Nuclear Localization of N-Ethylmaleimide Sensitive Factor

by

Marzena Serwin

A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate
Department of Cell & Systems Biology
University of Toronto

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Abstract

N-Ethylmaleimide Sensitive Factor (NSF) was first characterized as the protein responsible for restoring vesicle trafficking to N-ethylmaleimide (NEM) treated cells. Diverse lines of evidence indicate that NSF may function in the nucleus of cells, a previously unknown site of action for the protein. In this study Chinese hamster ovary (CHO) cells were used to examine the cellular localization of NSF in fibronectin-spread cells and in cells treated with NEM and leptomycin B (LMB). We show that NSF localizes to the nuclei of several cell lines and cell types and shows variability in the level of nuclear localization during cell spreading and drug treatments. Additionally, we have identified three putative nuclear export sequences (NES) within the NSF-D2 domain. Furthermore, immunoprecipitation identified Vimentin as a likely nuclear binding partner. Thus, for the first time, we report nuclear localization of NSF and an uncharacterized interaction of NSF with Vimentin.
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Finally, I would like to dedicate this thesis to my parents, without whom none of this would be possible.
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List of Abbreviations

µl.......................................................................................... Microliters
%
............................................................................. Percent
°C...................................................................................... Degrees Celsius
mg............................................................................... Milligrams
AAA.......................................................... ATPases Associated with Diverse Cellular Activities
BCA............................................................. Bicinchoninic Acid
CF.............................................................. Cytoplasmic Fraction
CHO cells................................. Chinese Hamster Ovary Cells
CRM1.................................................. Chromosome Region Maintenance 1
DAPI........................................................................ 4’,6-diamidino-2-phenylindole
DMEM.................................................. Dulbecco’s Modified Eagle Medium
DTT........................................................................ Dithiothreitol
FBS........................................................................ Fetal Bovine Serum
FN........................................................................ Fibronectin
GFP........................................................................ Green Fluorescent Protein
IP........................................................................ Immunoprecipitation
Jurkat............................................. Human T Cell lymphoblast-like cell line
kDa........................................................................ Kilodaltons
LMB........................................................................ Leptomycin B
MAPKK................................................ Mitogen Activated Protein Kinase Kinase
N........................................................................ Nuclear Fraction
N2A........................................................ Mouse Neuroblastoma N2A cells
NEM........................................................................ N-Ethylmaleimide
NES........................................................................ Nuclear Export Sequence
NIH3T3.................................................... Mouse embryonic fibroblast cell line
NLS........................................................................ Nuclear Localization Sequence
NPC........................................................................ Nuclear Pore Complex
NSF........................................................................ N-Ethylmaleimide Sensitive Factor
PC12…………………………..Rat adrenal pheochromocytoma cell line
PKIα………………………………..Protein Kinase Inhibitor α
SNAP…………………………………………Soluble NSF Attachment Protein
SNARE…………………………Soluble NSF Attachment Protein Receptors
TBP…………………………………………TATA Binding Protein
REAP……………………………………Rapid, Efficient and Practical
VAMP………………………………Vesicle Associated Membrane Protein
VCP……………………………………..Valosin-Containing Protein
W…………………………………………Whole Cell Fraction
Chapter 1
Nuclear Localization of N-Ethylmaleimide Sensitive Factor

1 Introduction

1.1 General

Within the cytoplasm of the eukaryotic cell reside organelles, each responsible for a unique function. Transport between and within these structures requires membrane fusion events. In the late 1970’s some key studies were conducted that addressed the questions surrounding vesicle transport and specifically, membrane fusion events. Siddiqi and Benzer (1976) first identified the *Drosophila melanogaster* comatose temperature-sensitive mutant. The flies were exposed to a mutagen, ethyl methanesulfonate, and the adult offspring of the mutagen-exposed parents were incubated at nonpermissive temperatures (Siddiqi, O. and Benzer, S., 1976). This resulted in conditional paralysis of some flies. However, when compared to other temperature-sensitive mutants, the *comatose* mutant required a longer time to reach paralysis and a longer time for recovery (Siddiqi, O. and Benzer, S., 1976). A few years later Novick *et al.* (1980) identified 23 gene products that are involved in vesicle secretion in the yeast *Saccharomyces cerevisiae*, among them *Sec18-1*. A decade later Block *et al.* (1988) purified N-Ethylmaleimide Sensitive Factor (NSF) from Chinese Hamster Ovary (CHO) cells, this protein would later be found to be responsible for the *comatose* and *Sec18-1* phenotype.

When Siddiqi and Benzer (1976) first characterized the *D. melanogaster* *comatose* mutant they noted that the mutant slowly reached paralysis at restrictive temperatures and slowly recovered when returned to permissive temperatures (Siddiqi, O. and Benzer, S., 1976). Although, it was not known at the time *D. melanogaster* has two NSF isoforms, unlike most organisms. Further characterization of the *comatose* mutant identified the temperature-sensitive alleles to be within *dNSF-1*, a gene coding for a NSF isoform (Pallanck, L. *et al.* 1995b). Despite there being an accumulation of vesicles at the synapse of the *comatose* mutant, they cannot undergo exocytosis (Siddiqi,
Similarly, mutagenesis studies conducted using *S. cerevisiae* mutants showed an accumulation of vesicles within the cytoplasm, suggesting that the 23 genes targeted are involved in vesicle trafficking (Novick, P. *et al.* 1980). Among them was *Sec18-1*, which gives rise to Sec18p, the yeast homolog of NSF (Novick, P. *et al.* 1980). Rothman and colleagues (1989) decided to test whether vesicle trafficking could be restored to the Golgi networks of NEM-treated CHO cells using cytosol extracted from *S. cerevisiae* (Wilson, D.W. *et al.* 1989). The presence of the NSF yeast homolog, Sec18p, restored vesicle transport (Wilson, D.W. *et al.* 1989). This confirmed the functional equivalence of NSF and Sec18p, and suggested that similar vesicle trafficking pathways exist in mammals and yeast (Wilson, D.W. *et al.* 1989).

Block *et al.* (1988) found that NSF was the catalytic protein capable of restoring vesicular transport to Golgi networks treated with N-ethylmaleimide (NEM). Several studies conducted over the next decade linked NSF to vesicle trafficking events from: the endoplasmic reticulum to the Golgi apparatus, within the Golgi networks, in the endocytic pathway, in neurotransmission and in neuroendocrine secretion (Beckers, C.J. *et al.* 1989; Malhotra, V. *et al.* 1988; Block, M. *et al.* 1988; Ikonen, E. *et al.* 1995; Diaz, R. *et al.* 1989; Pallanck, L. *et al.* 1995a; Moriyama, Y. *et al.* 1995). The data presented by Block *et al.* (1988) helped characterize the mechanism by which NSF functions. The study demonstrated that NSF was inhibited by the sulfhydryl alkylating agent, NEM, suggesting a cysteine residue must be either important to NSF function or, at the very least, near the functional site (Block, M.R. *et al.* 1988). Secondly, ATP-deprived cytosolic fractions did not restore vesicular transport to NEM treated membranes, suggesting that NSF requires ATP for its functioning (Block, M.R. *et al.* 1988).

NSF is a 78kDa ATPase Associated with diverse cellular Activities (AAA) protein with a homoheaxmeric structure. NSF contains three domains (NSF-N, NSF-D1 and NSF-D2) (Figure 1). NSF-N (residues 1-205) was identified as the site of α-soluble NSF attachment proteins (α-SNAPs) - Soluble NSF Attachment Protein Receptors (SNARE) binding (May, A.P. *et al.* 1999). NSF-D1 (residues 206-488) was found to be the most active ATPase domain of NSF, while NSF-D2 (residues 489-744) was found to be necessary for NSF hexamerization, the latter two identified NSF as a member of the AAA family (Tagaya, M. *et al.* 1993).
Figure 1. Domain Map of an NSF protomer. Shown are the N-terminal, D1 and D2 domains and the Walker B and Walker A motifs within the D1 and D2 domains, respectively. Putative NESs within the D2-domain are amplified.
1.2 ATPases Associated with Diverse Cellular Activities (AAA and AAA+) Family of Proteins

The proteins composing the AAA family are characterized by a 200–250 conserved amino acid ATP binding domain, the AAA domain (Hanson, P.I. and Whiteheart, S.W. 2005). Within this sequence lie the Walker A and Walker B motifs, which facilitate the ATPase activity of the AAA family members (Hanson, P.I. and Whiteheart, S.W. 2005). Further structural characterization of the classic AAA proteins has led to the establishment of a more diverse superfamily, now identified as the AAA+ family (Hanson, P.I. and Whiteheart, S.W. 2005). Present within all kingdoms, the AAA+ ATPases are oligomers and often undergo hexamerization (Lenzen, C.U. *et al.* 1998; Hanson, P.I. and Whiteheart, S.W. 2005).

There are some motifs that are present within the subfamily of AAA ATPases, but are not common to all AAA+ proteins. One example of a classic AAA motif is the second region of homology (SRH), positioned toward the C-terminal of the Walker B motif (Lupas, A.N. and Martin, J. 2002). NSF contains the SRH motif and so falls within the classification of the AAA family of proteins (Lupas, A.N. and Martin, J. 2002). However, common to all AAA+ proteins is the Sensor 1 motif positioned toward the C-terminal of the Walker B motif (Steel, G.J. *et al.* 2000). Additionally, the Walker A and B motifs are crucial for the ATPase activity of the AAA+ proteins (Neuwald, A.F. *et al.* 1999).

