throughout the entire cell (Fig. 8.2A). As well, GFP-PsaA-NES-1 and -2 were detected throughout the entire cell similar to GFP alone (Fig. 8.2B). During mitosis GFP-PsaA-ΔNES-1 redistributed from the nucleus to the cytoplasm (Fig. 8.3). During metaphase it was much less concentrated in the nucleus than in the cytoplasm (Fig. 8.3).

8.4.3 Localization of GFP-Snf12-ΔNES and GFP-Snf12-NES during interphase and mitosis

To determine if the putative NES within Snf12 is involved in nuclear export during mitosis cell lines were made expressing a GFP-fusion protein either lacking the putative NES (GFP-Snf12-
ΔNES) or expressing GFP-NES alone (GFP-Snf12-NES; Fig. 8.1). In both interphase and mitotic cells GFP-Snf12-ΔNES was expected to localize in a similar pattern as the full length GFP-Snf12 while GFP-NES was expected to localize throughout the cytoplasm but not in the nucleus. In fact, during interphase this was what was observed (Fig. 8.4A). However during mitosis both GFP-fusion proteins were detected throughout the entire cell, similar to the full length GFP-Snf12 (Fig. 8.4B; Chapter 5). A punctate pattern was sometimes observed with anti-GFP. This pattern was also observed when detecting GFP alone and thus is most likely an artifact of this antibody.

8.4.4 GFP-KRKR is more concentrated in the cytoplasm during anaphase

Residues $^{372}$KRKR$^{375}$ within Snf12 have previously been shown to be both necessary and sufficient for the nucleoplasmic and nucleolar localization of Snf12 (Chapter 5).
Figure 8.3. Localization of GFP-PsaA-ΔNES-1 during mitosis. GFP-PsaA-ΔNES-1 redistributed throughout the cytoplasm during prometaphase through telophase. During metaphase it was not detected in the nucleus (arrow). Merged image shows overlay of anti-GFP (green), α-tubulin (red), and DAPI (blue). Scale bar represents 2 µm.

Figure 8.4. Localization of GFP-Snf12-ΔNES and GFP-Snf12-NES during interphase and mitosis. A) GFP-Snf12-ΔNES was detected in the nucleoplasm in both live (left column) and fixed (next three columns) cells while GFP-Snf12-NES was detected throughout the entire cell during interphase (inter.). Merged image shows overlay of anti-GFP (green) and DAPI (blue). B) GFP-Snf12-ΔNES and GFP-Snf12-NES were detected throughout the entire cell in fixed cells during mitosis. Only telophase is shown (telo.). Merged image shows overlay of anti-GFP (green), α-tubulin (red), and DAPI (blue). Scale bar represents 2 µm.
This four residue sequence is sufficient to localize GFP to the nucleus (Chapter 5). It was therefore of interest to see if GFP-KRKR remains in the nucleus during mitosis. GFP-KRKR redistributed throughout the cytoplasm during mitosis (Fig. 8.5A). It was 14.6% more concentrated in the cytoplasm than in the nucleus during anaphase (Fig. 8.5B).

8.5 Discussion

During closed mitosis the nuclear envelope usually becomes permeable allowing the mixing of cytoplasm and nucleoplasm (De Souza and Osmani, 2007). *Dictyostelium* is a model eukaryote that undergoes closed mitosis however it is not known if the nuclear envelope becomes permeable during this time. PsaA, a nucleoplasmic protein, and Snf12, a nucleolar and nucleoplasmic protein, have previously been shown to redistribute throughout the entire cell during mitosis. It is not known whether this redistribution is due to nuclear export or increased nuclear envelope permeability. This study has shown that the nuclear export of PsaA and Snf12 is not due to their putative NESs since GFP-PsaA-ΔNES-1, -2, and GFP-Snf12-ΔNES still redistributed throughout the cytoplasm during mitosis. Fittingly, the Snf12 NLS/NoLS GFP-KRKR also exited the nucleus during mitosis. This shows that residues KRKR are sufficient to localize GFP to the nucleus but not to retain GFP in the nucleus during mitosis. These results strongly support the idea that nuclear membrane permeability increases during mitosis in *Dictyostelium*.