The P-loop, contained within the Walker A motif, contains a consensus sequence GXXXXGK[T/S], where X represents any amino acid. Point mutations of the lysine to an alanine (K to A) abolishes ATP binding within the Walker A motif (Babst, M. *et al.* 1998). Similarly, within the Walker B motif lies the ATP coordinating sequence hhhhDE (where h represents a hydrophobic residue). The aspartate coordinates the magnesium ion, while glutamate activates the water molecule necessary for the hydrolysis reaction. Point mutating glutamate at position 326 to glutamine (E326Q) results in an AAA+ protein that is capable of binding ATP, but not catalyzing its hydrolysis (Weibezahn, J. *et al.* 2003). The E/Q mutation is commonly used in the study of NSF function (Dalal, S. *et al.* 2004; Stewart, B.A. *et al.* 2001).
1.3 NSF and the SNARE Complex

Discovery of NSF ushered in rapid identification of proteins involved in vesicle trafficking and membrane fusion (Clary, D.O. et al. 1990; Söllner, T. et al. 1993a; Söllner, T. et al. 1993b). Among these discoveries, the Soluble NSF Attachment Protein (SNAPs) and the SNAP receptors (SNAREs) proved critical for development of the SNARE hypothesis (Söllner, T. et al. 1993b; Rothman, J.E. and Warren, G. 1994). The SNAREs, thought to be the key mediators of membrane fusion, are grouped into vesicle membrane associated SNARE (v-SNARE) and target membrane associated SNAREs (t-SNAREs). It is the association of Synaptobrevin, also known as vesicle associated membrane protein (VAMP), a v-SNARE, with Syntaxin and Synaptosomal Associated Protein 25 (SNAP-25), both t-SNAREs, that drives membrane fusion, leading to exocytosis. Upon fusion the v-SNARE and t-SNAREs reside as a protein complex in the same membrane; this complex is known as the cis-SNARE complex. In order for these proteins to participate in further rounds of exocytosis they must be unraveled from one another. NSF accomplishes this task via three adaptor protein α-SNAP per NSF hexamer, this has earned NSF the title of a chaperone protein, a protein that assists in the disassembly of protein complexes (Moeller, A. et al. 2012) (Figure 2).

1.4 NSF: Structure and Function

Crystal structures of the NSF-D2 (1.75 Å) and NSF-N (1.9 Å) domains have been determined and 11 Å electron cryomicroscopy and single-particle analysis data exist for the NSF-hexamer and the 20S particle (Yu, R.C. et al. 1998; Lenzen, C. et al. 1998; May, A.P. et al. 1999; Yu, R.C. et al. 1999; Hanson, P.I. et al. 1997; Hohl, T.M. et al. 1998; Furst, J. et al. 2003) (Figures 3 and 4). The NSF-hexamer is composed of three-layers, (from the N-terminal to the C-terminal) the N-domains, D1-domains and D2-domains with an approximate diameter of 115Å, though the D1- and D2-domains show changes in diameter between the ATP and ADP-bound states (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012). The D1- and D2-domains have a parallel arrangement with
Figure 2. Cartoon schematic showing stages of vesicle fusion. Vesicle docking and priming, membrane fusion followed by NSF-mediated SNARE complex dissociation and recycling is shown.
Figure 3. Surface features of the NSF-D2 domain. (A) N-terminal face of NSF-D2. (B) C-terminal face of NSF-D2. The crystal structure was resolved at 1.75 Å. Reprinted with permission (Lenzen, C. U., *et al.* 1998).
Figure 4. Surface features of the NSF-N domain. (B) Sequence conservation of exposed NSF-N surface. (C) Identified grooves on NSF-N surface, with groove 3 being a likely binding site of α-SNAP. The crystal structure was resolved at 1.9 Å. Reprinted with permission (Yu, R. C. et al. 1999).
respect to each other, much like the p97 D1- and D2-domains (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012). The 20S complex resembles a sparkplug, with the three α-SNAPs forming a tripod-like structure that binds the SNARE complex at its C-terminus, while its N-terminus is anchored by the N-domain (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012).

Though all of the details of NSF mediated SNARE disassembly are not clear, a recent study by Whiteheart and colleagues brought to light a possible mechanism (Moeller, A. et al. 2012). Structural modeling, using electron microscopy analysis, of the 20S particle in the ATP-bound pre-SNARE-disassembly state (using a non-hydrolyzable ATP-analog, AMP-PNP) and in the ADP-bound post-SNARE disassembly state, showed conformational changes occurring in the N- and D1-domains, with the D2-domain showing no change (Moeller, A. et al. 2012). Specifically upon ATP-hydrolysis, via the D1-domain, the N-domain residues identified as α-SNAP-binding sites move from an upward exposed position to a downward inaccessible position (Moeller, A. et al. 2012). This suggests that SNARE complex disassembly is strongly dependent on the nucleotide state of NSF-D1; an ATP-bound state facilitates SNAP-SNARE binding, while an ADP-bound state prevents it (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012).

Similarly to the Whiteheart group, Chang et al. (2012) also used structural analysis of NSF in various nucleotide states to analyze the mechanics of NSF-mediated SNARE complex disassembly. Using wild-type NSF from CHO cells the authors reconstructed the protein from unbound state NSF monomers and confirmed the NSF proteins were functional and formed hexamers in homogeneous ATPγS (a nonhydrolyzable ATP-analog), ADP-AlFx (a transition state ATP-analog) and ADP-bound states (Chang, L.-F. et al. 2012). Chang et al. (2012) identified an exposed, SNAP-SNARE competent upward binding state, and inaccessible downward state of the NSF-N domains, much like the changes seen by Moeller, A. et al. (2012).

Additionally, both groups confirmed a D1-domain rotation occurring during ATP-hydrolysis, suggesting this is the mechanical force yielded from ATP hydrolysis and required to pry the SNARE complex into its components (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012). The D1-N-linker shows a high degree of flexibility and likely serves to transfer the mechanical force derived from ATP-hydrolysis from the D1-
domain to the N-domain, which eventually pulls the SNARE complex apart resulting in the downward conformation; this motion has been referred to as the ‘power stroke’ (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012). Three-dimensional reconstructions of the D2-domain using electron microscopy imaging showed no changes in all three nucleotide states tested, suggestive of the rigidity of this domain (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012). In contrast, significant conformational changes were observed in the D1- and N-domains (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012). Therefore, the authors of both studies suggest that the D2-domain likely serves as a platform on which the disassembly occurs (Moeller, A. et al. 2012; Chang, L.-F. et al. 2012).

Although, much information has been accumulated regarding NSF’s structure and function there remain unaddressed results that leave inconsistencies in the NSF story. Several studies have reported NSF’s relocalization from the soluble to insoluble fraction (Block, M. et al. 1988; Mohtashami, M. et al. 2001; Liu, C. and Hu, B. 2004). N-Ethylmaleimide Sensitive Factor relocalization has been linked to the inactivation of NSF however no further studies have been conducted on insoluble NSF. Insoluble NSF may in fact be NSF that is associated with the nuclear membrane, as it was shown by Tagaya et al. (1996), rather than simply a dysfunctional NSF form (Tagaya, M. et al. 1996; Mashima, J. et al. 2000). Other possibilities to consider are that NSF may association with other proteins leading to the formation of insoluble complexes. Furthermore, no studies to date have examined the possibility of NSF nuclear localization, although data suggest this is feasible. These results and hypotheses require additional investigation to elucidate further NSF’s cellular roles.

1.5 Other Roles of NSF

In addition to its SNARE-dependent role, NSF also interacts with non-SNARE proteins and shares sequence similarity with other AAA proteins, suggestive of other roles it may play within the cell (Whiteheart, S.W. et al. 2004). In particular, the N-domains of p97 and valosin-containing protein (VCP) are highly conserved to that of NSF and all three play important roles in membrane fusion events in the cytoplasm (Whiteheart, S.W. et al. 2004; Partridge, J.J et al. 2003). Furthermore, p97/VCP has been found to act as a
chaperone for ubiquitinated proteins destined for proteasomal degradation following ubiquitination by E3 ubiquitin ligase gp78 (Fang, S. et al. 2001; Zhong, X. et al. 2004).

In 2004, Zhong et al. furthered our understanding of this interaction by showing that gp78 and p97/VCP physically interact (Zhong, X. et al. 2004). NSF has also been shown to interact with an E3 ubiquitin ligase, Highwire, responsible for regulation of Wallenda levels, a protein that plays a major role in synaptic growth (Collins, C. et al. 2006; Kaneuchi, T. et al. unpublished data). Loss-of-function Highwire mutants show synaptic overgrowth at the Drosophila melanogaster neuromuscular junction (Collins, C. et al. 2006). This phenotype is also shown in the NSF\(^{E/Q}\) mutant, which is capable of binding, but not hydrolyzing ATP (Kaneuchi, T. et al. unpublished data). Work done by Kaneuchi, T. et al. suggests that NSF acts in the same pathway as Highwire in the suppression of the synaptic overgrowth phenotype. The authors of this study postulate that NSF’s role in this pathway may involve the disassembly of Highwire from its substrates (Kaneuchi, T. et al. unpublished data). Additionally, Lowenstein and colleagues (2003) have demonstrated that S-nitrosylation of NSF inhibits secretion of Weibel-Palade bodies from endothelial cells (Matsushita K. et al. 2003). N-Ethylmaleimide Sensitive Factor has also been shown to mediate rapid surface expression of AMPA subunit, GluR2, thereby preventing long-term depression (Kamboj S.I. et al. 1998; Nishimune A. et al. 1998; Osten P. et al. 1998; Huang Y. et al. 2005).

Though many AAA+ proteins have been found to localize to the nucleus, AAA proteins were not known for their nuclear roles. In 2003, Indig and colleagues reported that p97/VCP localizes and is abundant in the mammalian nuclei, functioning as a regulator of nucleic acid recycling (Partridge, J.J. et al. 2003). In 1996 and 2002 Tagaya and colleagues reported the presence of NSF in the nuclear membrane of PC12 cells (Tagaya, M. et al. 1996; Mashima, J. et al. 2000). Additionally, these results were confirmed when isolated nuclei from bovine adrenal medulla showed NSF presence in the nuclear membrane (M. Tagaya and S. Mizushima, unpublished data). Therefore, although there are few reports on the role of nuclear NSF, the strong similarity and currently known cellular functions of NSF and p97/VCP suggest that localization of NSF to the nucleus is feasible. Moreover, it is highly unlikely that AAA proteins, which
show congruence in structure and function to NSF should play several cellular roles, while NSF is limited to SNARE disassembly.

1.6 Nucleocytoplasmic Shuttling

The nucleoplasm and cytoplasm are separated by the bilayered nuclear envelope (Kumeta, M. et al. 2012). Nuclear export and import occur through the nuclear pore complex (NPC), a 40-50nm in diameter channel that serves to exclude some material, while facilitating the transport of other (Güttler, T. and Görlich, D. 2011). The octameric NPC is composed of approximately 30 different proteins known as nucleoporins (Nups) (Xu, L. and Massagué, J. 2004; Kumeta, M. et al. 2012).

Nuclear import involves the binding of importins to the nuclear localizing sequence (NLS) of their cargo (Rexach, M. and Blobel, G. 1995; Görlich, D. et al. 1996). Once the dimer is formed it travels from the cytoplasm through the NPC into the nucleoplasm. There, the high concentration of RanGTP facilitates the displacement of the cargo with RanGTP (Rexach, M. and Blobel, G. 1995; Görlich, D. et al. 1996). Ran (or RAs-related Nuclear protein) is a 25kDa protein involved in nucleocytoplasmic transport (Kutay, U. et al. 1997; Floer, M. et. al. 1997). Ran exists in two states, RanGTP and RanGDP. The nucleus has a RanGTP concentration 1000-fold higher than that seen within the cytoplasm (Kutay, U. et al. 1997; Floer, M. et. al. 1997). Once the importin is RanGTP bound, it travels back to the cytoplasm via the NPC and the GTPase activity is stimulated allowing the dimer to dissociate (Rexach, M. and Blobel, G. 1995; Görlich, D. et al. 1996).

Nuclear export of larger molecules requires that the cargo contain a NES. The pathway that cargo travels when moving from the nucleoplasm into the cytoplasm depends on GTP-bound Ran (Kutay, U. et al. 1997; Floer, M. et. al. 1997). The high nuclear RanGTP concentration acts to stimulate exportin-cargo binding, similarly, cargo binding stimulates RanGTP binding (Kutay, U. et al. 1997; Floer, M. et. al. 1997; Güttler, T. and Görlich, D. 2011). This complex travels through the NPC. Once in the cytoplasm the intrinsic GTPase activity of Ran is stimulated by the binding of RanGTPase-activating protein (RanGAP) (Kutay, U. et al. 1997; Floer, M. et. al. 1997). However, RanGAP can itself only interact with exportin-bound RanGTP by binding to
Ran-binding proteins, also known as Ran-binding domains (RanBDs) (Kutay, U. et al. 1997; Floer, M. et. al. 1997). This results in the hydrolysis of GTP to GDP and disassembly of the export complex.


Our analysis revealed that NSF has three putative, previously unknown, nuclear export sequences (NESs) within the NSF-D2 domain (Figure 1; Xinping, Qiu, unpublished data) and BLAST sequence alignment of the putative NSF-NES sequences within five species was conducted (Figure 5). Conserved hydrophobic residues were identified at the 2nd, 6th and 9th position between all three NESs and across all species considered, as seen in the established NESs of Exportin 1 cargo: α-actin (NES-1/NES-2), PKIα and MAPKK (Wada, A. et al., 1998; Figure 6). When point mutations within the mammalian NSF-D2 domain of nuclear export sequence 3 were introduced, there was an increase of NSF nuclear signal within Drosophila Schneider 2 (S2) cells, when compared to wtNSF (Figure 7; Xinping, Qiu, unpublished data).

1.7 Further Data from the Stewart Lab

Genetic screens in our lab using a Gene Search collection of a Drosophila melanogaster-specific transposable element, P-element, insertion followed by more specific genetic crosses, have shown that NSF interacts with nuclear proteins (some examples include transcription factors: E2f, ovo, btd, btm, lola, dpdl, fos, jun and other such as DNA binding proteins such as: LanA and His2Av) (Laviolette, M.J. et al. 2005; Peyre, J.B. et al. 2006). Moreover, unpublished work on NSF-induced morphology phenotypes showed, that over-expression of Fos and Jun transcription factors suppressed overgrowth at the larval D. melanogaster neuromuscular junction (Qiu et al. unpublished). Altogether, several lines of indirect evidence suggest NSF may play a role in the nuclear function of the cell.
**Figure 5.** Sequence alignment of the putative NSF-NES sequences (1, 2, and 3) of *Homo sapiens*, *Cricetulus griseus*, *Drosophila melanogaster*, *Dictyostelium discoideum* and *Saccharomyces cerevisiae*. Conserved hydrophobic residues are boxed; residues that are not conserved are in red. Sequence alignment was conducted using BLAST.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td><em>H. sapiens</em> (NSF-NES1)</td>
<td>VVVDDIERLL</td>
</tr>
<tr>
<td><em>C. griseus</em> (NSF-NES1)</td>
<td>VVVDDIERLL</td>
</tr>
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**Figure 6.** Sequence alignment of the putative NSF-NES1 of *Drosophila melanogaster* and the NESs of α-actin, PKIα and MAPKK. Conserved hydrophobic residues are boxed. The NESs of α-actin (1 and 2), PKIα and MAPKK were reported in Wada, A. *et al.*, 1998.
Figure 7. Imaging of mammalian-NSF nuclear localization within *Drosophila* Schneider 2 (S2) cells. (A) Nuclear envelope staining using Lamin Dm0 (B) Mammalian wtNSF-EGFP (top) and mammalian NSF-EGFP with a point mutation within the NES3 sequence (I705A and I707A) (bottom) (C) Overlay of A and B (Xinping Qiu, unpublished data).
In order to more explicitly show nuclear localization of NSF and further
delineate NSF function, we turned to cell culture studies. In one series of experiments
that examined NSF in a cell-spreading model, we detected NSF in the nuclei of cells
only after spreading occurred. This suggested that NSF has a dynamic, previously
undetected localization in cells.

1.8 Hypothesis

In 1996 NSF was first shown to localize to the nuclear membrane of PC12 cells and
recently, we have identified three putative nuclear export sequences (NESs) within the
NSF-D2 domain (Figure 1; Xinping, Qiu, unpublished data). Continuing with this body
of work, from both cell culture and D. melanogaster studies, my working hypothesis is
that NSF is a dynamic nuclear protein that interacts with other nuclear proteins to
control cell function. To test this hypothesis I used a cell-fractionation approach to
examine nuclear localization in spread versus non-spread cells of NSF and in cells
treated with drugs known to inhibit nuclear export. Secondly, to identify potential NSF
interacting proteins, I immunoprecipitated NSF and used mass spectrometry to identify
its nuclear-specific binding partners. Thirdly, I used nuclear fractions originating from
several cell lines, in order to determine if NSF is present in the nuclei of a wide variety
of cell types. Lastly, I tested the physiological importance of nuclear NSF by examining
NGF’s interacting partners during specific phases of the cell cycle.

My results show that NSF localizes to the nuclei of Chinese hamster ovary
(CHO-K1) cells, neuroblastoma N2A cells, PC12 (rat adrenal pheochromocytoma) cells
and NIH 3T3 (mouse embryonic fibroblast) cells. Using immunoprecipitation, I have
identified Vimentin as a nuclear binding partner of NSF.

2 Materials and Methods

2.1 Culture Methods

Chinese hamster ovary (CHO) cells and mouse neuroblastoma N2A (N2A) cells were
grown at 36.5°C, 5% CO₂ and 65% humidity. CHO cells were grown in Dulbecco’s
Modified Eagle Medium (DMEM) (Sigma, Cat#D5546) containing 10% Fetal Bovine Serum (FBS) (Sigma, Cat#F6178). N2A cells were grown in high glucose DMEM (Invitrogen, Cat#10567-014) containing 10% FBS and 1% penicillin-streptomycin (Sigma, Cat#P0781). When cells reached 90% confluency the growth medium was removed and cells were trypsinized (Sigma, Cat#T4049). The trypsin was removed and 6mL of fresh growth medium was added to the flask. Immediately after, 1mL was drawn and a fresh flask containing 5mL of growth medium was inoculated to a maximum of 6 new culture flasks.

Stock cultures of CHO and N2A cells were harvested and stored at -80°C and when necessary, thawed and cultured. CHO cells were stored at -80°C in 40% DMEM, 50% FBS and 10% Dimethyl sulfoxide (DMSO) (Bioshop, Cat#DM5555). N2A cells were stored at -80°C in 40% high glucose DMEM, 50% FBS and 10% DMSO.

2.2 Fibronectin Treatment

CHO or N2A stock culture cells were split and two flasks were seeded. Cells were grown as described in Culture Methods until approximately 90% confluency. Cells were trypsinized and re-plated to a fibronectin-coated test plate or not-coated test plate and incubated for 3 hours at 36.5°C, 5% CO₂ and 65% humidity. Cellular fractionation followed as described in Cellular Fractionation.

2.3 Leptomycin B Treatment

CHO stock culture cells were split and two flasks were seeded. Cells were grown as described in Culture Methods until approximately 90% confluency. The growth medium was removed and 4mL of fresh medium was added to each flask. Leptomycin B (3.4x10⁻³ nM; Sigma, Cat#L2913) was added to one flask and the second remained as a control. Cells were incubated at 36.5°C, 5% CO₂ and 65% humidity for 4 hours. Cellular fractionation followed as described in Cellular Fractionation.

2.4 N-ethylmaleimide Treatment

CHO stock culture cells were split and two flasks were seeded. Cells were grown as described in Culture Methods until approximately 90% confluency. The growth medium
was removed and 5mL of ice-cold 1xPBS pH 7.4 was added to each flask. NEM (40µM/1mM/5mM; Sigma, Cat#E3876) was added to three experimental flasks and the fourth remained as a control. Cells were placed on a rocker on ice for 15 minutes followed by DTT (20mM/0.5M/25M; Sigma, Cat#D9779) quenching for 15 minutes on a rocker on ice. Cellular fractionation followed as described in Cellular Fractionation.

2.5 Cell Synchronization by Nocodazole Treatment

CHO stock culture cells were split and three flasks were seeded. Cells were grown as described in Culture Methods until approximately 80% confluency. Nocodazole (50ng/ml; Sigma, Cat#M1404) was added directly to the growth medium. Cells were incubated at 36.5°C, 5% CO₂ and 65% humidity for 10 hours. Cellular fractionation followed as described in Cellular Fractionation.

2.6 Cell Synchronization by Thymidine Treatment

CHO stock culture cells were split and four flasks and 19 test plates were seeded. Cells were grown as described in Culture Methods until approximately 50% confluency. Thymidine (2mM; Sigma, Cat#T1895) was added directly to the growth medium. Cells were incubated at 36.5°C, 5% CO₂ and 65% humidity for 24 hours. After the 24-hour incubation, the thymidine-containing medium was removed from the culture flasks and test plates and the cells were rinsed twice with growth medium. New growth medium was added to each rinsed flask and test plate. The cells progression through the cell cycle was monitored at each hour, with time 0 (T₀) marking the removal of thymidine, for a total of 18 hours. Cells grown on test plates were fixed with 4% formaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI) The cells were incubated at 36.5°C, 5% CO₂ and 65% humidity until they reached prophase. Cellular fractionation followed, for the cells grown in flasks, as described in Cellular Fractionation.