GFP-KRKR is more concentrated in the cytoplasm than in the nucleoplasm during anaphase, suggesting it is actively exported. It is possible that these residues are modified (possibly by phosphorylation) during mitosis changing their function from a NLS to a NES. Alternatively, this may be due to indiscriminant export of nuclear proteins, which is the simplest way to explain why a four amino acid long NLS, capable of localizing GFP to the nucleus during interphase, is
more concentrated in the cytoplasm during mitosis. As well, previous work has shown that N-GFP-PsaA and C-GFP-PsaA are also more concentrated in the cytoplasm during mitosis (Catalano et al., 2011). This was also observed here for GFP-PsaA-ΔNES-1.

The recently identified *Dictyostelium* nucleolar proteins NumA1 and CBP4a are two examples of nuclear proteins that do not leave the nucleus during mitosis (Chapter 2, 3). The disassembly of the nucleolus at this time leads to the redistribution of nucleolar proteins to non-nucleolar locales, not only in *Dictyostelium* but in other eukaryotes as well (Ginisty et al., 1999; Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). The intra-nuclear redistribution of NumA1 and CBP4a during mitosis is not an attribute of *Dictyostelium* nucleolar proteins since nucleolar Snf12 and FhkA redistribute throughout the entire cell (Chapter 5, 6).
In summary, during mitosis, proteins that reside within the nucleus during interphase either remain in the nucleus, concentrate in the cytoplasm, or diffuse freely between nucleoplasm and cytoplasm. The best model to fit such a scenario would be a permeabilized nuclear envelope through which nuclear proteins can diffuse. Some may have a nuclear retention signal while others may have a cytoplasmic retention signal. However this would not account for active nuclear export. Although more work needs to be done to better understand nuclear protein dynamics during mitosis the evidence presented here has given us insight into these events.
8.6 References


Chapter 9
Conclusion

9.1 Summary of goals obtained

After a general summary of which goals were reached the major findings and significance of this research will be discussed. There were five main goals for this thesis:

1. Verify if NumA1, CBP4a, and PsaA are nucleolar proteins.

   NumA1 was verified as a nucleoplasmic and nucleolar protein while CBP4a was predominantly nucleolar. PsaA was found to be nucleoplasmic and not nucleolar. This suggests that the Ca$^{2+}$-dependent interaction between NumA1 and CBP4a is linked to their nucleolar functions. In contrast, NumA1 and PsaA likely work together in the nucleoplasm.

2. Determine if Snf12 and FhkA are nucleolar proteins.

   Snf12 was verified as a nucleoplasmic and nucleolar protein localizing to a nucleolar subcompartment (NoSC2) within the nucleolus. The concentration of Snf12 in the nucleolus increased after AM-D treatment and heat shock demonstrating this protein is involved in stress response. FhkA was found to be nucleolar as well, localizing to the periphery of nucleolar patches, a region possibly representing another subcompartment, NoSC3.

3. Identify and, where possible, verify NLSs and NoLSs within the nucleolar proteins.

   Several NLSs were identified in NumA1 all of which were capable of targeting FITC to the nucleus however residues $^{61}$RPRK$^{64}$ were found to be responsible for targeting NumA1 to the nucleus and nucleolus. This sequence thus represents a NLS/NoLS. CBP4a residues $^{40}$KKCK$^{43}$ function as a NLS but it is unclear if this sequence targets CBP4a to the
nucleus/nucleolus. PsaA residues $^{680}$RKRF$^{683}$ and Snf12 residues $^{372}$KKRK$^{375}$ were identified as the functional NLS and NLS/NoLS for these proteins respectively.