2.7 Cellular Fractionation

Cellular fractionation was conducted as described earlier in Lamond Lab: Cellular Fractionation, (http://www.lamondlab.com/pdf/CellFractionation.pdf, Lamond Lab, University of Dundee, Dundee, Scotland) with some modifications. In brief, DMEM
was removed and cells were washed with ice-cold 1XPBS pH 7.4, and the solution was
gently removed. Ice-cold Buffer A (1mL) was added and cells were scraped off the
plates, transferred to an Eppendorf tube, kept on ice for 5 minutes and mechanically
disrupted using a Dounce homogenizer to create a whole cell lysate (Please refer to
Appendix A for Buffer A recipe). The whole cell lysate samples were aliquoted
(300µL), 60µL of 5X RIPA buffer was added to the samples, sonicated for 10 seconds
on ice (Duty Cycle: 20%; Timer: 2; Output Control: 3) (Branson Sonifier 250, VWR
Scientific), followed by centrifugation at 2800 g for 10 minutes at room temperature
using an Eppendorf table top Micromax centrifuge. A whole cell lysate sample was then
transferred to a fresh Eppendorf tube and stored at -80°C.

The remaining whole cell lysate sample was centrifuged at 228 g for 5 minutes at
room temperature and the supernatant was collected as the cytoplasmic fraction
(300µL). 60µL of 5X RIPA buffer was added to the cytoplasmic fraction and the sample
was centrifuged at 2800 g for 10 minutes at room temperature. The cytoplasmic sample
was then transferred to a fresh Eppendorf tube and stored at -80°C.

The remaining pellet was resuspended in S1 (600µL) solution and S3 (600µL)
solution was gently layered on top (Please refer to Appendix A for S1 and S3 recipe).
The sample was centrifuged at 2800 g for 10 minutes at room temperature. The
supernatant was collected and stored at -80°C, while the resulting nuclear pellet was
resuspended in 1X RIPA buffer (150µL) and then sonicated for 10 seconds on ice (Duty
Cycle: 20%; Timer: 2; Output Control: 3) (Branson Sonifier 250, VWR Scientific)
followed by centrifugation at 2800 g for 10 minutes at room temperature. This nuclear
sample was then transferred to a fresh Eppendorf tube and all samples (whole cell,
cytoplasmic, and nuclear fractions) were stored at -80°C.

Nuclear lysates originating from the human epithelial carcinoma cell line (HeLa;
Abcam, Cat#ab14655) and rat adrenal pheochromocytoma cell line (PC12; Abcam,
Cat#ab14884), mouse embryonic fibroblast cell line (NIH 3T3; Abcam Cat#ab14874),
and human T cell lymphoblast-like cell line (Jurkat; abcam, Cat#ab14844) were
purchased and resolved on a 10% SDS-PAGE gel followed by immunoblotting. Nuclear
lysates originating from CHO cells and N2A cells (N2A cells were a gift from Dr. Mary
Cheng’s Lab at the University of Toronto Mississauga) were generated using the Cellular Fraction protocol.

### 2.8 Immunoprecipitation

Protein G resin (50µL; Pierce, Cat#20398) was transferred to an Eppendorf tube, washed with cold lysis buffer (450µL) and centrifuged at 10,000 g for 30 seconds. The supernatant was discarded and the resin was resuspended in 50µL of cold lysis buffer with complete, EDTA-free protease inhibitors (Roche Applied Science, Cat#05056489001) and the nuclear or cytoplasmic fraction (500µL) was added and the sample(s) were rocked on ice for 1 hour. After the 1 hour incubation the sample(s) was centrifuged at 10,000 g for 2 minutes and the supernatant was divided equally between two Eppendorf tubes containing newly washed protein G resin suspended in 50µL of cold lysis buffer with complete, EDTA-free protease inhibitors. Special care was taken so as to not disturb the pelleted protein G resin. Anti-NSF or anti-Vimentin (3µL; Sigma, Cat#V6630) was added to one sample. A second sample was used as a negative control and no antibody was added. The samples were incubated with rocking for 12-24 hours at 4°C.

After the incubation period the samples were centrifuged at 10,000 g for 1 minute and the supernatant was removed and kept as the flow through sample. The beads were resuspended in 500µL of lysis buffer with complete, EDTA-free protease inhibitors (Roche Applied Science, Cat#05056489001) and subsequently centrifuged at 10,000 g for 1 minute. The supernatant was removed and kept as wash 1; this was repeated two more times and each time the supernatant was removed and kept as wash 2 and wash 3. After the last wash the protein G resin pellet was resuspended in 50µL of 1x protein loading buffer and the sample was vortexed. Once the whole cell lysate fraction (used as a positive control), flow through, wash 1, wash 2 and wash 3 samples were aliquoted, 2x protein loading buffer was added to each sample and all samples were boiled at 100°C for 10 minutes. The samples were then centrifuged at 10,000 g for 20 seconds and resolved by performing an SDS-PAGE (10% gel) followed by immunoblotting. The blots were incubated with primary antibodies, anti-NSF and anti-
Vimentin. Blots were subsequently washed and incubated with appropriate secondary antibodies and signals revealed with ECL plus reagents.

2.9 Immunoblot Quantification

Protein concentrations were determined using a Bicinchoninic Acid (BCA) Protein Assay kit (Pierce, Cat#23227) and their absorbencies measured with a nanodrop spectrophotometer (Nanodrop Technologies, NanoDrop ND-1000 Spectrophotometer). Samples of equal protein concentration, determined using BCA assay were resolved by performing an SDS-PAGE (10% gel) followed by immunoblotting. The blots were incubated with the appropriate primary antibodies: anti-N-Ethylmaleimide Sensitive Factor (anti-NSF; Cell Signaling Technology, 1:5000, Cat#2145), anti-Vimentin (3μL; Sigma, Cat#V6630), anti-Tubulin (E7 anti-Tubulin; dilution factor 1:200, Developmental Studies Hybridoma Bank), anti-Actin (mAb JLA-20, 1:200, Developmental Studies Hybridoma Bank) and anti-TATA Binding Protein (anti-TATA BP; dilution factor 1:1000, Fisher-Scientific Cat#MA125427). Blots were subsequently washed and incubated with appropriate secondary antibodies and signals revealed with ECL plus reagents (GE Healthcare).

Separate immunoblotting of the whole cell, cytoplasmic and nuclear fractions facilitated the use of ImageJ in order to measure NSF levels across the subcellular fractions. Whole cell, cytoplasmic and nuclear NSF levels were quantified and any treated fraction was divided by the corresponding control, yielding a quotient. When the quotient value was 1, NSF levels remained unchanged between the treated and control cells, when the quotient value was >1 there was an increase in NSF levels during the treatment, and if the quotient value was <1 there was a decrease in NSF levels during the treatment. It should be noted that the purification steps involved in the separation of the cytoplasmic and nuclear fractions require several transfers of the sample. This reduces contamination of the fractions, but results in a loss of sample. Consequently, changes in the whole cell lysate best reflect the overall changes, however they do not necessarily correspond to the changes seen in the cytoplasmic and nuclear fractions.
3 Results

3.1 Optimization of Cellular Fractionation Protocol

The first objective of my research was to establish localization of NSF into the nucleus of CHO cells using western blotting techniques. In order to determine this, I performed cellular fractionation. Several protocols were tested: Abcam: Nuclear Fractionation Protocol; Bendeck Lab: Nuclear Fractionation; REAP: A two minute cell fractionation method and Lamond Lab: Cellular Fractionation (Abcam: http://www.abcam.com/ps/pdf/protocols/Nuclear%20fractionation%20protocol.pdf; Chow, W., et al. 2008; Suzuki, K. et al., 2010; Lamond Lab, University of Dundee, 2007). It took several trials to establish the protocol (Figure 8, 9 & 11A). The Bendeck Lab: Nuclear Fractionation protocol produced poor isolation of the nuclear fraction as evidenced from the lack of TBP staining in the nuclear fraction (Figure 8). Therefore, it was necessary to use an alternate protocol. The REAP protocol, resulted in strong NSF bands, however there was poor separation of the nuclear and cytoplasmic fraction as TBP appears in all three fractions (Suzuki, K. et al., 2010; Figure 9). Consequently, the Lamond Lab: Cellular Fractionation protocol was considered (Figure 11A).

In the Lamond Lab protocol no detergent was used until after fraction separation. The hypotonic buffer used prior to dounce homogenization of cells, facilitated cell lysis without dissolving the nuclear membrane, as a detergent buffer would do. The cellular fractionation protocol provided by the Lamond Lab produced the most reliable results and after optimizing this protocol for my needs and introducing some modifications, I produced replicable results.