4. Investigate nucleolar protein dynamics during mitosis.

During mitosis NumA1 redistributed to the nuclear envelope region and spindle fibers region while CBP4a redistributed to intranuclear islands and the metaphase plate region. Snf12 and FhkA redistributed throughout the entire cell while FhkA was also detected at the nuclear envelope and spindle fibers region. PsaA redistributed throughout the cell but was more concentrated in the cytoplasm. Each nucleolar protein thus redistributed differently during this time.

5. Determine if nucleolar subcompartments exist in *Dictyostelium*.

At least two nucleolar subcompartments were identified; NoSC1 (housing CBP4a) and NOSC2 (housing Snf12). NoSC2 is found within NoSC1. NumA1, eIF6, and Bud31 localize throughout the entire nucleolus. FhkA and Hsp32 localize to the nucleolar periphery which may represent NoSC3.

9.2 Major findings and significance

9.2.1 Five nucleolar proteins identified using a variety of techniques

This study has led to the identification of five new nucleolar proteins for a total of eight in *Dictyostelium*. Nucleomorphin isoform NumA1, the NumA1 binding partner CBP4a, SWI/SNF complex member BAF60a homologue Snf12, and Rad53/Chk2 homologue FhkA were verified as nucleolar proteins by colocalization with DAPI unstained nuclear regions and by their absence from nucleoli after treatment with the rDNA transcription inhibitor AM-D. Bud31 was verified as a nucleolar protein by colocalization of GFP-Bud31 with DAPI unstained nuclear regions and
with nucleolar proteins NumA1 and CBP4a. The NumA1 binding partner PsaA is not a nucleolar protein however it was characterized and examined in order to learn more about NumA1.

A variety of techniques were used to determine the localization of these proteins. First, polyclonal antibodies were produced against CBP4a, PsaA, Snf12, and FhkA (anti-NumA1 already existed, O’Day et al., 2009) and immunolocalization was performed. Second, cells expressing GFP-fusion proteins were made for CBP4a, PsaA, Snf12, eIF6, and Bud31. Both live and fixed cells were examined. Except for eIF6 and Bud31, both N- and C-terminal GFP-fusion proteins were made for each of these proteins to control for possible effects of GFP on localization. Third, when possible, cells expressing a GFP-fusion protein were fixed and probed with the corresponding antibody (for PsaA and Snf12). This verified antibody specificity and proper GFP-fusion protein expression simultaneously.

The identification of NumA1, CBP4a, Snf12, FhkA, and Bud31 as nucleolar proteins increases the total number of nucleolar proteins in Dictyostelium from three to eight. These eight proteins now provide a starting point for researchers interested in studying the relationship between the nucleolus and nucleolar proteins in Dictyostelium. There are now enough proteins to allow patterns to be drawn. For example, CBP4a, Snf12, and FhkA all reside in a nucleolar subcompartment and all bud from the nucleus after AM-D treatment whereas eIF6 and NumA1 are not located in a subcompartment and do not bud from the nucleus (Table 9.1).
Table 9.1. Attributes of all *Dictyostelium* nucleolar proteins.

<table>
<thead>
<tr>
<th>Previously identified</th>
<th>Identified in this study</th>
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<tbody>
<tr>
<td></td>
<td>Hsp32</td>
</tr>
<tr>
<td>Localization</td>
<td>NoSC3</td>
</tr>
<tr>
<td>NLS</td>
<td>No</td>
</tr>
<tr>
<td>NoLS</td>
<td>(partial)</td>
</tr>
<tr>
<td>Mitosis loc.</td>
<td>Nu</td>
</tr>
<tr>
<td>AM-D Bud</td>
<td>No</td>
</tr>
</tbody>
</table>

No; nucleolus, Nu; nucleus, Cyt; cytoplasm, loc; location, poss; possible. (Balbo and Bozzaro, 2006; Moerman and Klein, 1998; Yamaguchi et al., 2005).