3.2 NSF Shows Nuclear Localization and is Affected by Fibronectin Mediated Cell Spreading

The purpose of this work was to investigate the nuclear localization of NSF and to identify its binding partner(s) in efforts to characterize its nuclear role. Our confocal imaging data had previously shown an increase in the nuclear accumulation of NSF in fibronectin spread cells. Additionally, a study published by Skalski et al. (2005)
Figure 8. A representative blot generated using the Bendeck Protocol (Chow et al., 2008). Whole cell (W), cytoplasmic (C) and nuclear (N) fractions from fibronectin (FN) spread or not-spread CHO cells. Cells were lifted and plated on FN coated or not-coated plates and incubated for 3 hours prior to fractionation. Fractionation was conducted using two different detergents in efforts to assess which one performed better; both showed comparable results. SDS-PAGE was performed followed by a western blot. White arrows indicate molecular weights in kDa.
Figure 9. A representative blot generated using the REAP Protocol (Suzuki, K. et al., 2010). Whole cell (W), cytoplasmic (C) and nuclear (N) fractions from fibronectin (FN) spread or not-spread CHO cells. Cells were lifted and plated on FN coated or not-coated plates and incubated for 3 hours prior to fractionation. SDS-PAGE was performed followed by a western blot. White arrows indicate molecular weights in kDa.
Figure 10. Transmitted light microscopy (Eclipse Nikon; lens Nikon Fluor 60x/1.00W) of non-spread (A) and spread (B) CHO cells. Cells were trypsinized and transferred to a non-coated test plate (A) or to a test plate coated with immobilized fibronectin (B); 2 hours later plates was imaged. Bars represent 20µm.
Figure 11. Whole cell (W), cytoplasmic (C) and nuclear (N) fractions from fibronectin (FN) spread or not-spread CHO cells. Cells were lifted and plated on fibronectin coated or not-coated plates and incubated for 3 hours prior to fractionation. SDS-PAGE was performed followed by a western blot (A) 16µg of protein was loaded. (B) 10µg of protein was loaded. White arrows indicate molecular weights in kDa. (C) NSF band intensity of fibronectin spread cells divided by control NSF band intensities within the whole cell lysate, cytoplasmic and nuclear cellular fractions. Bars represent quotient means determined from 3 experiments with standard deviations based on these means.
showed that NSF\textsuperscript{E/Q} mutants inhibited SNARE-mediated $\alpha_5\beta_1$ integrin trafficking and thereby cell spreading. Therefore, I sought to determine whether nuclear localization could be determined using cellular fractionation and immunoblotting. CHO cells were transferred to test plates coated with immobilized fibronectin and imaged 2-hours later. Cell spreading was evident in cells plated on fibronectin when compared to cells transferred to non-coated plates (Figure 10). Cellular fractionation and immunoblotting showed an overall increase in whole cell NSF levels (quotient value of 1.21), with no overall change to nuclear NSF levels (quotient value of 1.00) and a subtle decrease in cytoplasmic levels (quotient value of 0.75) (Figure 11). This data verifies the previously unreported nuclear localization of NSF, and corresponds well with the positive nuclear control, TBP. Furthermore, although the data is not significant, the trend demonstrates an upregulation of whole cell NSF levels during cell spreading.

### 3.3 Bioinformatics Analysis of NSF

Exportin 1 recognizes and binds to leucine-rich NESs, thereby facilitating nuclear export. N-Ethylmaleimide Sensitive Factor has three putative NESs, with NES-1 containing two Leu and one Ile, NES-2 containing six Leu and NES-3 containing three Leu and three Ile (Figure 1).

In order to better identify where the three NESs are found within the NSF structure, I obtained an NSF structural model (residues 224-742) from the protein data bank (PDB), and using RasMol highlighted the three regions of interest (Figures 12 and 13). Using MOLMOL I determined the percentage of the total surface area of each residue that is solvent accessible, within each putative NES (Figure 12B, C, D). Additionally, we have previously shown that point mutations of I705A and I707A in NES-3 result in nuclear-NSF accumulation (Figure 7). Taken together, the data suggests that NSF is shuttled out of the nucleus by Exportin 1.

### 3.4 NEM Affects Cellular Localization of NSF

When Block et al. (1988) treated Golgi networks of CHO cells with NEM, transport was inhibited; it was this study that first led to the isolation of NSF (Block et al. 1988). Consequently, we were interested in studying the effect of NEM on NSF localization,
Figure 12. (A) N-ethylmaleimide Sensitive Factor (residues 224-742 at a resolution of 1.75Å) illustrating the three nuclear export sequences (NES) and (B, C, D) percentage of solvent accessible surface area. (A) The structural model was obtained from the protein data bank (Model ID: c1d6b18bb389199c6837f37889952f34) and using RasMol (version 2.7.5) the three putative NES were highlighted. (B, C, D) Using MOLMOL (version 2K.2) the percentage of the solvent accessible surface area of each residue was determined.

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**NES 1: 600-609**

**NES 2: 621-630**

**NES 3: 705-714**
Figure 13. (A) N-ethylmaleimide Sensitive Factor D2 monomer (residues 489-742 at a resolution of 1.75Å) illustrating the three nuclear export sequences (NES). The structural model was obtained from the protein data bank (Model ID: c1d6b18bb389199c6837f37889952f34) and using RasMol (version 2.7.5) the three putative NES were highlighted. (B) N-Ethylmaleimide Sensitive Factor D2 hexameric structure with boxed monomer in green reflecting D2 monomer orientation in (A). Image (B) was reprinted with permission (Yu, R.C. et al., 1998).
specifically nuclear NSF localization. Furthermore, NEM has been shown to act as a chromosome region maintenance 1 (CRM1 or Exportin 1) inhibitor by alkylating Cys529 of Exportin 1 (Kudo, N. et al. 1999). Therefore, NEM treatment of CHO cells was conducted to examine its effect on 1) NSF localization and 2) Exportin 1 activity. In order to address these questions I examined nuclear NSF levels following cellular fractionation and immunoblotting. Immunoblotting of NEM treated CHO cells showed small overall changes in whole cell NSF levels, at all three NEM concentrations tested (quotient values of: 0.99 at 0.04mM, 0.88 at 1.0mM and 1.02 at 5mM) (Figure 14A and B). However, at a NEM concentration of 0.04mM, cytoplasmic and nuclear NSF levels are higher than their respective control levels (quotient values of 1.10 and 1.20, respectively). At 1mM NEM, cytoplasmic levels appear unchanged (quotient value of 1.01), while nuclear levels decrease (quotient value of 0.61). At 5mM NEM, cytoplasmic levels show a subtle decrease (quotient value of 0.92), while nuclear levels remain unchanged (quotient value of 1.01).

Although the data is not significant, the trend demonstrates that at high concentrations of NEM whole cell, cytoplasmic and nuclear NSF levels remain largely unchanged from control levels. At the lowest NEM concentration (0.04mM), cytoplasmic and nuclear NSF levels increased, suggestive of Exportin 1 inhibition and nuclear accumulation of NSF. However, the largest changes of whole cell and nuclear NSF levels occur at a NEM concentration of 1mM, when NSF levels decreased (Figure 14B). These results are somewhat counterintuitive, suggesting an alternate route for NSF export. N-ethylmaleimide appears to have an effect on nuclear NSF localization, however the effect is not linear and requires further investigation in order to better understand the mechanism in effect.

3.5 LMB Does Not Affect Nucleocytoplasmic Shuttling of NSF

Leptomycin B is used extensively in molecular biology as an inhibitor of Exportin 1. Like NEM, it interferes with Exportin 1 activity by alkylating Cys529 (Kudo, N. et al. 1999). Therefore, I employed LMB treatment of CHO cells as a second method in determining whether NSF depends on Exportin 1 for nuclear export. Imaging data
Figure 14. Whole cell (W), cytoplasmic (C) and nuclear (N) fractions from NEM treated or not-treated CHO cells. Cells were incubated either in the absence or presence of NEM prior to fractionation. SDS-PAGE was performed followed by a western blot (A) 16µg of protein was loaded. White arrows indicate molecular weights in kDa. (B) NSF band intensity of 0.04mM, 1.0mM and 5mM n-ethylmaleimide (NEM) treated cells divided by control NSF band intensities in the whole cell lysate, cytoplasmic and nuclear cellular fractions. Bars represent quotient means determined from 3 experiments with standard deviations based on these means.
Figure 15. Imaging of wtNSF-EGFP in CHO cells in the absence, presence and post-LMB (Xinping Qiu, unpublished data). CHO cells were treated with 200ng/mL of LMB and incubated for 4 hours. Cells were then washed, fixed and stained with DAPI so as to view the localization of wtNSF-EGFP with respect to the nucleus.
showed a very subtle increase in nuclear localization of NSF during LMB treatment of CHO cells when compared to controls (Figure 15; Xinping, Qiu, unpublished data). Similarly, western blotting techniques indicated that at a LMB concentration of 1ng/mL, whole cell and nuclear NSF levels showed a mild decrease from control levels (quotient values of 0.90 and 0.92, respectively), while cytoplasmic levels increased (quotient value of 1.21) (Figure 16A and C). At 10ng/mL of LMB whole cell NSF levels show a strong increase (quotient value of 1.53), however cytoplasmic and nuclear NSF levels are close to control values (quotient values of 1.05 and 0.94, respectively) (Figure 16A and C). At 100ng/mL of LMB whole cell NSF levels remain unchanged (quotient value of 0.97), however cytoplasmic NSF levels increased (quotient value of 1.30), while nuclear NSF levels showed a decrease (quotient value of 0.60) when compared to control levels (Figure 16A and D). With a two-fold increase in LMB concentration, whole cell and cytoplasmic NSF levels increased (quotient values of 1.55 and 1.18, respectively), while nuclear NSF levels remained unchanged from control levels (quotient value of 0.99) (Figure 16B and D). A representative blot of all LMB concentrations tested is shown (Figure 16A and B). LMB concentrations of 1ng/mL and 10ng/mL were only tested once. In summary, at 1ng/mL, 10ng/mL and 200ng/mL of LMB, nuclear NSF levels are unchanged (quotient values approximately equaling 1.0), however, at a LMB concentration of 100ng/mL nuclear NSF levels dropped. These results in conjunction with the imaging data conducted by Xinping Qiu, imply that inhibition of Exportin 1 by LMB does not interfere with nuclear export of NSF, indicating that NSF does not rely on Exportin 1 for nucleocytoplasmic shuttling.

3.6 NSF Shows Nuclear Localization Across Several Cell Lines

In order to determine if NSF localizes to the nucleus in other cell lines, nuclear lysates from four different cell lines were purchased. In addition to CHO cells, mouse neuroblastoma (N2A) cells were cultured and cellular fractionation was performed. Protein immunoblotting was performed using nuclear lysates derived from HeLa and PC12 cells (Figure 17A). NSF was present in the PC12 nuclear lysate, though none was detected in the nuclear fraction derived from HeLa cells (Figure 17A). Protein
Figure 16. Whole cell (W), cytoplasmic (C) and nuclear (N) fractions from LMB treated or not-treated CHO cells. Cells were incubated either in the absence or presence of LMB for 4 hours, prior to fractionation. SDS-PAGE was performed followed by a western blot (A) 8.2µg of protein was loaded. (B) 8.0µg of protein was loaded. White arrows indicate molecular weights in kDa. (C) NSF band intensity of 1ng/mL and 10ng/mL LMB treated cells divided by control NSF band intensities in the whole cell, cytoplasmic and nuclear cellular fractions. (D) NSF band intensity of 100ng/mL and 200ng/mL LMB treated cells divided by control NSF band intensities in the whole cell, cytoplasmic and nuclear cellular fractions. Bars represent quotient means determined from 2 experiments with standard deviations based on these means.
Figure 17. Immunostaining of NSF within nuclear lysate originating from several cell types. (A) HeLa and PC12 nuclear lysates; immunoblotting was conducted for the presence of NSF and TBP; tubulin served as a positive control (37.5\(\mu\)g of protein was loaded). (B) CHO, N2A, NIH3T3, PC12 and Jurkat nuclear lysates; immunoblotting was conducted for the presence of NSF and TBP; tubulin and actin served as a positive control (30\(\mu\)g of protein was loaded). SDS-PAGE was performed followed by a western blot. White arrows indicate molecular weights in kDa.
immunoblotting was then performed using nuclear lysates derived from: CHO, N2A, NIH 3T3, PC12 and Jurkat cells (Figure 17B). Cell lines showed varying levels of nuclear NSF, with CHO cells showing the highest levels and Jurkat cells showing absence of NSF (Figure 17B). It should be noted that nuclear lysates originating from HeLa, PC12, NIH 3T3 and Jurkat cells were purchased, while nuclear lysates derived from CHO and N2A cells were prepared using the Cellular Fractionation protocol. These results confirm NSF nuclear localization in several cell lines and suggest that the role nuclear NSF is executing is not exclusive to a specific cell type.

3.7 Nuclear NSF Interacts with Vimentin

In order to elucidate the nuclear function of NSF, I chose an immunoprecipitation strategy to identify the binding partner(s) of nuclear-NSF. N-Ethylmaleimide Sensitive Factor was immunoprecipitated from a CHO nuclear fraction and the resulting sample was analyzed by mass spectrometry (Figure 18A). The mass spectrometry analysis confirmed the presence of NSF in the sample and identified a number of co-immunoprecipitated proteins (Figure 18B). Among these, the intermediate filament protein Vimentin showed the highest number of unique peptides (31 unique peptides, Figure 18C). In addition, several other interacting proteins were identified (for a sample of co-immunoprecipitated proteins with a protein identification probability of >99.9% see Figure 18D). Among these were exclusively nuclear proteins, histones H2A and H3, and the cytoskeletal protein, actin (Figure 18D).

In order to confirm the NSF-Vimentin interaction the reverse IP approach was taken using an anti-Vimentin antibody. Initially SyproRuby staining confirmed the presence of protein in the nuclear IP sample and the absence of protein in the control (Figure 19A). Immunoprecipitation from cytoplasmic and nuclear fractions was performed, followed by immunoblotting (Figure 19B and C). Although NSF did not immunoprecipitate with Vimentin from the cytoplasmic fraction, I did detect an NSF-Vimentin interaction in the nuclear fraction (Figure 19B and C). These results confirm the NSF-Vimentin interaction and suggest that the NSF-Vimentin interaction is exclusive to the nucleus.
**Figure 18.** N-Ethylmaleimide Sensitive Factor IP from CHO cells. (A) NSF IP from CHO cell nuclear fraction showing whole cell lysate (W), IP pull-down, flow through (FT), and wash 1 (W1) samples in the presence (+) and/or absence (-) of the NSF antibody. SDS-PAGE was performed followed by a western blot. To confirm NSF presence immunoblotting was performed; the actin stain served as a positive control. White arrows indicate molecular weights in kDa. (B) NSF IP mass spectrometry results and (C) Vimentin IP mass spectrometry results. IP samples were sent to Sick Kids Proteomics Center for mass spectrometry analysis. Results confirmed the IP of NSF and revealed that Vimentin co-immunoprecipitated. Yellow highlights indicate 80-94% probability of residue identification and green highlights indicate over 95% probability of residue identification. (D) N-Ethylmaleimide Sensitive Factor co-IP results as determined from mass spectrometry analysis conducted by Sick Kids Proteomics Center. All results show a >99.9% protein identification probability.
**Figure 19.** Vimentin IP from CHO cells. (A) Vimentin IP from CHO cell nuclear fraction showing whole cell lysate (W), IP pull-down, flow through (FT) and wash 1 (W1) samples in the presence (+) or absence (-) of the Vimentin antibody. SDS-PAGE was performed followed by SYPRO Ruby staining. (B) Vimentin IP from CHO cell cytoplasmic and (C) nuclear fractions showing whole cell lysate (W), flow through (FT), wash 1 (W1) sample and IP pull-down samples in the presence (+) and absence (-) of the Vimentin antibody. SDS-PAGE was performed followed by a western blot. To confirm NSF and Vimentin’s presence immunoblotting was performed. White arrows indicate molecular weights in kDa.
3.8 NSF is Present in the Nuclei of Prophase Cells

Our unpublished data indicates that NSF nuclear dynamics may be associated with the cell cycle, therefore, I sought to investigate the NSF nuclear profile in a population of synchronized cells. Nocodazole is commonly used in molecular biology as a tool for cell synchronization (Poxleitner, M.K. et al. 2008; Matsui, Y. et al. 2012). Initially, to confirm that the NSF immunoprecipitated from the nuclear fraction was truly nuclear, CHO cells were treated with nocodazole for 10-hours. This should have arrested cells, however, cell synchronization was not achieved as only 20% of cells reached chromosomal condensation, suggestive of prophase (Figure 20A).

As previously described, a cytoplasmic and nuclear Vimentin IP was performed followed by immunoblotting (Figure 20B and C). As before, NSF was not associated with the Vimentin IP from the cytoplasmic fraction, however both proteins were present in the nuclear Vimentin IP (Figure 20B and C). These results confirm the nuclear NSF-Vimentin interaction.

In order to ascertain that the identified nuclear NSF is truly nuclear, rather than mistakenly present within the nuclei of newly formed cells still requiring reorganization, it was necessary to perform nuclear localization prior to the disintegration of the nuclear membrane. In order to accomplish this it was necessary to perform cellular fractionation on early prophase cells. I, therefore, employed an alternate cell synchronization protocol. Chinese hamster ovary cells were treated with thymidine for 24 hours. Upon removal of thymidine, cells grown on test plates were fixed, stained with DAPI and imaged at each hour, with time 0 marking the removal of thymidine, to a total of 18 hours (Figure 21). At 14.5 hours approximately 80% of the nuclei showed early chromatin condensation, suggestive of early prophase (Figure 21B). At this point cellular fractionation was performed followed by an NSF-IP of the cytoplasmic and nuclear fractions in efforts to determine the localization of NSF during prophase (Figure 22). The IP results show that NSF is present in both the cytoplasmic and nuclear fractions. It should be noted that the nuclear NSF band appears at a lower molecular weight than the whole cell fraction NSF band (Figure 22B). This phenomenon is also evident in Figures 11, 14 and 16. Likely these differences are due to post-translational modifications of NSF, although further work is necessary to confirm this. However, this
may prove to be a useful marker for distinguishing cytoplasmic and nuclear NSF in future studies.
Nocodazole mediated CHO cell synchronization. (A) DAPI staining of CHO cells after a 10-hour nocodazole (50ng/mL) treatment. Black arrow indicates a cell entering mitosis as evidenced by chromosomal condensation. (B) Vimentin IP from CHO cell cytoplasmic and (C) nuclear fractions following a 10 hour nocodazole (50ng/mL) treatment, showing whole cell lysate (W), IP pull-down, flow through (FT), wash 1 (W1) and wash 3 (W3) samples in the presence (+) and absence (-) of the Vimentin antibody. SDS-PAGE was performed followed by a western blot. To confirm NSF and Vimentin’s presence immunoblotting was performed. White arrows indicate molecular weights in kDa.
Figure 21. Thymidine mediated CHO cell synchronization. Cells were incubated with 2nM thymidine for 24 hours. After incubation, thymidine was removed, cells were washed and their progression through the cell cycle was monitored for 18 hours, with $T_0$ marking removal of thymidine, $T_1$ marking one hour after thymidine removal and so forth. (A) Cells from $T_0$ to $T_5$ (B) from $T_6$ to $T_{12.5}$ and (C) from $T_{13.5}$ to $T_{18}$. 
Figure 22. N-Ethylmaleimide Sensitive Factor IP from thymidine synchronized CHO cells. (A) NSF-IP from the cytoplasmic and (B) nuclear fractions following a 24 hour thymidine (2mM) treatment, showing whole cell lysate (W), flow through (FT), wash 1 (W1), wash 3 (W3) and IP pull-down samples in the presence (+) and absence (-) of the NSF antibody. SDS-PAGE was performed followed by a western blot. To confirm NSF’s presence immunoblotting was performed. White arrows indicate molecular weights in kDa.
4 Discussion

4.1 Overview

The present study was undertaken to investigate the nuclear localization of NSF and to identify its nuclear binding partner(s) in efforts to characterize its nuclear role. I hypothesized that NSF is a dynamic nuclear protein that interacts with other nuclear proteins to control cell function. To test this hypothesis I first attempted to use different means to bias nuclear-NSF localization: fibronectin induced CHO cell spreading and drug induced Exportin 1 inhibition. To identify nuclear binding proteins, I immunoprecipitated NSF and used mass spectrometry to identify any co-immunoprecipitated products. I then tested nuclear-NSF localization within several cell types. Lastly, I used drug-induced cell synchronization methods to identify nuclear NSF localization at the subcellular level during specific cell cycle phases.

N-Ethylmaleimide Sensitive Factor whole cell and nuclear levels fluctuated during cell spreading and NEM/LMB treatments. Immunoprecipitation identified NSF’s putative nuclear binding as Vimentin and NSF was found to localize to the nucleus of CHO, N2A, PC12 and NIH 3T3 cells.