9.2.2 First NoLS and NLS/NoLS identified in *Dictyostelium* using a variety of techniques

In order to enter the nucleus and localize to the nucleolus proteins must possess both a NLS and a NoLS which in some cases is the same (NLS/NoLS; Birbach et al., 2004; Kakuk et al., 2008; Ueki et al., 1998; Wang et al., 2005). This study identified the first NoLSs (NumA1; \(^61\text{RPRK}^{64}\), Snf12; \(^{372}\text{KRKR}^{375}\) in *Dictyostelium*. These sequences also represent the first NLS/NoLSs identified in *Dictyostelium*. The Snf12 NLS/NoLS (\(^{372}\text{KRKR}^{375}\)) is both necessary and sufficient for nuclear/nucleolar localization.

To date only one NLS had previously been identified in *Dictyostelium*: \(^{1019}\text{KKPKR}^{1023}\) in SMEK, a protein involved in chemotaxis and gene expression (Mendoza et al., 2005). This study has identified several additional NLSs in *Dictyostelium*. A functional NLS (\(^{680}\text{RKRF}^{683}\)) within PsaA has been identified, necessary for nuclear localization. Several other NLSs have been identified including those found in NumA1 as well as CBP4a residues \(^{40}\text{KKCK}^{43}\). These sequences can localize either GFP or FITC to the nucleus however it is unclear if they are functional within the protein.
A variety of techniques was used to identify functional NLSs and NoLSs. For some a peptide equivalent to the sequence was conjugated to FITC (NumA1, CBP4a) while for others a cell line expression a GFP-fusion protein with the sequence was made (PsaA, Snf12). Alternatively, cell lines expressing a GFP-fusion protein lacking the specific sequence was made (CBP4a, PsaA, Snf12).

9.2.3 Nuclear/nucleolar morphogenesis after AM-D treatment

AM-D treatment led to the redistribution of nucleolar CBP4a, Snf12, and FhkA to the cytoplasm. The redistribution of nucleolar proteins to non-nucleolar locales after AM-D treatment is well established in the literature (Balbo and Bozzaro, 2006; Kawashima et al., 1979). However these three proteins translocated along with a piece of the nucleus, an event which has not previously been observed. The nucleolar patches appeared to bud from the nucleus, eventually separating to become cytoplasmic circles. CBP4a and Snf12 were detected within the bud/circle while FhkA was detected around the edge of the bud/circle. These proteins thus maintained their same relative positions in the cytoplasmic circles as in nucleoli. Nucleolar aggresomes, which bud from the nucleolus, have been observed in mammals and represent proteins destined for degradation (Latonen, 2011; Latonen et al., 2011). Although different than aggresomes, the budding observed in this study may represent a novel mechanism by which nucleoplasmic proteins are send to the cytoplasm for degradation.

9.2.4 First analysis of nucleolar protein dynamics during mitosis in Dictyostelium

During mitosis nucleolar proteins redistribute to non-nucleolar locales (Ginisty et al., 1999; Kieffer-Kwon et al., 2004; Lam et al., 2005; Ochs et al., 1983). Accordingly, NumA1, CBP4a, Snf12, and FhkA all redistribute to non-nucleolar locales at this time. This study represents the
first time nucleolar dynamics during mitosis have been examined in *Dictyostelium* and suggest there are previously unidentified intranuclear domains during mitosis as well.

### 9.2.5 Identification of nucleolar subcompartments in *Dictyostelium*

This study has also discovered that the *Dictyostelium* nucleolus contains subcompartments. CBP4a resides in a novel subcompartment named NoSC1. Snf12 resides in another novel subcompartment named NoSC2 which is contained within NoSC1. FhkA and Hsp32 localize to what may be a third subcompartment, NoSC3 (Moerman and Klein, 1998). The Snf12 NLS/NoLS localizes to NoSC2 and thus represents the first NoSCLS identified in *Dictyostelium*. It should be noted however that this NoSCLS is not specific for NoSC2 since it also localizes throughout the nucleoplasm. It is possible that proteins residing in a specific nucleolar subcompartment may have a similar function.

### 9.2.6 Evidence for nuclear envelope permeability, nuclear retention, and active export of nuclear and nucleolar proteins during mitosis

In some eukaryotes that undergo closed mitosis the nuclear envelope has been shown to become permeable (De Souza and Osmani, 2007). Fittingly, the evidence presented here suggests that the nuclear envelope in *Dictyostelium* also becomes permeable at this time. Some nuclear proteins are appear to be actively exported (PsaA), some remain in the nucleus (NumA1 and CBP4a), and some diffuse throughout the entire cell (Snf12 and FhkA).

### 9.2.7 Does *Dictyostelium* possess an inside-out nucleolus?

In most organisms the FC region is located in the center of the nucleolus while the DFC and GC subcompartments are located around the FC region (Boisvert et al., 2007). However the nucleolar subcompartments identified here are contained within the region equivalent to the FC (the periphery of the nucleolar patches; Moerman and Klein, 1998; Simon and Olins, 1994).
Such a nucleolus may be termed “inside-out”. Fittingly, the nucleolar patches in *Dictyostelium* are located adjacent to the nuclear envelope, outside of the nucleoplasm, whereas in most other organisms the nucleolus is embedded within the nucleoplasm. An inside-out nucleolus may pose a problem since ribosomal subunits would be produced in the center of the nucleolus and must therefore migrate through the nucleolus to exit into the nucleoplasm. Alternatively, since NoSC1 and 2 are adjacent to the nuclear envelope, newly formed ribosomal subunits may exit directly through the nuclear envelope into the cytoplasm without travelling through the nucleolus or nucleoplasm. This would be an efficient process since it would eliminate the need for these subunits to travel through the nucleoplasm, as is the case in other eukaryotes. It would be interesting to determine if there are differences between NPCs adjacent to the nucleoplasm versus those adjacent to nucleoli. If the aforementioned pathway is true, nucleolar NPCs may be specialized for the transport of ribosomal subunits.

### 9.3 Future studies

#### 9.3.1 Deletion constructs and RNAi

In order to determine the function of these nucleolar proteins deletion constructs should be made for NumA1, CBP4a, Snf12, and FhkA. However null mutations for the other nucleolar proteins Hsp32, eIF6, and TRAP1 are thought to be lethal suggesting the same may be true for the nucleolar proteins identified here. If deleting these genes proves unsuccessful, RNAi may be performed in order to observe the effect of decreased mRNA for each protein. Effects to examine after RNAi treatment include changes in cell size and morphology, cell culture growth rate, fruiting body development, and localization and abundance of other nucleolar proteins. These results would help to better understand the function of these proteins.
9.3.2  Ultrastructural immunological analysis

In order to learn more about the nucleolar subcompartments identified in this study the localization of NumA1, CBP4a, Snf12, and FhkA would be examined at the ultrastructural level. This would be done via immunogold with the same antibodies used here. This would lead to a more precise delineation of these subcompartments which may aid in understanding their function. The dynamics of the AM-D-induced nuclear budding would also be examined and at this level of detail the morphological changes taking place would be clearer.

9.3.3  Investigate the relationship between microtubules and nuclear budding

Given the relationship between microtubules and nucleolar shape and position it is proposed that microtubules may direct the nuclear budding observed after AM-D treatment (Sameshima et al., 1991). To investigate this relationship cells would be treated with both AM-D and the microtubule polymerization inhibitor, nocodazole. This may result in a lack of or partial budding in these cells compared to those treated with AM-D alone. The original AM-D treatment experiment should also be redone in order to track the cytoplasmic circles after 8 h. It would be interesting to see if these circles persist in the cytoplasm, disappear, or merge with the nucleus. These experiments should be performed in cells treated with AM-D for more than 8 h as well as cells that have recovered from 4 h of AM-D treatment (cells that have recovered still possess cytoplasmic circles after 8 h).
9.4 References


Copyright Acknowledgements

Chapter 2 was published in:

I performed the research and writing while my supervisor contributed to experimental design and data analysis and provided editorial input. This chapter was published exactly ‘as is’ except for formatting changes.