Earlier data has shown localization of NSF to the nuclear membrane in PC12 cells (Tagaya, M. et al. 1996; Mashima, J. et al. 2000). Additionally, unlike golgi-localized NSF, nuclear membrane localized NSF was not released when incubated with Mg$^{2+}$-ATP (Tagaya, M. et al. 1996; Mashima, J. et al. 2000). There also exists evidence for NSF’s role in the formation of the nuclear envelope and assembly of the NPC (Baur, T. et al. 2007). The current data surrounding the diverse and multiple roles played by AAA+ proteins suggests that NSF is likely not limited to SNARE disassembly (for a review of the AAA+ superfamily functions see: Snider, J. et al. 2008). In fact, there is a strong line of evidence that shows NSF does play many roles and is not limited to the cytoplasm (Tagaya, M. et al. 1996; Kamboj S.I. et al. 1998; Nishimune A. et al. 1998; Osten P. et al. 1998; Mashima, J. et al. 2000; Matsushita, K. et al. 2003; Huang Y. et al. 2005; Baur, T. et al. 2007). My data supports this hypothesis and suggests of a novel nuclear role for NSF.
4.2 Bioinformatics Analysis of NSF

Three putative nuclear export sequences (NESs) were identified within the NSF D2-domain (Figure 1). Blast sequence alignment of all three NESs showed conserved hydrophobic residues between *Homo sapiens*, *Cricetulus griseus*, *Drosophila melanogastor*, *Dictyostelium discoideum* and *Saccharomyces cerevisiae* (Figure 5). Furthermore, hydrophobic residues at the specified positions show conservation between NESs of different proteins (Figure 6). Identification of putative NESs facilitated studies where point mutations were introduced into the putative NESs (Figure 7, Xinping, Qiu; unpublished data). When two point mutations (I705A and I707A) were introduced into the third NESs of mammalian NSF, transfected Schneider 2 cells of *Drosophila melanogastor* showed a strong NSF signal within the nucleus. Taken together this data strongly supports the correct identification of the NESs.

4.3 Fibronectin-Mediated Cell Spreading Affects NSF Localization

Fibronectin is a cell adhesion protein whose receptors are part of integrin-family of proteins (Wennerberg, K. *et al.* 1997). Spreading of CHO cells plated on immobilized fibronectin coated test-plates was evident after 2 hours (Figure 10). Immunoblotting data revealed elevated whole cell NSF levels, although nuclear NSF levels did not show an increase (Figure 11C). Cytoplasmic NSF levels, did however, show a decrease (Figure 11C). The increase in whole cell NSF levels should be accompanied with an increase in nuclear and/or cytoplasmic NSF levels, however, the data does not show this. The loss of sample is inevitable during the purification of the cytoplasmic and nuclear fractions; these steps are omitted when preparing the whole cell fraction. It is the loss of sample that likely gave rise to the incongruity in the results.

Nevertheless, when considering the whole cell fraction the data suggests that during integrin activation, when much cytoskeletal reconstruction occurs within the cell, NSF is upregulated within the cell. This is in agreement with other studies that found the E329Q-NSF mutant to be an inhibitor of SNARE-mediated α3β1 integrin trafficking and consequently cell spreading (Tayeb, M. A. *et al.* 2005; Skalski, M. and Coppolino, M. G., 2005; Skalski, M. *et al.* 2011).
4.4 NEM Affects the Subcellular Localization of NSF

N-ethylmaleimide treatment revealed an increase in nuclear-NSF at a NEM concentration of 0.04mM, suggestive of Exportin 1 inhibition by NEM (Figure 14A and B). Inhibiting nuclear export via leptomycin B results in the nuclear accumulation of tubulin due to Exportin 1 inhibiton; NEM has been shown to inhibit nucleocytoplasmic shuttling by the same mechanism (Kudo, N., 1999; Akoumianaki, T. et al. 2009). Consequently, tubulin was used as a positive control for both NEM and LMB treatments. Nuclear tubulin increased at 0.04mM NEM (Figure 14A). However, there is a decrease in nuclear NSF and tubulin levels at a NEM concentration of 1mM (Figure 14A and B). This result is counterintuitive, as one would expect an increase in nuclear accumulation of both proteins with an increase in Exportin 1 inhibition. Interestingly, at 5mM NEM whole cell, cytoplasmic and nuclear NSF levels return to control levels. It may be speculated that the decrease of nuclear NSF and tubulin at 1mM NEM, despite the increase at 0.04mM NEM, is due to an alternate export machinery.

4.5 Exportin 1 is Likely Not Responsible for Nuclear-NSF Export

Imaging data of CHO cells treated with LMB and protein immunoblotting revealed a very mild increase in the nuclear localization of NSF (Figure 15 and 16). These results are comparable to those seen with the NEM treatments (Figure 14). The data at 1mM NEM and 100ng/mL LMB implies that Exportin 1, which has been classified as an exportin that shuttles leucine rich NES cargo, may not be responsible for nuclear export of NSF (Pemberton, L.F. et al. 2005). Alternatively, NSF may be shuttled out of the nucleus by Exportin 1, but not depend on the LMB sensitive motif of Exportin 1 for this transport (Connor, M.K., et al. 2003). To further investigate whether the mild effect of NEM and LMB on NSF is due to an alternate nuclear export route, imaging analysis of NSF-NES mutants may be used. NSF-NES mutants with point mutations introduced at positions of leucine residues could be imaged for nuclear NSF accumulation, as a function of time, during NEM/LMB treatment in order to monitor nuclear NSF accumulation with Exportin 1 inhibition. Alternatively, the putative NESs of NSF may be tagged onto a green fluorescence protein (GFP) and microinjected into the nuclei of
CHO cells. Cells may be treated with NEM and LMB and the localization of the NES-tagged GFP monitored. This experiment would help to 1) further validate the correct identification of the NESs and 2) ascertain whether Exportin 1 is responsible for NSF export.

### 4.6 Evidence for Cytoplasmic Post-Translational Modification of NSF

It is quite evident that nuclear-NSF resolves at a slightly lower molecular weight than cytoplasmic-NSF (Figures 11, 14 and 16). This phenomenon may be due to posttranslational modifications of NSF such as S-nitrosylation or phosphorylation. S-nitrosylation of NSF has been reported as a means of regulation during AMPA receptor recycling and during exocytosis of Weibel-Palade bodies (Huang, Y. et al., 2005; Matsushita, K. et al. 2003). Phosphorylation of NSF by Pctaire1 on serine 569 in the D2 domain has been reported as a means of regulating NSF’s oligomerization (Liu, Y. et al., 2006). While phosphorylation by protein kinase C (PKC) at serine 237 in the D1 domain results in NSF’s inability to bind to the SNAP-SNARE complex (Matveeva E.A. et al., 2001). Though it has been reported that the sulfur-nitric oxide bond is cleaved during SDS-PAGE manipulation of a sample, a phosphate group remains and can be detected on a western blot (Torta, F. et al., 2008; Kocinsky, H.S. et al., 2005). Further experiments will have to be conducted to test whether the differences observed in the way nuclear versus cytoplasmic-NSF resolve on the SDS-PAGE gel, relates to its phosphorylation. If indeed, cytoplasmic and nuclear NSF resolve at distinct molecular weights on account of post-translational modifications, this difference may be exploited in distinguishing the levels of cytoplasmic versus nuclear NSF.

### 4.7 NSF Localizes to the Nuclei of Various Cell Lines & Cell Types

Immunoblotting revealed NSF is present in nuclear lysates originating from PC12, CHO, N2A and NIH3T3 cell lines (Figure 17A and B). The nuclear lysate derived from CHO cells had the highest level of nuclear NSF. An anti-TBP antibody was used as a positive control, however the success of the stain varied across the cell lines. This is
likely due to the fact that the host animal, mouse, will show better reactivity with mouse, rat and hamster-originating samples than human samples, and this is indeed the result. Nonetheless, TBP is present across all nuclear samples tested.

The presence of NSF in nuclear lysates of other cells is an important contribution to the current study as it demonstrates NSF’s nuclear localization between species and across a range of cell lines. A previous report on the nuclear localization of NSF by Tagaya, M. et al. (1996) demonstrated the presence of NSF in the nuclear membrane of PC12 cells. The authors confirmed that the nuclear membrane-associating NSF was not released when treated with Mg\(^{2+}\)-ATP, unlike the golgi-associating NSF, which was released (Tagaya, M. et al. 1996; Mashima, J. et al. 2000). Additionally, the molecular weight of the resolved NSF bands varies between the cell lines tested (Figure 17B). The CHO, N2A, NIH 3T3 and PC12 nuclear lysates all appear to have two bands detected by the NSF antibody, one appearing at approximately 78kDa and the other at 70kDa (Figure 17B). These differences between samples may result from differences between the cell lines themselves. Because most of the cell lines tested have differentiated (NIH 3T3 cells differentiate further), their roles within an organism vary, and the levels of nuclear versus cytoplasmic NSF may, consequently, also vary (Wang, Z. et al. 2011). Further studies will have to investigate the level of nuclear NSF localization within a range of cell lines, as well as the differences in molecular weights, as our data suggests these factors may be variable.

4.8 Vimentin: A Putative Nuclear Binding Protein of NSF

Once nuclear localization of NSF was confirmed, and the extent of its sensitivity to NEM and LMB treatments within the nuclei of CHO cells was demonstrated, IP of nuclear NSF was employed in order to determine if there are nuclear-specific binding partner(s) (Figure 18A). Mass spectrometry analysis of IP samples was conducted by the Sick Kids center for proteomics (Figure 18B and C). Mass spectrometry analysis confirmed the IP of nuclear NSF and identified several proteins that co-immunoprecipitated (Figure 18B and D). Among those identified, the intermediate filament, Vimentin, had the highest number of unique peptides (Figure 18C). I confirmed the NSF-Vimentin interaction using a reverse IP approach (Figure 19).
Because I compared co-IP of NSF and Vimentin in cytoplasmic and nuclear fractions, and only found an interaction in the nuclear fraction, my data strongly suggest that the NSF-Vimentin interaction is restricted to the nucleus.

Vimentin is a member of the group of proteins that make up intermediate filaments. Intermediate filaments are a family of close to 70 proteins, divided into six groups based on their sequence similarity, which fall intermediate in diameter size (10 nm) when compared to actin filaments (7 nm) and microtubules (25 nm) (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011). Intermediate filaments are not as dynamic in their assembly and disassembly as actin filaments and microtubules (Cooper, G.M. 2000). Additionally, unlike actin filaments and microtubules, they are not responsible for cell movement but serve to provide structural support to the cell, and phosphorylation of intermediate filaments affects their assembly and organization (Cooper, G.M. 2000). Within the family of intermediate filaments the six classes show differences in expression and in intracellular organization. For example, type I and II intermediate filaments form only heterodimers, while type III intermediate filaments may form homodimers or heterodimers (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011).

Vimentin is a 57-kDa, highly conserved, type III intermediate filament and it is often used as a mesenchymal cell marker (Wu, Y. et al. 2007). Additionally, overexpression of vimentin is correlated with poor prognosis in cancer patients (Satelli, A. and Li, S. 2011). Vimentin shows differential expression in several cell lines due to the varying roles it plays (Satelli, A. and Li, S. 2011). Like all intermediate filaments it is apolar, with a central α-helix, necessary for intermediate filament assembly (Cooper, G.M. 2000).

Vimentin forms polymers, either with itself or with other type III/IV intermediate filaments (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011). This polymerization is facilitated by the conserved central α-helix and is common within the intermediate filament proteins (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011). During polymerization, two Vimentin polypeptides align in parallel and their α-helices intertwine, this dimer associates with a second Vimentin dimer in an anti-parallel orientation forming an apolar tetramer (Cooper, G.M. 2000). The linear alignment of several tetramers forms an apolar protofilament (Cooper, G.M. 2000). The regulation of

Initially, Vimentin was known for its cytoplasmic roles and thought to reside in the cytoplasm. However, when fluorescently labeled Vimentin was coinjected with single-stranded DNA and circular DNA into the cytoplasm of cultured cells it localized to the nucleus (Hartig, R., et al., 1998). Recently, Vimentin has been shown to play a nuclear role in the regulation of p21Waf1 expression in neuroblastoma cells, though the mechanism by which it operates is still not well understood (Mergui, X., et al., 2010). Additionally it has been shown to reduce activating transcription factor 4 (ATF4) activity, thereby inhibiting osteocalcin transcription (Lian, N., et al., 2009). Vimentin’s diverse cytoplasmic roles suggest its nuclear roles will reflect the same diversity and vary from cell-line to cell-line, however much more investigation is required.

Vimentin has been linked to several cancers, such as prostate, breast and gastrointestinal tract cancers just to name a few, and its expression pattern varies among them (Lang, S.H. et al., 2002; Hu, L. et al., 2004; Gilles, C. et al., 2003). However, in all cancers its over-expression is associated with poor prognosis due to the metastatic and invasive phenotype of the resulting cancer cells (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011). The mechanism by which Vimentin facilitates these phenotypes is still under investigation (Satelli, A. and Li, S. 2011). Despite this, it is becoming apparent that Vimentin may prove to be a marker for cancer prognosis and a possible target for future chemotherapeutic agents.

4.9 NSF is Present in the Nuclei of Prophase Cells

Nocodazole is used extensively in cell synchronization protocols in efforts to bring a pool of cells into mitosis but arrest them at metaphase (Harper, J. V. 2005; Matsui, Y. et al. 2012). Upon removal of nocodazole, cells should continue to progress normally through the cell cycle (Harper, J. V. 2005). Correspondingly, nocodazole treatment was
used in efforts to synchronize the cell cycle and to rule out the possibility that the detected nuclear-NSF was not truly cytoplasmic NSF that had not been shuttled out of a newly formed nucleus of a G1 cell. However, upon treatment it is difficult to say whether cell synchronization was achieved (Figure 20A). Nocodazole treatment of CHO cells resulted in approximately 20% of cells reaching chromosomal condensation, suggestive of prophase (Figure 20A). Therefore, cell synchronization was not achieved.

Nonetheless, an IP of Vimentin from the nocodazole treated cytoplasmic and nuclear fraction was performed, and an NSF signal, albeit weak, was detected in the nuclear IP sample (Figure 20B and C). No NSF signal was detected in the cytoplasmic IP fraction (Figure 20B). These results are in congruence with the Vimentin IP results of nocodazole untreated CHO cells (Figure 19). However, it should be noted that studies have shown that although nocodazole disrupts microtubule formation, it does not arrest cells in a single stage of the cell cycle (Cooper, S. et al. 2006).

Other forms of cell synchronization that have been shown to be effective with CHO cells involve treatment with DMSO (Fiore, M. et al. 2002). Fiore, M. et al. (2002) found that CHO cells grown with 1-2% DMSO for 4 days were synchronized at the G1 phase. Thymidine is also added to cell cultures as a means to prevent nucleotide synthesis and consequently, DNA synthesis, arresting cells in the S phase of interphase (Jackman, J. and O’Connor, P. 2001; Harper, J. V. 2005). Therefore, to further confirm the above results, a thymidine block was used to arrest CHO cells at the S-phase (Figure 21).

After removal of thymidine cells were monitored at each hour until early prophase was reached by approximately 80% of cells (Figure 21C, T14.5). At this point cellular fractionation was performed followed by immunoprecipitation. Immunostaining revealed that NSF is present in the cytoplasm and nuclear fractions of prophase cells (Figure 22). Therefore, because the Vimentin-IP revealed an absence of NSF in the cytoplasmic fraction, but presence of NSF in the corresponding nuclear fraction, the data suggests that the NSF-Vimentin interaction is exclusively nuclear (Figure 19B and C). Taken together, this data indicates that the NSF immunoprecipitated in this and earlier experiments is truly nuclear.
5 Summary and Conclusion

Recent studies have shown the nuclear effects of Vimentin include regulation of p21Waf1 expression in neuroblastoma cells and down-regulating the activity of activating transcription factor 4 (ATF4) resulting in the inhibition of osteocalcin transcription (Mergui, X., et al., 2010; Lian, N., et al., 2009). The over-expression of Vimentin has been linked with poor prognosis in several cancers (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011). The resulting cancer cells tend to exhibit invasive and metastatic behaviors (Cooper, G.M. 2000; Satelli, A. and Li, S. 2011). Unfortunately, the pathway leading to this phenotype is still not understood (Satelli, A. and Li, S. 2011). Although more work is required to confirm NSF’s nuclear role and its nuclear interaction with Vimentin, it is becoming apparent that Vimentin, and possibly NSF, may prove to be markers for cancer prognosis and possible targets for future chemotherapeutic agents. Considering NSF’s well-studied role as a chaperone protein, serving to dissociate the SNARE complex, I speculate that NSF’s nuclear role may involve the disassembly of a complex, which Vimentin associates with.

It is worth contemplating why earlier studies have not detected nuclear-NSF. Searching through the literature provides some answers to this conundrum. Although, there are studies that examined imaging and co-localization of NSF with several other proteins, no study to current date has examined nuclear localization of NSF and no study conducted cellular fractionation followed by immunoblotting to test for the presence of NSF within the nucleus (Song, I. et al. 1998; Mukhopadhyay, S. et al. 2008; Zhao, C. et al. 2010). Imaging of NSF will not reveal nuclear-NSF, as shown in our results (Figures 7 and 15). This is likely due to the levels of nuclear-NSF when compared to those of cytoplasmic-NSF. Without a doubt, there is a higher concentration of NSF within the cytoplasm than that within the nucleoplasm. This surely reduced the chances of nuclear-NSF detection and consequently, cellular fractionation and immunoblotting was not pursued.

In conclusion, using immunoblotting techniques I have shown that NSF localizes to the nucleus of several cell lines and using CHO cells I have demonstrated that its nuclear levels change in the presence of LMB and NEM and during fibronectin-mediated cell spreading. Immunoprecipitation and reverse co-IP of nuclear-NSF
facilitated the identification of its putative nuclear binding partner, Vimentin. I have shown preliminary data that suggests that this interaction is exclusive to the nucleus. Additionally, I have shown that nuclear NSF is present in early prophase cells, ones with intact nuclear membranes, arguing against cytoplasmic NSF contamination. Determining the nature of the nuclear NSF-Vimentin interaction and its function will lead to the characterization of a novel role for NSF. Ultimately, this mechanism will add to the list of functions NSF and other AAA+ ATPases are responsible for.
References


Goto, H., Tanabe, K., Manser, E., Lim, L., Yasui, Y. and Inagaki, M.


**Matsui, Y., Nakayama, Y., Okamoto, M., Fukumoto, Y. and Yamaguchi, N.** Enrichment of cell populations in metaphase, anaphase, and telophase by


Appendix A

Recipe of Necessary Buffers for Cellular Fractionation Protocol as per Lamond Lab (University of Dundee, Dundee, Scotland)

**Buffer A (10 ml)**

_Hypotonic buffer that causes the cells to swell so that they can be efficiently broken open by dounce homogenizing._

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume (ml)</th>
<th>mM (final)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M HEPES, pH 7.9</td>
<td>0.1</td>
<td>10</td>
</tr>
<tr>
<td>1 M MgCl2</td>
<td>0.015</td>
<td>1.5</td>
</tr>
<tr>
<td>2.5 M KCl</td>
<td>0.04</td>
<td>10</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>0.005</td>
<td>0.5</td>
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<tr>
<td>dH2O</td>
<td>Up to 10 ml</td>
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</tr>
<tr>
<td>Protease inhibitors</td>
<td>1 Mini EDTA-free COMPLETE tablet</td>
<td></td>
</tr>
</tbody>
</table>

**S1 (0.25 M Sucrose, 10 mM MgCl2) 20 ml**

_Sucrose buffer used to fractionate nuclei._

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume (ml)</th>
<th>mM (final)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 M Sucrose</td>
<td>1.96</td>
<td>0.25</td>
</tr>
<tr>
<td>1 M MgCl2</td>
<td>0.2</td>
<td>10</td>
</tr>
<tr>
<td>dH2O</td>
<td>Up to 20 ml</td>
<td></td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>1 EDTA-free COMPLETE tablet</td>
<td></td>
</tr>
</tbody>
</table>

**S3 (0.88 M Sucrose, 0.5 mM MgCl2) 20 ml**

_Sucrose buffer used to fractionate nucleoli._

<table>
<thead>
<tr>
<th>Stock</th>
<th>Volume (ml)</th>
<th>mM (final)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 M Sucrose</td>
<td>6.9</td>
<td>0.88</td>
</tr>
<tr>
<td>1 M MgCl2</td>
<td>0.01</td>
<td>0.5</td>
</tr>
<tr>
<td>dH2O</td>
<td>Up to 20 ml</td>
<td></td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td>1 EDTA-free COMPLETE tablet</td>
<td></td>
</tr>
</tbody>
</table>

* All tables were adapted from: