Identification and characterization of *Drosophila* Snurportin reveals a role for the import receptor Moleskin/Importin-7 in snRNP biogenesis

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ABSTRACT

AMANDA HICKS NATALIZIO: Identification and characterization of *Drosophila* Snurportin reveals a role for the import receptor Moleskin/Importin-7 in snRNP biogenesis
(Under the direction of A. Gregory Matera)

Biogenesis of small nuclear ribonucloceoproteins (snRNPs) is biphasic. Small nuclear RNAs (snRNAs) are exported to the cytoplasm for assembly into pre-snRNPs where they are hypermethylated, forming a trimethylguanosine (TMG) cap, and then transported back into the nucleus via the import adaptor, snurportin1 (SPN) and the import receptor importin-β. I have identified CG42303 as dSNUP, the *Drosophila* orthologue of human SPN (hSPN). Strikingly, the importin-β binding (IBB) domain, which is essential for SPN-mediated snRNP import in humans, is not conserved in dSNUP.

Consistent with the lack of an IBB, dSNUP did not interact with the *Drosophila* importin-β orthologue, Ketel. Despite this fact, dSNUP localized to the nucleus, indicating that there is an alternative dSNUP import pathway or that dSNUP is imported indirectly through importin-β bound snRNPs. I excluded the latter possibility since, in contrast to human cells, snRNPs did not associate with importin-β in *Drosophila* cells. Previous results suggested that hSPN interacts indirectly with a known import receptor, importin-7. I tested the possibility that the

Drosophila orthologue of importin-7, known as Moleskin (Msk), interacts with dSNUP and snRNPs.

I discovered that Msk physically associates with both dSNUP and U snRNPs, while snRNP components failed to bind importin-β. Furthermore, Msk null mutant larvae had a significant *in vivo* reduction of the snRNP component survival motor neuron (SMN), and the snRNP specific nuclear Cajal body marker coilin. Additionally, Msk null mutants exhibited cytoplasmic accumulation of TMG cap signal in the Malpighian tubules, indicating that the import of TMG capped snRNAs is inhibited in the absence of Msk. The reduction of SMN protein was dramatic enough to be detected by western blotting, suggesting a vital role for Msk in the stability of SMN. Interestingly, Msk also localized to snRNP specific nuclear Cajal bodies. In sum, these data indicate that importin-β does not play a role in snRNP import in *Drosophila* and implicate a crucial function for Msk in fruit fly snRNP biogenesis. Future experiments will be needed to determine the precise function of importin-7/Moleskin in both fruit fly and human snRNP biogenesis.

To my parents, my sister, Cassandra, my brother, Randy,

my uncle Dwight, my aunt Tomma, my husband, Tony,

and the light of my life and son, Alaric.

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A special thanks to my husband, Tony. Having a supporting husband that believes in my abilities, even when I'm feeling lost, is something that carried me through to the end. Last, but certainly not least, I want to thank my son, Alaric. Although he may have slowed down the pace of my Ph.D., my son allowed me to finish it with my sanity still intact. Coming home to Alaric's smiling face was always able to wash away the day's stresses and disappointments from lab.

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LIST OF ABBREVIATIONS

Å	Angstom
aa	Amino acids
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAS	Cellular apoptosis susceptibility protein
CB	Cajal body
CBC	Cap binding complex
CBP	Cap binding proteins
DAPI	4',6-diamidino-2-phenylindole
dSmB	*Drosophila melanogaster* Smith B
dSMN	*Drosophila melanogaster* survival of motor neurons
DNA	Deoxyribonucleic acid
dSNUP	*Drosophila melanogaster* Snurportin
dsRNA	Double stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
Ex	Exon
EXO	Exonulcease
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein

GST	Glutathione S-transferase
GTP	Guanosine triphosphate
h	Hours
HEAT	α-helical repeats
hIBB	*Homo sapiens* importin-β binding domain
hSPN	*Homo sapiens* Snurportin
IF	Immunofluorescence
IBB	Importin-β binding domain
Imp7	Importin-7
Impα	Importin-α
Impβ	Importin-β
IP	Immunoprecipitation
IPTG	Isopropyl-beta-D-Thiogalactopyranoside
kDa	Kilodalton
LMB	Leptomycin B
LR	Leucine-rich
m7G	7-methylguanosine
MDa	Megadalton
min	Minutes
mRNA	Messenger RNA
Msk	Moleskin
NES	Nuclear export signal
NGS	Normal goat serum

NLS	Nuclear localization signal
NP40	Nonidet-40
NPC	Nuclear pore complex
Nup153	Nucleoporin 153
ORF	Open reading frame
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with0.1% Triton 100x
PCR	Polymerase chain reaction
PRMT5	Protein arginine methyltransferase 5
PHAX	Phosphorylated adaptor for snRNA export
RIPA	Modified radioimmunoprecipitation assay
RNA	Ribonucleic acid
RNAi	RNA interference
RNP	Ribonucleoprotein
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
SMN	Survival of motor neurons
snRNA	Small nuclear RNA
snRNP	Small nuclear RNP
SPN1	Snurportin1

TMG	2,2,7-trimethylguanosine
tRNA	Transfer RNA
U snRNA	Uridine-rich small nuclear RNAs
VFP	Venus fluorescent protein
WT	Wild type
Xpo1	Exportin1/C

CHAPTER I

INTRODUCTION

Cellular Compartmentalization

The evolutionary advantage to compartmentalization of eukaryotic cells cannot be overstated. Division of the cell into organelles and sub-domains offers greater regulatory control of cellular processes than their prokaryotic counterparts. Most cellular compartments are surrounded by a lipid membrane and are organelles, such as mitochondria, lysosomes, the Golgi apparatus, or the cell nucleus. Compartmentalization provides several functions. It increases the membrane area without increasing the size of the cell, provides local environments that facilitate metabolic functions that may otherwise be inhibited by other processes within the cell, and enables the regulation of many key processes between various cellular regions.

The cytoplasm and the nucleus are two major compartments within the eukaryotic cell. The nuclear envelope, consisting of a lipid bilayer, separates the nuclear and cytoplasmic compartments. The nucleus contains the genomic material of the cell. Since prokaryotic cells are not compartmentalized, their

genomic material is unregulated, and DNA, RNA, and protein synthesis all occur in the cytosol. Unlike, prokaryotic cells, eukaryotic cells must have a means to transport molecules across the nuclear envelope for intercompartmental communication and regulation of their genomic material. Nuclear and cytoplasmic compartmentalization allows for the uncoupling of nuclear DNA/RNA synthesis from cytosolic protein synthesis, which enhances the cell's regulatory capabilities at the transcriptional and translational levels. Examples of this are numerous, from transcriptional repressors being sequestered to the cytoplasm to enhance a particular gene's expression to mRNA degradation within the nucleus to prevent export and translation in the cytoplasm.

The high levels of regulation seen in eukaryotic cells are not without expense. Eukaryotic cells must invest a substantial amount of energy to properly maintain such a complex regulatory and structural system. Often, energy is required for the various compartments to communicate. Molecules and macromolecules must be transported between compartments to facilitate this communication. Importantly, this intercompartmental communication must be appropriately regulated to avoid negative consequences for the cell.

Cellular Transport

The fundamental eukaryotic process of macromolecule transport into and out of the nucleus is a highly regulated process, involving several factors. Numerous transport factors and cofactors regulate the association between these transport components and the molecule or cargo being transported. Most

importantly, an environment conducive to transport is also needed and is provided by nuclear pore complexes (NPCs).

Macromolecular transport between the nucleus and the cytoplasm is routed through NPCs (Figure 1.1). NPCs transport a vast array of molecules across the nuclear envelope, including, proteins, mRNAs, tRNAs, ribosomal subunits, small nuclear ribonucleoprotein (snRNP) complexes, and even DNA in particular instances. The mechanistic complexity of nuclear transport is evidenced by the diverse array of molecules transported by NPCs.

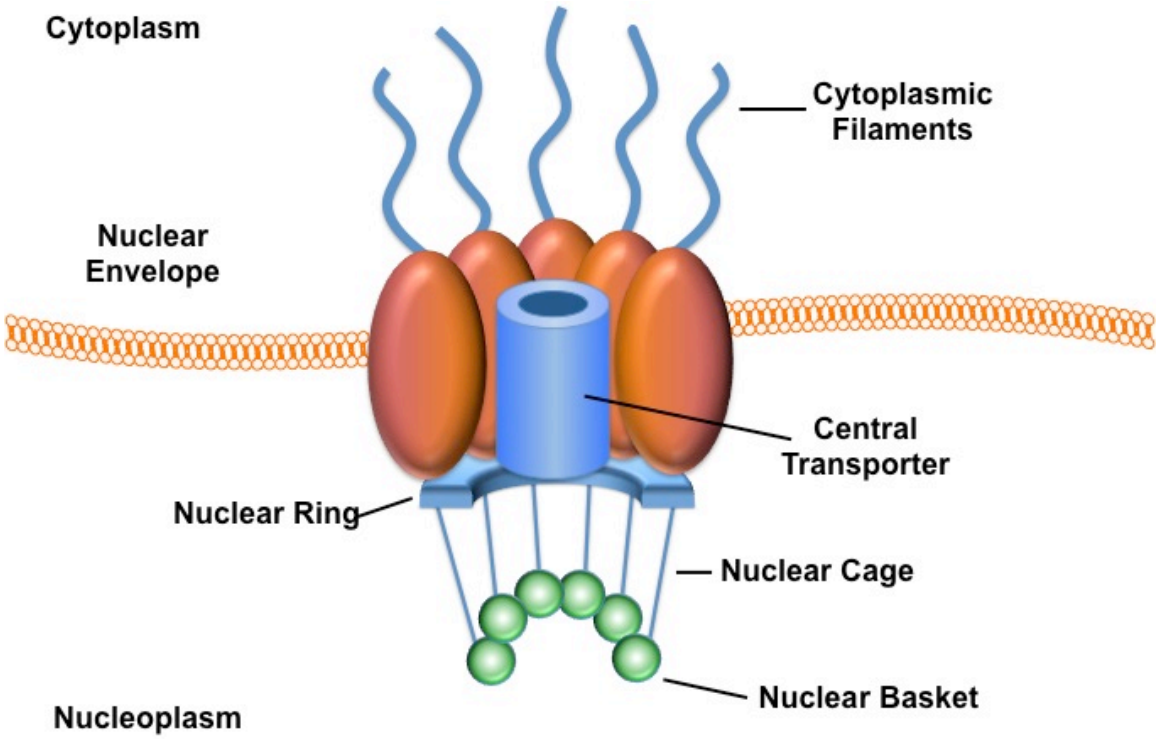


Figure 1.1. Structure of the nuclear pore complex. The nuclear pore complex (NPC) is embedded in the lipid bilayer nuclear envelope. The NPC is a cylindrical structure with a central core transporter that connects the nucleoplasm to the cytoplasm. The protein components of the NPC are termed nucleoporins (nups). Many nups contain FG repeats, which mediate interactions with hydrophobic binding sites on the surface of transport receptor proteins. Peripheral NPC structures include the nuclear basket and cage, and cytoplasmic filaments.

NPCs are large supramolecular structures (~125 MDa in vertebrates) that span the nuclear envelope and thus, connect the cytoplasm to the nucleoplasm (Figure 1.1); (Stoffler et al, 1999). Vertebrate cells have, on average, 2000 NPCs per cell that can individually conduct 1000 translocations per second. The NPC is a cylindrical structure embedded in the lipid bilayer and consists of >30 distinct protein components called nucleoporins (nups). Many of these nups contain FG repeats (phenylalanine-glycine repeats) that are needed to interact with the dual α-helical repeats (HEAT repeats) of transport receptor proteins such as importin-β (Impβ). The NPC also contains several peripheral structures including, the nuclear basket and cage, and cytoplasmic filaments. The cytoplasmic ring moiety of the NPC has eight cytoplasmic filaments, while the nuclear ring moiety has eight tenuous filaments that form a distinct nuclear basket. (Figure 1.1).

The central pore of the NPC provides an aqueous channel that smaller molecules (<40 kDa) can passively diffuse through in an energy independent manner. However, the movement of macromolecules (proteins and/or RNAs) is typically energy and signal dependent, and mediated by transport receptors (Mattaj & Englmeier, 1998); (Gorlich & Kutay, 1999).

Transport receptors, which are also called karyopherins, recognize specific nuclear localization signals (NLSs) and nuclear export signals (NESs). NLS-containing proteins bind to either the import receptor, Impβ, directly (e.g. ribosomal proteins) or the import adaptor, importin-α (Impα), whose N-terminal Impβ binding domain (IBB) binds Impβ. Impβ interacts with components of the

NPC, and once in the nucleus, it binds RanGTP, facilitating cargo and/or Impα release. When not bound to Impβ, Impα is thought to fold in on itself through interactions between its N-terminus and C-terminus, which facilitates cargo release into the nucleus (Fried & Kutay, 2003). Impα is then free to form an export complex with the cellular apoptosis susceptibility protein (CAS) and RanGTP. The Impα/CAS/RanGTP complex and Impβ bound RanGTP are export competent, and are recycled back to the cytoplasm through the NPC. Once in the cytoplasm, Ran dissociates, allowing Impβ and Impα to participate in additional rounds of active transport (Figure 1.2).

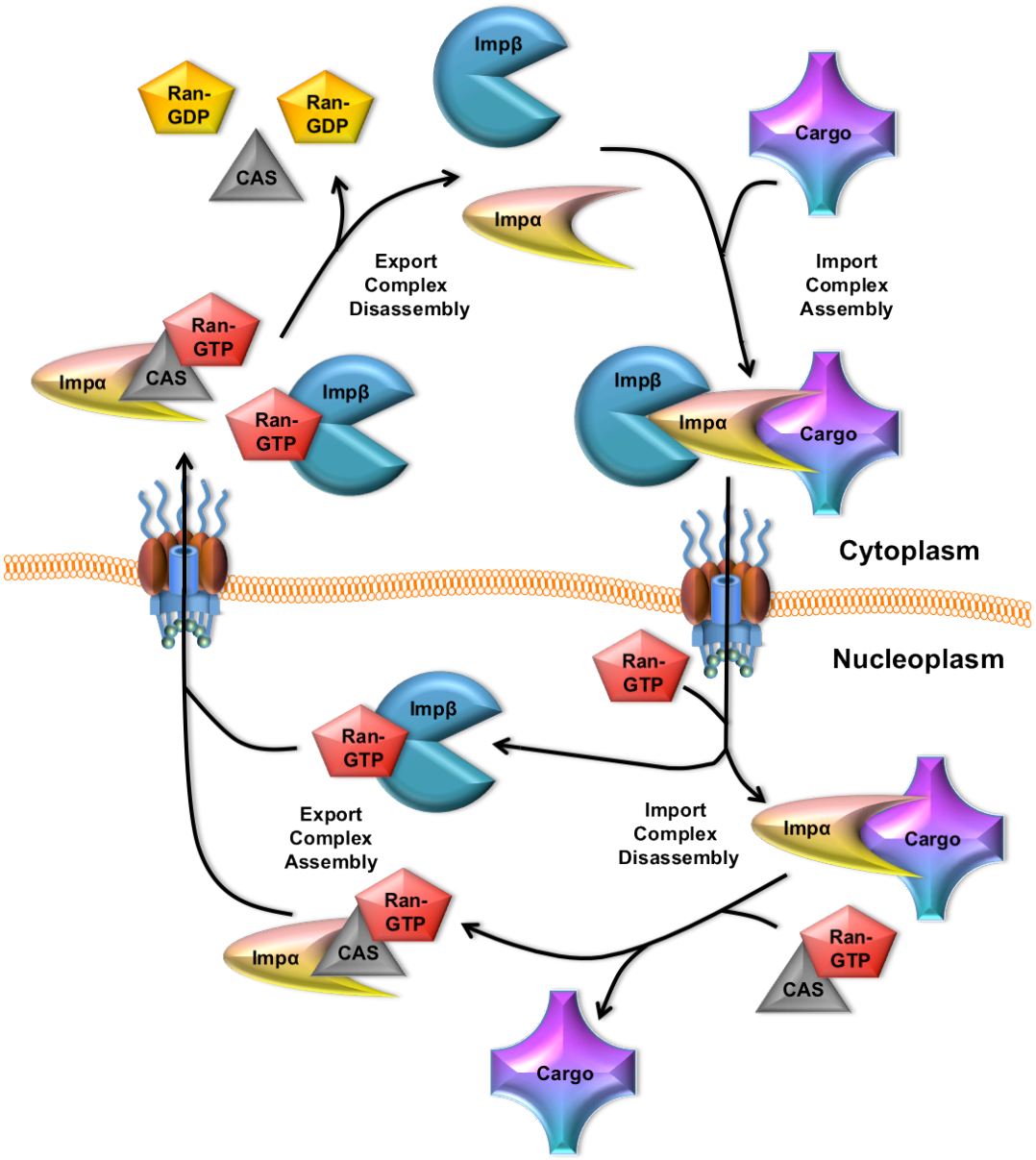


Figure 1.2. Classical NLS-dependent import. The import adaptor, Impα, binds to the NLS of a cargo molecule. Subsequently, Impβ binds to Impα to mediated cargo nuclear import through the NPC. Upon nuclear entry, the import complex is disassembled following RanGTP binding to Impβ, and CAS/RanGTP binding to Impα. These export complexes are then exported to the cytoplasm where they are disassembled by RanGTP hydrolysis.

RanGTP Gradient

RanGTP has an asymmetric distribution across the nuclear envelope, which is essential for the regulation of nucleocytoplasmic transport. RanGTP is enriched in the nucleus, and Ran regulatory proteins maintain the RanGTP energy gradient. Ran's guanine nucleotide exchange factor (RanGEF) is an exclusively nuclear, chromatin-associated protein that catalyzes the conversion of Ran from its GDP- to GTP-bound form. Ran GTPase activating protein (RanGAP) is exclusively cytoplasmic, and stimulates RanGTP hydrolysis. The strict compartmentalization of RanGEF and RanGAP are essential in maintaining the RanGTP gradient. The NPC itself provides no directional cues for transport. The RanGTP gradient is the sole determinant of the directionality of protein transport, proven by the fact that if you reverse the GTP gradient, protein flow is inverted (Fried & Kutay, 2003).

Active transport through the NPC requires energy, typically derived from RanGTP hydrolysis, although Ran-independent transport can occur (e.g. diffusion). The binding of RanGTP to export receptors (exportins) promotes the association of exportins with substrates, and the binding of RanGTP to import receptors facilitates cargo release and recycling of the importin for subsequent rounds of import. Another example of Ran-independent cargo import is Uridine-rich small nuclear RNAs (U snRNAs).

snRNP Biogenesis

Uridine-rich small nuclear RNAs (U snRNAs) are non-coding RNAs that play many roles in RNA metabolism in the nucleus, most notably in splicing (Mattaj et al, 1993); (Tarn & Steitz, 1997). The Sm-class U snRNAs form the core components of the spliceosome. Two distinct classes of spliceosomes exist: the 'major' spliceosome, responsible for >99% of intron splicing in the human genome, and the 'minor' spliceosome, which removes the remaining <1% of introns (Levine & Durbin, 2001). The major spliceosome is comprised of the major-class snRNAs U1, U2, U4 and U6, and the minor spliceosome is made up of the minor-class snRNAs U11, U12, U4atac and U6atac. The spliceosomal snRNA, U5, is a component of both spliceosomes (Patel & Steitz, 2003). The major-class snRNAs are ~100 fold more abundant than the minor-class snRNAs, consistent with a greater requirement for the major spliceosome (Zieve & Sauterer, 1990).

Small nuclear RNAs of the Sm-class are transcribed by RNA polymerase II in the nucleus, undergo 3' end cleavage by the integrator complex (Int.), and acquire a 7-methylguanosine (m_7G) cap (Cougot et al, 2004). After 3' end processing, the pre-snRNA is bound by the cap binding complex (CBC); (Izaurralde et al, 1995) and transits through Cajal bodies (CBs) where it is subsequently bound by the phosphorylated adaptor for RNA export (PHAX); (Frey & Matera, 1995); (Frey et al, 1999); (Ohno et al, 2000); (Frey & Matera, 2001); (Suzuki et al, 2010). PHAX then forms an export complex with the pre-

snRNA and Xpo1/RanGTP, and transits through the NPC where it is released into the cytoplasm upon phosphorylation (Figure 1.3); (Ohno et al, 2000).

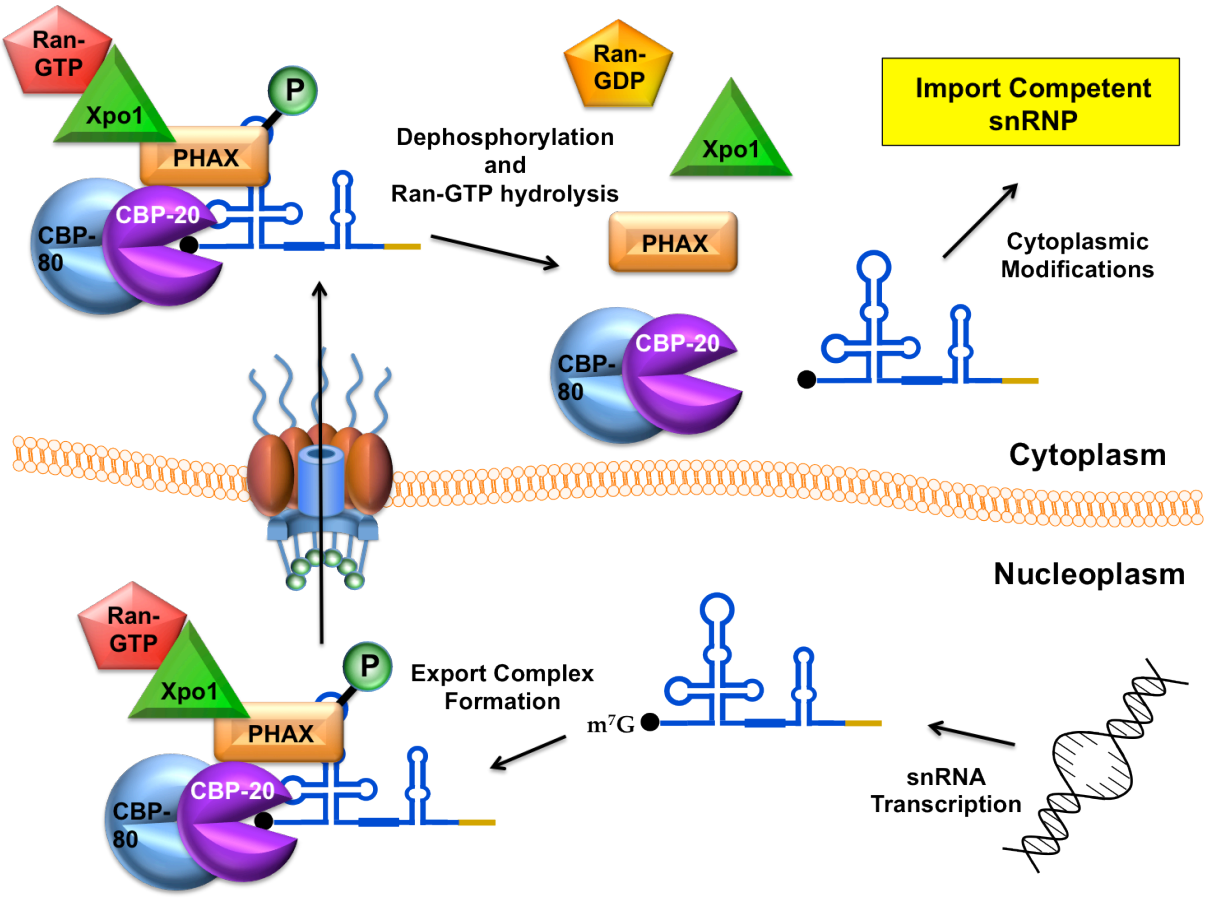


Figure 1.3. Vertebrate snRNA export pathway. U snRNAs are transcribed and m^7G capped in the nucleoplasm. The 5′ cap is bound by cap binding proteins (CBP) 20 and 80, which form the cap binding complex (CBC). Phosphorylated PHAX binds the CBC bound snRNA, and an export complex is formed by the binding of PHAX to Xpo1/RanGTP. U snRNAs are then exported to the cytoplasm where PHAX dephosphorylation and RanGTP hydrolysis promote complex disassembly. U snRNAs are then modified in the cytoplasm, resulting in import competent snRNP particles.

Once in the cytoplasm, the survival motor neuron (SMN) complex mediates the assembly of snRNPs by loading seven Sm proteins, SmB/B', SmD1, SmD2, SmD3, SmE, SmF and SmG, onto a conserved motif of the pre-snRNA called the 'Sm-site (Meister et al, 2002); (Pellizzoni et al, 2002b); (Yong et al, 2004); (Golembe et al, 2005); (Paushkin et al, 2002). This reaction requires energy in the form of adenosine triphosphate (ATP). Although the assembly of the Sm core onto the snRNA can occur spontaneously and non-specifically *in vitro,* the SMN complex provides specificity and improves the kinetics of this reaction (Pellizzoni et al, 2002b).

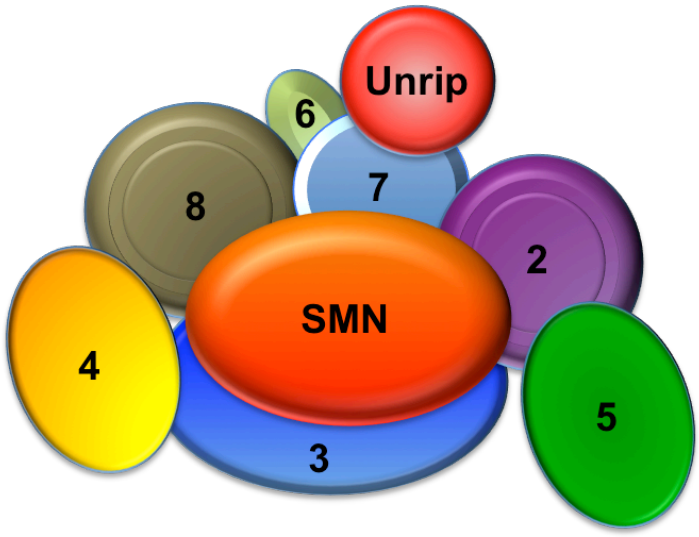


Figure 1.4. Vertebrate SMN complex. The numbered ovals in the complex represent Gemins2-8. Gemins2, 3, 7 and 8 make direct contacts with SMN. Unrip is only present in the cytoplasmic SMN complex. Based on Praveen, 2012.

The SMN complex is a large multimeric complex consisting of eight additional proteins: Gemin2, Gemin3, Gemin4, Gemin5, Gemin6, Gemin7,

Gemin8 and Unrip (Baccon et al, 2002); (Carissimi et al, 2006a); (Charroux et al, 1999); (Charroux et al, 2000); (Grimmler et al, 2005); (Gubitz et al, 2002); (Pellizzoni et al, 2002a). Unrip is a cytoplasmic specific member (Figure 1.4). The SMN complex serves as a scaffold upon which Sm proteins and snRNA are assembled, and this ensures that the Sm proteins only assemble specifically onto snRNAs. The role SMN plays in snRNP assembly is crucial, because without Sm core assembly, snRNPs are incapable of import, and thus cannot participate in active splicing within the nucleus. Importantly, all of the proteins of the SMN complex are required for snRNP assembly.

The Sm proteins, SmB, SmD1, and SmD3, contain RG rich C-terminal domains. These RG repeats are hypermethylated by the protein arginine methyltransferase 5 (PRMT5) complex, consisting of PRMT5, pICln and WD45 (Mep50); (Brahms et al, 2000); (Brahms et al, 2001); (Friesen et al, 2001); (Meister et al, 2001); (Friesen et al, 2002). These methylation marks enhance the binding of the Sm proteins to SMN, but are not necessary for the snRNP assembly process (Gonsalvez et al, 2008).

After Sm core assembly, the 3' end of the pre-snRNA is trimmed by an exonulcease (EXO); (Kleinschmidt & Pederson, 1987); (Seipelt et al, 1999); (Will & Luhrmann, 2001), and the 5'-end methylguanosine cap structure of the snRNA is hypermethylated to form a trimethylguanosine (TMG) cap by trimethylguanosine synthase (Tgs1); (Mouaikel et al, 2002); (Verheggen et al, 2002). SMN is thought to recruit Tgs1 because it physically interacts with SMN (Mouaikel et al, 2003), and SMN is present both before and after cap

hypermethylation (Narayanan et al, 2002). The TMG cap is bound by the import receptor, Snurportin1 (SPN); (Palacios et al, 1997); (Mattaj & De Robertis, 1985); (Hamm et al, 1990); (Fischer et al, 1993). Subsequently, Impβ binds SPN and imports the partially assembled pre-snRNP, along with the SMN complex, into the nucleus (Figure 1.5); (Palacios et al, 1997); (Huber et al, 1998).

The process of snRNP assembly and import is rapid, taking place in approximately one hour as shown by pulse chase (Gonsalvez et al, 2007). Once in the nucleus, pre-snRNPs localize to CBs, are released from the SMN complex, modified, and bound by other snRNP-specific proteins. Mature snRNPs can then be stored in nuclear domains, speckles, or go on to active transcription sites in perichromatin fibrils to participate in splicing (Figure 1.5); (Sleeman & Lamond, 1999).

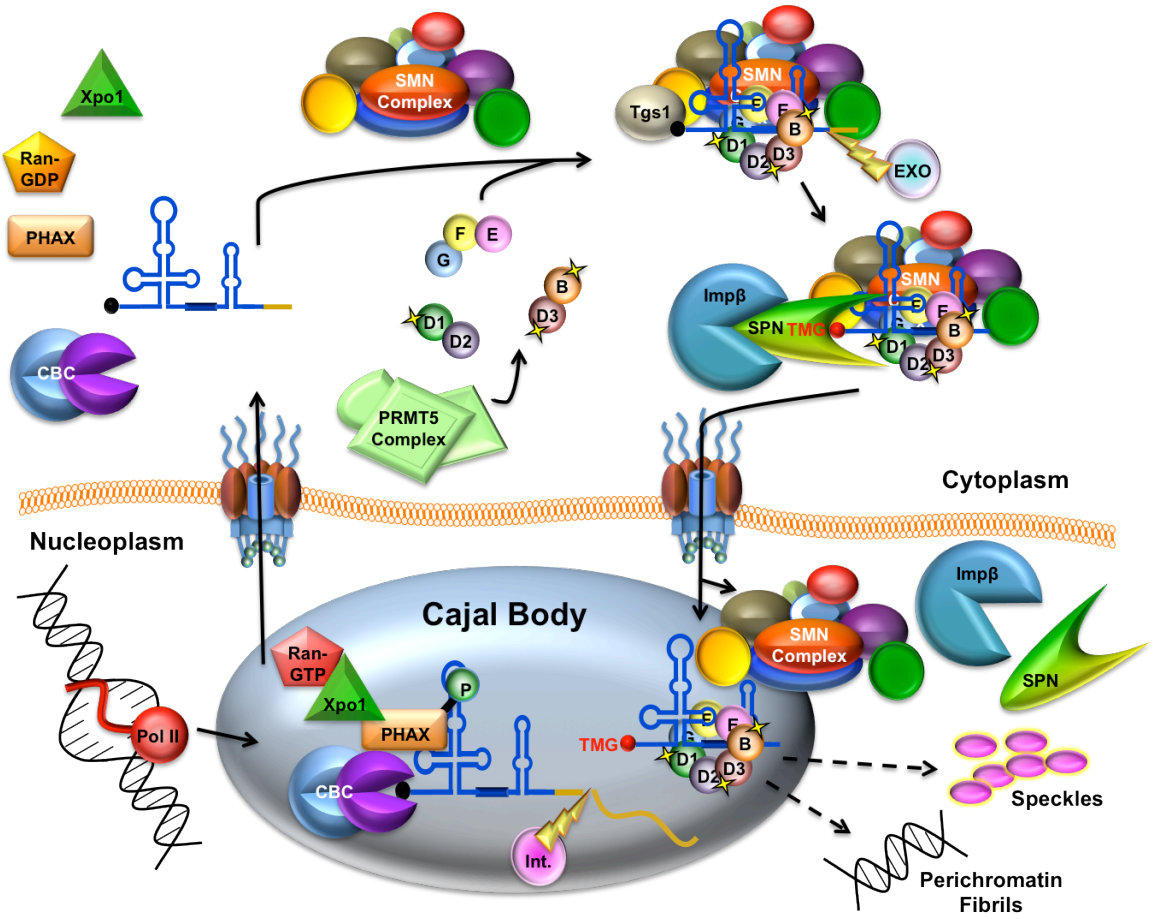


Figure 1.5. Vertebrate snRNP biogenesis overview. RNA polymerase II transcribes the snRNA gene which then undergoes 3' end cleavage by the integrator complex (Int.) and is bound by the cap binding complex (CBC) and PHAX en route to the Cajal body (CB). Xpo1/RanGTP is recruited to export the pre-snRNA through the NPC. Once in the cytoplasm, the export complex is disassembled, and the SMN complex facilitates assembly of the seven membered ring of Sm proteins onto the pre-snRNA. A subset of Sm proteins are symmetrically dimethylated by the PRMT5 complex. The 3' end of the snRNA is trimmed by an exonulcease (EXO), and the 7-methyl guanosine (m^7G) cap is hyper methylated to a trimethylguanosine (m_3G;TMG) cap by trimethylguanosine synthase (Tgs1). The TMG cap of the snRNA is bound by the import adaptor, Snurportin (SPN), and import receptor Importin-β (Impβ). Subsequently, the pre-snRNP is imported into the nucleus along with the SMN complex where it localizes to the CB. In the CB, the snRNA binds other proteins and acquires further modifications before localizing to speckles for storage or to the perichromatin fibrils for active splicing.

Identification of Snurportin

NLS-dependent transport is the most well characterized nuclear import mechanism, but U snRNPs do not appear to have a classical NLS. In contrast to classical NLS-mediated import, U snRNP import does not require Impα. *In vitro* snRNP import assays led to the conclusion that Impβ was necessary for snRNP import (Palacios et al, 1997). These studies revealed that Impβ alone was unable to support U snRNP import, which suggested that Impβ does not directly recognize U snRNPs. This finding indicated that there was an unidentified import adaptor that played a vital role in cap-dependent snRNP import.

The identification of this cap-dependent snRNP import adaptor, Snurportin (SPN), was an essential element to our understanding of snRNP nuclear import. Prior to SPN identification, we knew that the 5' TMG caps of U1 and U2 snRNAs were required for snRNP nuclear transport in *Xenopus* oocytes, suggesting that a cytoplasmic transport factor that bound to TMG caps was yet to be identified. Using this logic, Huber et al. (1998) incubated HeLa cell cytoplasmic lysate with a chemically synthesized, radiolabeled TMG cap oligo and then UV-crosslinked bound proteins. SDS-PAGE analysis revealed a 45 kDa protein band, which was later purified by size exclusion chromatography, followed by affinity chromatography using TMG cap oligo. This suspected snRNP import adaptor was then microsequenced. Several peptide sequences were identified which corresponded with expressed sequence tags (ESTs) of SPN (Huber et al, 1998).

Subsequent studies showed that TMG caps alone, with no other snRNP protein or RNA components, were able to effectively compete for SPN binding.

This finding supported the conclusion that SPN was a snRNP specific import adaptor, as it specifically bound to the TMG cap. Furthermore, snRNPs lacking the 5' TMG cap did not interfere with SPN binding to TMG cap oligo alone, and the addition of SPN significantly accelerated U1 import in both *Xenopus laevis* oocytes and permeabilized HeLa cells. Additionally, they showed that SPN contained an N-terminal Impβ binding domain (IBB), which was needed for SPN interaction with Impβ (Huber et al, 1998).

SPN-mediated snRNP Import

An import complex containing SPN, snRNP cargo, and Impβ facilitates snRNP import (Huber et al, 2002). To form this pre-import complex, SPN must not only bind to the TMG cap of the snRNP, but also simultaneously bind to Impβ. SPN has three functional domains, consisting of an ill-defined Xpo1 binding region, a centrally located TMG cap-binding domain, and an N-terminal IBB motif (Figure 1.6).

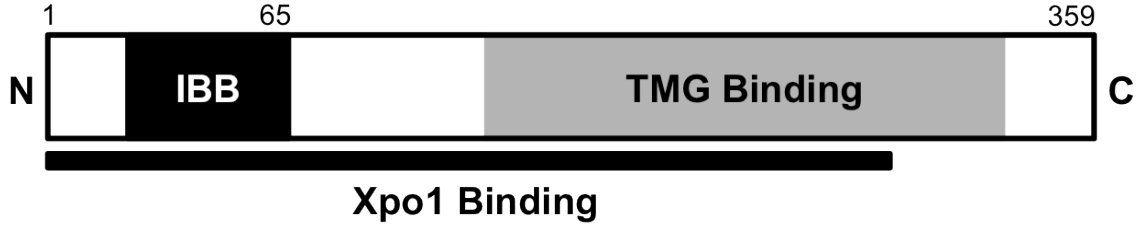


Figure 1.6. Schematic of SPN. A cartoon of SPN indicating the Impβ binding domain (IBB; shown in black), and the export receptor (Xpo1; black bar) and trimethylguanosine (TMG; shown in gray) cap-binding domains. Based on Ospina et al., 2005a.

These functional domains were better defined by Ospina et al. (2005a). Mutational analysis of SPN revealed specific residues within both the IBB and TMG binding domains that are required for SPN function (Ospina et al, 2005a). Mutation of a single arginine residue within the IBB domain (R27) of SPN disrupted its interaction with Impβ, but preserved its ability to bind to Xpo1 or TMG caps. Interestingly, this Impβ binding point mutant is unable to support snRNP import, but is able to shuttle between the nucleus and the cytoplasm (the reasons for which are discussed under the "snRNP import pathways" section). This study also discovered conserved tryptophan residues outside of the IBB that are required for TMG binding, and that SPN was capable of nuclear import without being bound to snRNP cargo (Ospina et al, 2005a).

The TMG cap is required for SPN binding, but recent experiments combining UV cross-linking with tandem mass-spectrometric analysis have identified additional contact sites between U1 snRNP and SPN outside of the known TMG cap interactions. Protein-protein and protein-RNA interactions were uncovered, which supports the idea that there is a larger interaction area between SPN and snRNPs than previously envisioned. SPN was shown to interact with Sm proteins B and D3 and stem-loop III of U1 snRNA (Kuhn-Holsken et al, 2010). These newly identified SPN-snRNP contact sites suggest that snRNP import complexes form only when the Sm proteins are bound to the cognate Sm site in the snRNA and arranged in the proper orientation.

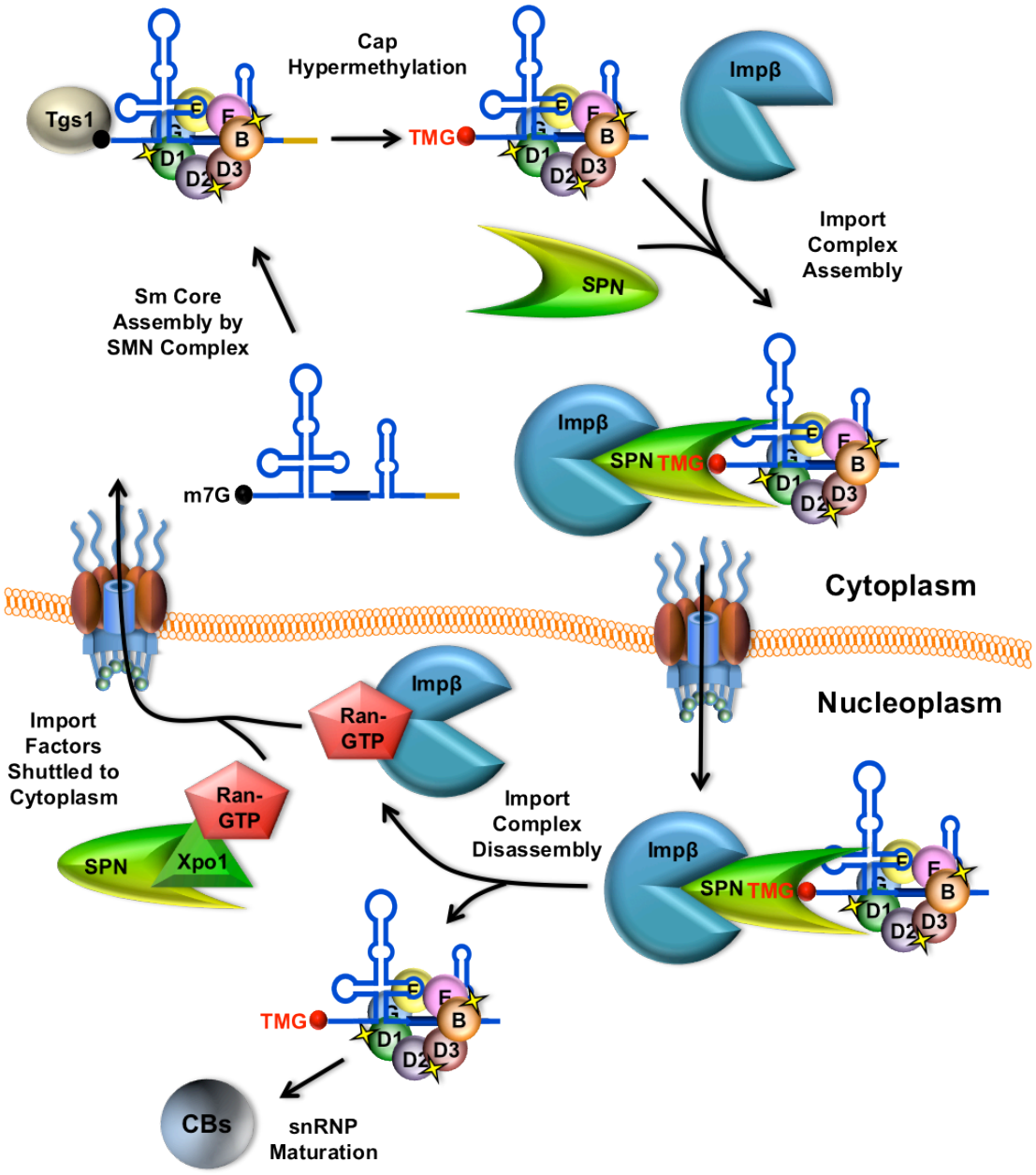


Figure 1.7. Vertebrate SPN-mediated snRNP Import Pathway. The assembly of the Sm core onto the snRNA by the SMN complex serves as a signal for trimethylguanosine synthase (Tgs1) hypermethylation of the m^7G cap. The hypermethylated m_3G (TMG) cap is specifically recognized by the import adaptor, Snurportin (SPN). The import receptor, importin-β, binds to SPN to mediate snRNP transport through the NPC. Following import, RanGTP hydrolysis and additional unknown factors initiate complex disassembly. SPN and Impβ are then shuttled back to the cytoplasm to mediate additional rounds of snRNP import. Newly imported snRNPs then undergo additional Cajal body-specific and nucleoplasmic modifications to form mature snRNPs.

The precise order of the snRNP import complex formation has yet to be elucidated, but the Sm core is thought to be needed for proper TMG cap formation (Mattaj & Englmeier, 1998); (Luhrmann et al., 1990). We also know that TMG capping must precede SPN binding since SPN specifically recognizes the TMG cap (Figure 1.7). After SPN binding, it is unclear whether additional steps are needed before Impβ binding and cargo import. Furthermore, the SMN complex plays a role in import after Sm core assembly (discussed later), but its possible that it plays additional roles in the snRNP biogenesis pathway that are not fully understood.

snRNP Import Pathways

The SPN/TMG cap mediated U snRNP import is the better characterized transport pathway, but a TMG cap-independent import mechanism exists as well, which also impinges upon the Impβ pathway (Figure 1.7); (Fischer & Luhrmann, 1990); (Fischer et al, 1993); (Palacios et al, 1997); (Gorlich & Kutay, 1999); (Hamm & Mattaj, 1990). U snRNPs have two known NLSs: the TMG cap and the Sm core (Figure 1.8). As discussed in the previous section, SPN is the import adaptor for the TMG cap NLS, but the import adaptor for the Sm core NLS has not been identified. This Sm core pathway provides an explanation for the curious finding that the Impβ binding deficient SPN point mutant (R27) was capable of nucleocytoplasmic shuttling (Ospina et al, 2005a). The SPN R27 retains both its Xpo1 and TMG cap binding capabilities. Therefore, SPN R27 likely gains access to the nucleus through the Sm core pathway via TMG capped snRNPs, and can be exported via Xpo1 (Ospina et al, 2005a).

SMN or a component of the SMN complex is thought to be the import adaptor for the Sm core NLS (Narayanan et al, 2002). It has been shown in HeLa cells that SMN is present in an import-competent snRNP complex with Impβ *in vivo.* Additionally, GST-tagged SMN can interact directly with His-tagged Impβ (Narayanan et al, 2002). These data are consistent with the hypothesis that SMN is the Sm core NLS import adaptor. Moreover, in the absence of import competent SPN, the SMN complex and Impβ can rescue snRNP import in a nuclear transport assay. However, SMN alone with Impβ is not sufficient for TMG cap-independent snRNP import (Ospina et al, 2005a). This observation suggests that other members of the SMN complex are necessary for the nuclear transport of snRNPs using the Sm core NLS import pathway.

Our understanding of U snRNP import is further complicated by the fact that particular U snRNPs possess different import requirements. While the TMG cap is required for U1 and U2 snRNA import in oocytes, it is not required in somatic cells or for U4 and U5 snRNA import in *Xenopus* oocytes (Fischer et al, 1993). The presence of a TMG cap improves the rate of snRNP import in somatic cells and oocytes, but is not required for U snRNP import in somatic cells. We also know that U1 import in HeLa cells is temperature dependent, saturable, dependent upon Sm core assembly, and independent of the TMG cap (Fisher et al., 1994). The TMG cap dependence observed in oocyte snRNP import is cell specific rather than species specific (Fischer et al, 1994), but we do not know at what point in development the TMG cap requirement changes or the factors mediating this change.

When *Xenopus* egg extract is supplied to somatic cells, U1 and U2 import becomes TMG cap dependent, suggesting that soluble cytosolic factors mediate the TMG cap dependence of U1 and U2 import (Marshallsay and Luhrmann, 1994). The interaction of the U2 snRNP with this cytosolic factor is saturable with TMG cap analogs in oocytes, but not HeLa cells (Fischer et al, 1994). Impβ depletion from *Xenopus* egg extract can also significantly inhibit snRNP import, suggesting that either Impβ is required for snRNP import or that the unidentified cytosolic factor is co-depleted with Impβ. Additionally, we know that over expression of an Impβ binding deficient SPN (SPNΔIBB) in *Xenopus* oocytes blocks snRNP import, but not in mammalian somatic cells (Huber et al, 2002). In sum, these results suggest that the SPN-mediated import pathway is required in the germline, but not in somatic cells.

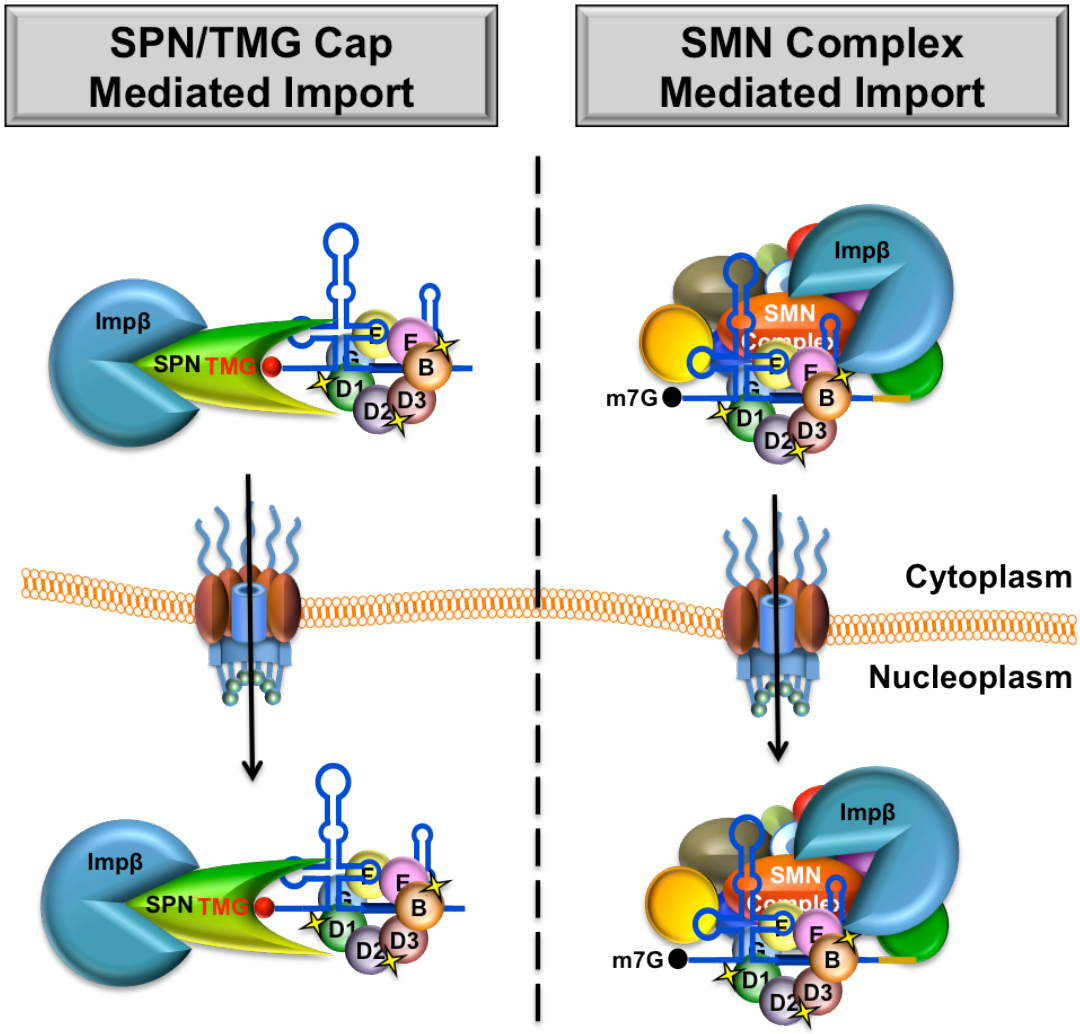


Figure 1.8. Vertebrate snRNP Import Pathways. Two independent snRNP import mechanisms exist in vertebrates, both of which require Impβ. The predominant import pathway is mediated by Snurportin (SPN) and requires the TMG cap of the snRNA. This is the only known snRNP import mechanism to exist in the germline, but an additional pathway has been shown to function in somatic cells. In somatic cells, Impβ can import uncapped snRNPs in the absence of SPN through its interaction with the SMN complex.

TMG cap independent import is thought to be due to the direct interaction of SMN with Impβ in the cytoplasm, thus serving as an Sm core NLS receptor, but SMN has not been conclusively proven to be the import adaptor for the TMG cap independent import pathway, since other members of the SMN complex are

required for SPN-independent import (Narayanan et al, 2002). Recombinant SPN and Impβ are necessary and sufficient for U1 snRNP import in permeabilized HeLa cells, and this import is Ran independent for U1 and U5 (Huber et al, 2002). Irrespective of the TMG cap or SPN binding, nuclear import is mediated via Impβ in the vertebrate system, and this observation fails to explain why we see cell specific differences in snRNP import requirements.

The difference in U1 and U2 versus U4 and U5 TMG cap dependence is not likely to be due to Sm core NLS differences because they compete for the same transport receptor, Impβ. It is possible that the structure and size of the snRNA accounts for the differences in TMG cap dependence since U1 and U2 are longer than U4 or U5, although assembled U5 snRNP is much larger (Fischer et al, 1994). Understanding how these two pathways function in an *in vivo* model system will help elucidate the significance of the need for two independent snRNP import pathways.

snRNP translocation through the NPC

We know that single U1 or U5 snRNP import events are both Ran and energy independent (Huber et al, 2002). Using *in vitro* import assays, Huber *et al.* showed that by mutating the SPN IBB to that of Impα, U1 snRNP import became Ran dependent, while mutating the IBB of β-galactosidase (normally Ran dependent import) to that of SPN allowed Ran independent import (Huber et al, 2002). This illustrated that the Ran and energy independent nature of single round SPN-mediated U1 snRNP import is completely accounted for by the way in which Impβ binds to the IBB of SPN.

Neither the translocation of snRNP cargo through the NPC nor its release from the nuclear basket is dependent on Ran. The snRNP cargo must be released into the nucleoplasm where they can undergo further maturation. Disassembly of the snRNP import complex is not fully understood, and the factors involved in this disassembly have yet to be identified. It is not known whether Ran is a requirement; however, we do know that the affinity of Impβ for SPN is reduced upon binding of RanGTP (Paraskeva et al, 1999). RanGTP binding may promote complex disassembly directly or indirectly by destabilizing the import complex.

Nuclear import of SPN is mediated by the N-terminal IBB (residues 1-65), and this region alone is necessary and sufficient to promote nuclear import in the absence of Ran and energy (Huber et al, 1998); (Huber et al, 2002); (Ospina et al, 2005a). Two distinct binding determinants for Impβ were discovered in the IBB of SPN. The bipartite IBB domain of SPN includes a region of homology to Impα IBB spanning residues 25-65, and a second region that shows homology to the nucleoporin 153 (Nup153) spanning residues 1-24 of SPN (Mitrousis et al, 2008). This same region of Nup153 binds to Impβ with high affinity (Bednenko et al, 2003). *In vitro*, these two binding regions within the IBB of SPN synergize to reduce the nanomolar binding affinity for Impβ (Mitrousis et al, 2008). When bound to Impβ, the Nup153 homology region (residues 1-24) of SPN makes Impβ more sensitive to RanGTP, which displaces Impβ. Mitrousis et al. hypothesized that this promotes the translocation of U snRNPs into the nucleus since RanGTP promotes import complex disassembly (Mitrousis et al, 2008).

Import Complex Disassembly

Mitrousis et al. (2008) also found that the Nup153 homology region of SPN was needed for Xpo1 binding. Previous studies suggest that Xpo1 may play a role in complex disassembly and that the binding of SPN to Xpo1 and snRNP cargo may be mutually exclusive (Paraskeva et al, 1999); (Ospina et al, 2005a). The 2.9 Å crystal structure of SPN bound to Xpo1 revealed that SPN binds to Xpo1 in a bipartite manner through both an amino-terminal leucine-rich nuclear export signal (LR-NES) and a nucleotide-binding domain (Dong et al, 2009b). Like the bipartite IBB of SPN, the bipartite Xpo1 binding region increases the affinity of Xpo1 for SPN. This multipartite nature, combining energetically weak and strong epitopes, is also found in nuclear localizations signals. This principal is thought to broaden substrate specificity by amplifying signal diversity and allows for rapid evolution in nuclear trafficking in both directions (Dong et al, 2009a). It is likely that in addition to RanGTP hydrolysis, Xpo1 plays a role in complex disassembly, but additional unknown factors could initiate complex disassembly as well. Whether import complex disassembly is needed for further snRNP modifications is undetermined.

SPN Recycling

Once SPN transports its snRNP cargo into the nucleoplasm, it must be disassembled so that it can be recycled back into the cytoplasm to mediate additional rounds of snRNP import. Immediately after the discovery of SPN as the snRNP import adaptor, the SPN export receptor, Xpo1, was discovered (Paraskeva et al, 1999). Xpo1 is not limited to SPN export, as it can export a

variety of proteins containing NESs (e.g. PHAX bound pre-snRNAs, Impβ, etc.). Xpo1 binds directly to the leucine-rich NES motif (not clearly defined in SPN), where it can then transport its cargo through the NPC into the cytoplasm. SPN does not contain a consensus NES, and the region mediating the interaction between SPN and Xpo1 has been difficult to determine, even with extensive SPN mutational analysis (Ospina et al, 2005a).

Mutational analysis by Ospina *et al.* (2005a) uncovered a potential auto-inhibitory interaction within SPN. The N- and C-terminal domains of SPN were found to interact, which suggests that SPN may have an auto-regulatory function similar to that of Impα. It is possible that the binding of Impβ or TMG capped snRNAs would increase accessibility to snRNP cargo or Impβ, respectively. This increased binding capacity would facilitate snRNP biogenesis under conditions when snRNP demand is high (e.g. increased transcription). Alternatively, when demand for snRNPs is low, this intramolecular interaction could sequester SPN molecules via steric hindrance. Additionally, it would prevent SPN from aberrantly binding newly imported snRNPs, and enable the regulation of snRNP biogenesis through the modulation of import complex formation (Figure 1.9); (Ospina et al, 2005a).

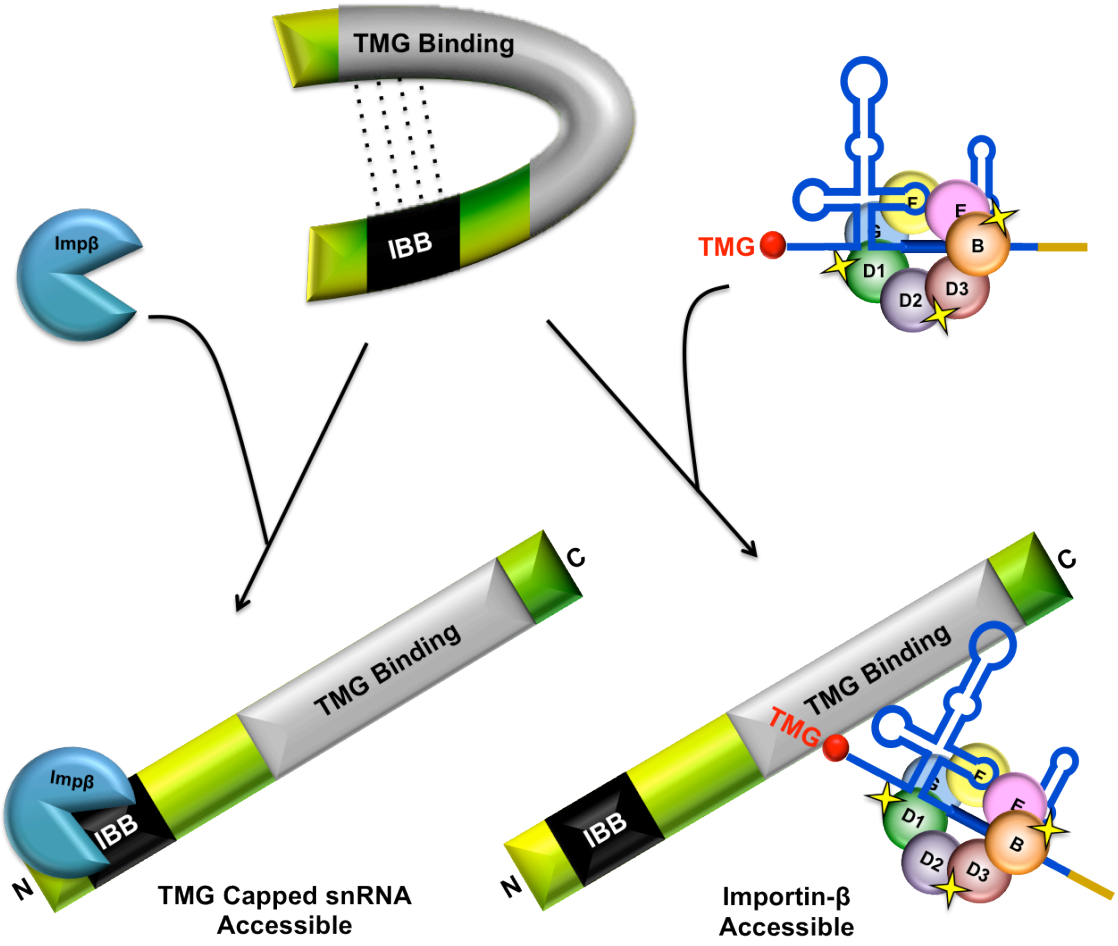


Figure 1.9. Model of Snurportin auto-regulatory snRNP import function. The N- and C-terminus of Snurportin has been shown to interact. This interaction could sequester SPN ability to bind to snRNPs or Impβ, effectively disrupting snRNP biogenesis. Upon increased demand, the binding of Impβ or snRNP cargo might increase access to snRNP cargo or Impβ, respectively. Adapted from Ospina et al., 2005a.

This SPN auto-regulatory hypothesis was later supported by the 2.9 Å crystal structure of SPN bound by Xpo1. SPN IBB bound by Xpo1 has a very different structure compared to the Impβ bound structure (Bhardwaj & Cingolani, 2010). When bound to Impβ, the C- and N-termini of SPN are predicted to be far apart. When residues 1-16 of SPN IBB are bound to Xpo1, the remaining IBB

domain of SPN wraps around the nucleotide binding C-terminus (Dong et al, 2009a). This is consistent with the idea that intramolecular interactions within the C- and N-terminus of SPN while bound to Xpo1 inhibit its ability to aberrantly bind TMG caps in the nucleoplasm.

In addition to its role in import complex disassembly, RanGTP also plays an essential role in the export and recycling of SPN. Binding of RanGTP to Xpo1 dramatically increases its affinity for SPN, which facilitates the formation of an export competent complex (Paraskeva et al, 1999). The directionality of transport is dependent on the RanGTP gradient, and export complex formation is likely to only occur in the nucleoplasm where the majority of Ran is in the GTP-bound form.

RanGTP binding increases the affinity of Xpo1 for NESs like the one found in SPN. The 2.9 Å crystal structure of SPN-bound to Xpo1 also revealed that little conformational change is needed for SPN-bound Xpo1 to bind to RanGTP, which explains the high affinity of Xpo1 for the GTPase (K_d~15 nM); (Dong et al, 2009a); (Paraskeva et al, 1999); (Petosa et al, 2004). Xpo1 substrate affinity was further elucidated by Fox et al. (2011). Mutations within the C-terminal helix of Xpo1 did not result in large scale changes in Xpo1 conformation, suggesting that local electrostatic interactions are mediating the NES affinity of Xpo1. In the absence of RanGTP, the Xpo1 NES binding site is in a close conformation, facilitating the release of cargo into the cytoplasm (Fox et al, 2011).

After SPN is transported back into the cytoplasm by Xpo1, the export complex must be disassembled so that SPN is free to participate in additional

rounds of snRNP import. Disassembly of export complexes is typically catalyzed by RanGAP, which hydrolyzes GTP. GTP hydrolysis decreases the substrate affinity of Xpo1, and low-affinity substrates are released into the cytoplasm. Even after Ran dissociation, high-affinity substrates, like SPN, remain stably associated with Xpo1 (Dong et al, 2009a); (Paraskeva et al, 1999); (Engelsma et al, 2004). Other factors in the cytoplasm are thought to be needed for complete disassembly to occur.

The residues in SPN required for TMG cap binding are also needed for Xpo1 binding (Huber et al, 1998); (Paraskeva et al, 1999). Therefore, Xpo1 and TMG cap binding are mutually exclusive. Additionally, binding of Impβ also decreases the affinity of Xpo1 for SPN; so the combination of TMG capped snRNPs and Impβ effectively dissociate the export complex once it is in the cytoplasm (Dong et al, 2009a).

Nuclear snRNP Modifications and Cajal Bodies

Once snRNPs reach the nucleoplasm, they must be further modified to form mature snRNPs that can be assembled into the spliceosome (Yu et al, 1998). Currently, pseuouridinylation and 2' O-methylation are the only known snRNA modifications after nuclear import (Darzacq et al, 2002); (Jady et al, 2003); (Richard et al, 2003). These modifications are highly conserved across species, and are known to participate in and influence snRNP and spliceosomal assembly. snRNPs imported from the cytoplasm first appear in Cajal bodies (CBs), suggesting that the final maturation steps take place there, but snRNA modifications not exclusively restricted to CBs. Evidence exists that snRNAs can

be modified throughout the nucleoplasm (Zhao et al, 2002); (Deryusheva & Gall, 2009); (Liu et al, 2009). Even so, CBs can be used as markers of ongoing snRNP biogenesis.

Cajal bodies are a non-membrane bound nuclear suborganelle, and historically, they are identified by the presence of coilin (Andrade et al, 1991). In addition to coilin, SMN has been shown to localize to CBs. SMN also interacts with coilin genetically and physically, but the importance of this interaction has yet to be determined (Tucker et al, 2001); (Hebert et al, 2001). The accumulation of SMN and other snRNP markers, such as SPN, in CBs has been shown to be dependent upon post-translational dimethylation of specific arginine residues within coilin (Hebert et al, 2001); (Boisvert et al, 2002).

Much effort has been focused on understanding the significance of nuclear bodies, yet we still do not know the exact function CBs perform in the cell. However, we do know that CBs contain high concentrations of factors involved in pre-mRNA splicing, ribosome biogenesis, and telomere maintenance (Matera, 1999). Although CBs are not essential structures (Liu et al, 2009), it is likely that they facilitate the pre-assembly of factors that carry out these cell essential functions.

CBs are prominent in some cell types, so they are easily identified. Ectopic expression of snRNP proteins has been shown to increase CB formation, even in cells that do not typically display them (Sleeman & Lamond, 1999). Cells highly engaged in transcription and translation require a large abundance of snRNPs to carry out pre-mRNA splicing and thus, have more prominent CBs

(Shpargel & Matera, 2005). Moreover, disruptions in snRNP biogenesis have been shown to break down Cajal bodies (Shpargel & Matera, 2005). The dynamic nature of CBs and their close association with ongoing snRNP biogenesis make them an excellent marker to study disruptions in snRNP biogenesis.

Research Objectives

One of the many distinguishing characteristics of eukaryotes is their ability to remove intervening sequences, introns, from pre-messenger RNA (pre-mRNA). This process is known as splicing and it is critical for proper gene function and protein diversity. In eukaryotic cells, the majority of splicing is carried out by the spliceosome.

The spliceosome is a large dynamic complex consisting of five small nuclear (sn) RNAs (U1, U2, U4, U5, and U6) and numerous protein components. snRNAs must be assembled with other proteins to form small nuclear ribonucleoproteins (snRNPs) before assembly into the spliceosome. snRNPs are essential for spliceosome function, so it is important that we understand the complexities of snRNP biogenesis.

The proper targeting of snRNPs to the nucleoplasm is central to snRNP biogenesis. snRNP processing is biphasic, where snRNPs are assembled in the cytoplasm and then must be imported into the nucleus for their assembly into the spliceosome. The transport of large macromolecular molecules such as RNPs is a highly regulated process mediated by import factors. snRNPs contain two independent import signals. One import signal consists of a 5′ RNA cap structure and is bound by the transport adaptor, snurportin1 (SPN). The Sm core serves as an alternate import signal and requires the SMN complex.

These multicomponent signals are specific for RNP import, and it ensures that only functional RNPs are imported into the nucleus. The complexity of these multicomponent signals is illustrated by the fact that each import pathway is

dependent upon the cytosolic factors present in a particular cell type, and various U snRNPs have different import requirements. Further studies will be needed to fully understand the nature of the Sm core and TMG cap import mechanisms.

All studies done thus far on snRNP import have been conducted *in vitro*. To fully understand the cell type specific differences we observe, an *in vivo* model of snRNP import is needed. My work is aimed at developing an *in vivo* *Drosophila* snRNP import model so that it may help us to understand the fundamental differences and requirements in these two pathways.

As a first step toward developing an *in vivo* model system of snRNP nuclear import, I identified and characterized *Drosophila* Snurportin (dSNUP). Like its human counterpart, dSNUP binds to snRNAs and to dSmB and dSMN in an RNA dependent manner, and localizes to CBs. Surprisingly, dSNUP lacks an IBB and did not bind to Impβ (known as Ketel in flies). Furthermore, Impβ/Ketel failed to interact with snRNAs. In an effort to determine the *Drosophila* snRNP import receptor, I discovered that a previously published hSPN interaction with a known import receptor, Imp7 (Imp7), is conserved in *Drosophila*. I show that the Imp7 *Drosophila* ortholog, Moleskin (Msk), interacts with dSNUP and snRNPs and can be found in CBs. My work demonstrates a conserved interaction of SPN with a known import receptor, Imp7/Msk, that has not previously been associated with the snRNP biogenesis pathway. These results implicate a conserved function for Imp7/Msk in snRNP biogenesis.

CHAPTER II

Identification and characterization of *Drosophila* Snurportin reveals a role for the import receptor Moleskin/Importin-7 in snRNP biogenesis[1]

Overview

Nuclear import is an essential step in small nuclear ribonucleoprotein (snRNP) biogenesis. Snurportin1 (SPN1), the import adaptor, binds to trimethylguanosine (TMG) caps on spliceosomal small nuclear RNAs (snRNAs). Previous studies indicate that vertebrate snRNP import requires importin-β, the transport receptor that binds directly to SPN1. We have identified CG42303*/snup* as the *Drosophila* orthologue of human *snurportin1 (SNUPN)*. Interestingly, the importin-β binding (IBB) domain of SPN1, which is essential for TMG cap-mediated snRNP import in humans, is not well conserved in flies. Consistent with its lack of an IBB domain, we find that *Drosophila* SNUP (dSNUP) does not interact with Ketel/importin-β. Fruit fly snRNPs also fail to bind Ketel, however, the importin-7 orthologue, Moleskin (Msk), physically associates with both dSNUP and spliceosomal snRNPs and localizes to nuclear Cajal bodies.

[1] Natalizio AH and Matera AG (2013) Identification and characterization of *Drosophila* Snurportin

Strikingly, we find that *msk* null mutants are depleted of the snRNP assembly factor, survival motor neuron (SMN) and the Cajal body marker, coilin is disrupted. Consistent with a loss of snRNP import function, long-lived *msk* larvae show an accumulation of TMG cap signal in the cytoplasm. These data demonstrate that Ketel/importin-β does not play a significant role in *Drosophila* snRNP import and implicate a crucial function for Msk in snRNP biogenesis.

INTRODUCTION

Biogenesis of uridine-rich small nuclear ribonucleoproteins (U snRNPs) is biphasic, taking place in two distinct cellular subcompartments (reviewed in Matera *et al.,* 2007). Small nuclear RNAs (snRNAs) of the Sm-class are transcribed by a specialized form of RNA polymerase II (Hernandez and Weiner, 1986) and then exported to the cytoplasm for assembly into pre-snRNPs by the export adaptor, PHAX (Ohno *et al.,* 2000). Once in the cytoplasm, the survival motor neuron (SMN) complex mediates the assembly of the Sm core RNP by loading seven Sm proteins onto the snRNA (Meister *et al.,* 2001; Pellizzoni *et al.,* 2002).

After Sm core assembly, the 5'-end methylguanosine cap structure of the snRNA is hypermethylated to form a trimethylguanosine (TMG) cap by trimethylguanosine synthase (Tgs1; Mouaikel *et al.,* 2002), and this modification is thought to be a signal for nuclear import (Palacios *et al.,* 1997; Mattaj *et al.,* 1985; Hamm *et al.,* 1990; Fischer *et al.,* 1993). The partially-assembled snRNPs are then transported back into the nucleus via the import adaptor, snurportin1 (SPN1) and the import receptor, importin-β (Impβ; Huber *et al.,* 1998; Palacios *et*

al., 1997). SPN1 contains two coplanar β-sheets linked by two crossing β-strands (Strasser *et al.,* 2005) that selectively bind the TMG cap. Once in the nucleus, snRNPs undergo additional maturation steps within the nucleoplasm and/or in Cajal bodies (Jady *et al.,* 2003). RNP import is a crucial step in the biogenesis of snRNPs, as these factors cannot participate in active splicing without proper import into the nucleus.

U snRNPs do not contain a classical nuclear localization signal (NLS). Instead, U snRNP import depends on two non-canonical signals: the TMG cap and the Sm core (Fischer *et al.,* 1993; Marshallsay and Luhrmann, 1994). SPN1 is the import adaptor for the TMG cap pathway (Huber *et al*., 1998), whereas the SMN complex (or some component thereof) is thought to function as the import adaptor for the Sm core pathway (Narayanan *et al.,* 2004). Thus, bipartite import signals are thought to ensure that only functional RNPs are imported into the nucleus.

U snRNP import is complicated by the fact that individual U snRNPs have distinct import requirements. Although the TMG cap is required for U1 and U2 snRNP import in frog oocytes, it is neither required in somatic cells nor for U4 and U5 snRNPs in oocytes (Fischer *et al.,* 1991*;* 1993; Wersig *et al.,* 1992). The observed TMG cap dependence of snRNP import is cell-type specific rather than species specific (Fischer *et al.,* 1994). In digitonin-permeabilized human cells, recombinant SPN and Impβ are necessary and sufficient for U1 snRNP import (Huber *et al.,* 2002). Moreover, a SPN mutant that is incapable of binding to Impβ does not interfere with U1 import via the Sm core dependent pathway (Ospina *et*

al., 2005). These observations show that the two import pathways are redundant *in vitro*, but they fail to elucidate the need for two independent snRNP import pathways. An *in vivo* model system of snRNP import is therefore needed to fully dissect the complex nature of this pathway.

As a first step toward developing an *in vivo* model system of snRNP nuclear import, we identified and characterized *Drosophila* Snurportin (dSNUP). We found that, like its human counterpart, dSNUP binds to snRNAs and to dSmB and dSMN in an RNA dependent manner. Surprisingly, dSNUP lacks a discernable IBB and fails to bind to Impβ *in vivo* and *in vitro*. Furthermore, fruit fly Impβ does not interact with snRNAs. We also identified Moleskin (Msk), the *Drosophila* orthologue of the vertebrate transport factor importin-7 (Imp7), as the putative snRNP import receptor. Msk localizes to Cajal bodies and physically interacts snRNPs. Additionally, we discovered that Msk null mutant larvae display a significant accumulation of TMG capped RNAs in the cytoplasm of larval Malpighian tubules and reduced levels of snRNP biogenesis markers coilin and dSMN. These results demonstrate a novel and conserved interaction between Snurportin and Msk/Imp7. Implications for studies of vertebrate nuclear import are discussed.

RESULTS

Identification and Characterization of *Drosophila* Snurportin

Bioinformatic analysis of the *Drosophila* genome predicts that the fruit fly orthologue of human *Snurportin1* maps to the computed gene locus CG42303/CG42304, near band position 62E on chromosome 3L. The current

FlyBase gene model predicts the existence of a dicistronic transcript with two non-overlapping open reading frames (ORFs) present within CG42303. RT-PCR and 5'-RACE data from the modEncode project (Sue Celniker Lab; http://www.modencode.org/celniker/) support the existence of two transcription start sites (Figure 2.1A), one for each ORF.

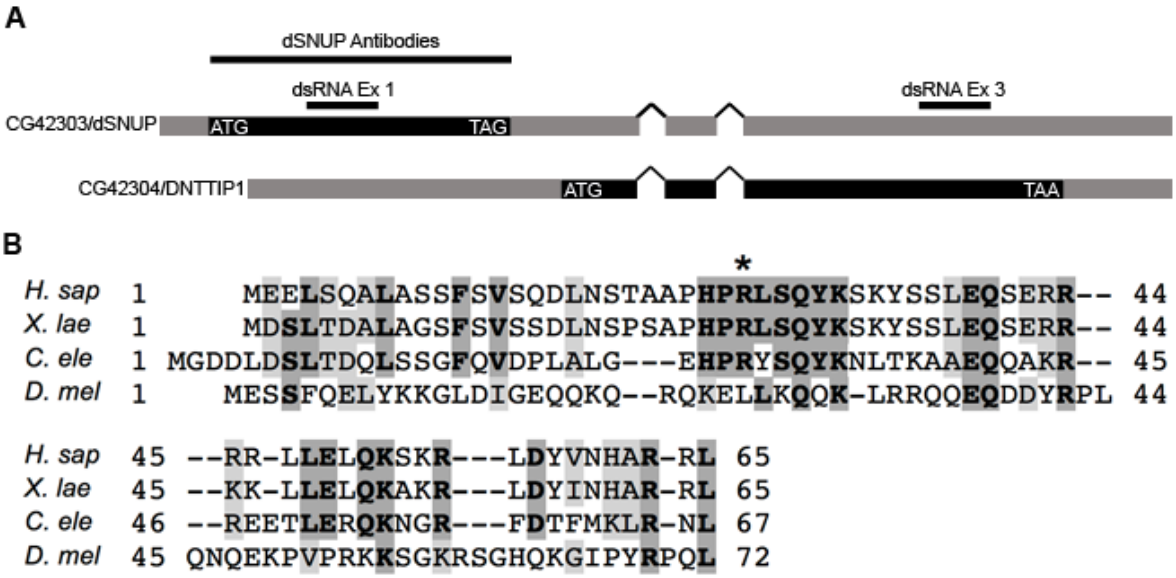


Figure 2.1. CG42303 is the *Drosophila* Snurportin orthologue. (A) Cartoon of bicistronic transcripts predicted from dSNUP/DNTTIP1 locus. Translated regions are shown in black and untranslated regions in gray. Black bars indicate regions targeted by dsRNAs or dSPN antibodies. (B) Alignment of N-termini of SPN orthologues. The IBB of hSPN is defined as amino acid residues 26–65, based on similarity with the IBB of importin-α (Huber *et al.,* 1998). *Homo sapiens, Xenopus laevis, Caenorhabditis elegans,* and *Drosophila melanogaster* SPN proteins are aligned, with identities in dark gray and similarities in light gray. Asterisk indicates human residue R27, mutation of which abolishes importin-β binding (Ospina *et al.,* 2005).

The protein predicted by CG42303 is encoded by a single exon and is 35% identical to SPN1. The CG42304 protein product is also highly similar (37% identity) to the human terminal deoxynucleotidyltransferase interacting factor 1

(TdIF1 or DNTTIP1), which binds and negatively regulates the activity of terminal deoxynucleotidyltransferase (Kubota *et al.,* 2007). This same genetic architecture exists in all other sequenced *Drosophilid* genomes, but is not conserved in *Anopheles gambiae, Apis mellifera,* or *Caenorhabditis elegans*, as SPN1 and DNTTIP1 homologs are unlinked in these organisms. These data suggest that the two genes have become linked sometime after the *Drosophila* radiation from other Diptera such as *Anopheles* or *Apis*.

Alignment of CG42303 with human SPN1 reveals extensive similarity throughout the length of the two sequences, especially within the TMG cap-binding domain (Huber *et al.,* 1998; Strasser *et al.,* 2005; Ospina *et al.,* 2005, and data not shown). Perhaps the most striking feature is that critical residues known to interact with Impβ in the N-terminal region of human SPN1 are missing from the putative fruit fly protein (Figure 2.1B; Ospina *et al.,* 2005). Human *SPN1* encodes a 360 aa protein of 43 kDa molecular weight; the SPN1-like ORF in CG42303 is predicted to generate a protein of 351 aa and 42 kDa. Using bacterially expressed protein targeting the upstream ORF in CG42303, we generated two polyclonal antibodies (one in rabbit, one in guinea pig) and tested them by western blotting. As shown in Figure 2.2A (lane 1), the rabbit antiserum recognizes a prominent 42 kDa band, along with three other minor polypeptides. The guinea pig antiserum does not recognize endogenous snurportin in westerns, but does detect recombinant and exogenously expressed fly snurportin; it also works in other assays (see below).

Previous gene models had suggested different architectural scenarios for CG42303 and CG42304. One model posited the existence of two completely separate transcripts (CG1247, CG1248), whereas the other (CG32297) suggested there is a single mRNA that generates a fused ORF encoding a predicted protein of ~100 kDa. To examine the specificity of our antibody and to test the various gene models, we designed double stranded RNAs targeted against putative exons 1 and 3 of CG42303 (Figure 2.1A). RNA interference (RNAi) analysis in *Drosophila* Schneider 2 (S2) cells shows that the 42 kDa band is specifically depleted by dsRNAs targeting either exon 1 or exon 3 (Figure 2.2A). This result supports the prediction that the CG42303 transcript contains a relatively long 3' flanking region, and shows that the 90 kDa band on the western is not a fused SPN1/DNTTIP1-like chimeric protein. We conclude that the exonic organization in the CG42303 gene model is correct. The mRNA encoding the downstream CG42304 protein product is thus likely to originate from an alternative transcription start site (Figure 2.1A).

The CG42303 protein product is expressed during all stages of development, most prominently in embryos (Figure 2.2B). We found that although it does not work well for detection of endogenous dSNUP by western blotting, guinea pig anti-dSNUP was functional in immunoprecipitation assays, as shown in Figure 2.2D. Using GST-pulldowns and co-immunoprecipitation assays from S2 cell cytoplasmic lysates, we show that CG42303 interacts with both RNA and protein components of snRNPs, as well as with the snRNP biogenesis factor, dSMN (Figures 2.2C and D). Furthermore, RNase treatment of the S2

lysate abolishes these protein interactions, demonstrating that they are RNA-dependent (Figure 2.2E). These results provide strong evidence that CG42303 is the *Drosophila* orthologue of human SPN1. To avoid confusion with the

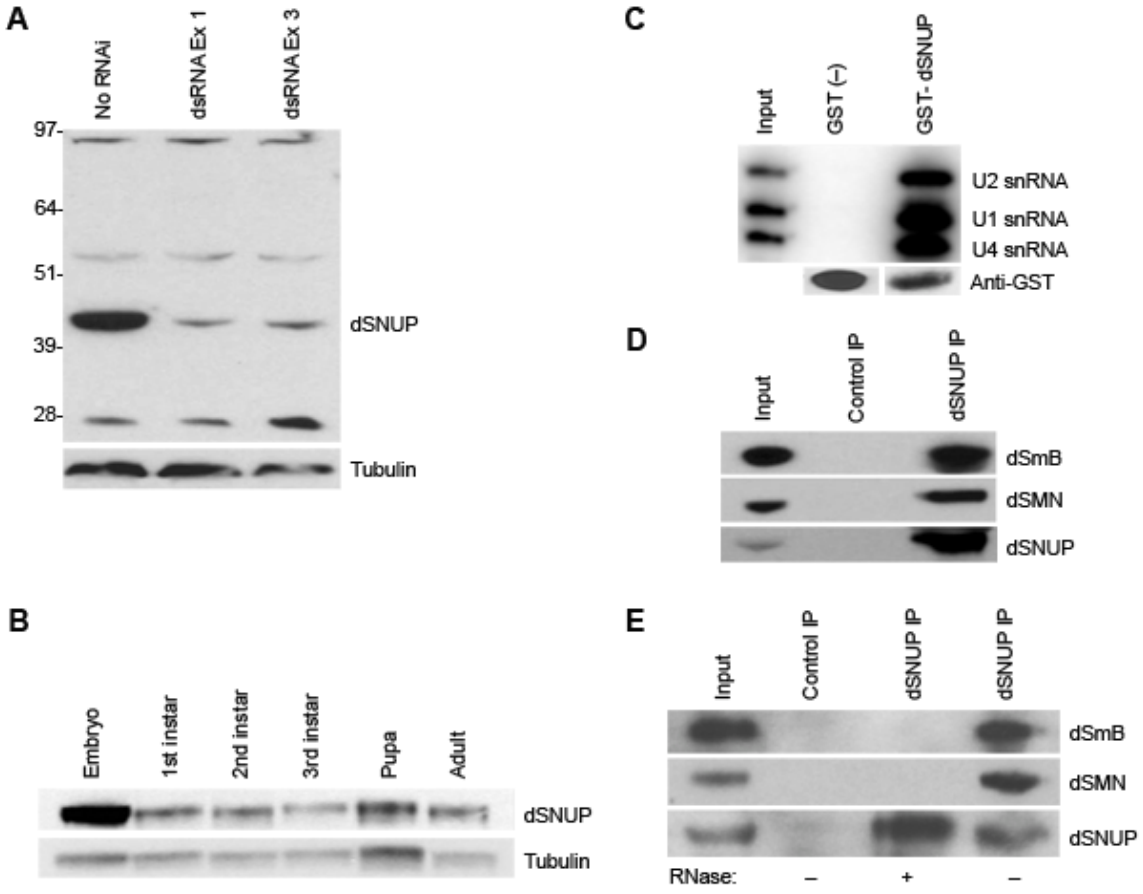


Figure 2.2. *Drosophila* Snurportin interacts with snRNPs. (A) dSNUP RNAi. Predicted 42 kDa band recognized by dSNUP rabbit antibody is specifically knocked down by dsRNAs targeting dSNUP Exons 1 or 3 in S2 cell culture. (B) Developmental Western Blot. dSNUP is expressed at all *Drosophila* developmental stages. (C) GST IP-Northern Blot. Bacterially purified GST-dSNUP interacts with U1, U2, and U4 snRNAs from S2 cell cytoplasmic lysate. (D) dSNUP Guinea pig IP. Guinea pig dSNUP antibody co-immunoprecipitates dSMN and dSmB in S2 cell cytoplasmic lysate. (E) RNase dSNUP Guinea pig IP. RNase treatment of cytoplasmic S2 lysate abolishes interaction of dSNUP with dSmB and dSMN.

abbreviations for the *Spinophilin* gene *(Spn)* and the *spindle* gene family *(spn-A, spn-B, etc.)* in *Drosophila*, we decided to designate the CG42303 gene as *Snurportin (Snup)*.

Previously, we showed that human SPN1 primarily localizes to the cytoplasm, concentrating around the nuclear periphery and sometimes in nuclear Cajal bodies (Narayanan *et al.,* 2002; Ospina *et al.,* 2005). Using the UAS-Gal4 system (Brand and Perrimon, 1993), we expressed Venus Fluorescent Protein (VFP) tagged dSNUP in transgenic flies and analyzed its localization by fluorescence microscopy. Using a variety of Gal4 drivers, we find that VFP-dSNUP localizes to the nucleus and the cytoplasm, with a pronounced accumulation at the nuclear periphery (Figure 2.3A). Notably, VFP-dSNUP localizes to snRNP-rich structures that co-stain with anti-dSmB in the oocyte germinal vesicle (Figure 2.3B) and in the nurse cell cytoplasm, where it accumulates in U bodies (Liu *et al.,* 2007) identified by anti-dSMN (Figure 2.3C). Additionally, dSNUP enrichment in U bodies was also confirmed in the follicle cell cytoplasm of egg chambers, visualized by anti-dSmB (Figure 2.3D). In larval Malpighian tubules, VFP-dSNUP frequently localizes to Cajal bodies (Figure 2.3A). This localization pattern is similar to that of human SPN mutants that contain deletions or substitutions in the IBB domain (Narayanan *et al.,* 2002; Ospina *et al.,* 2005). We therefore decided to examine the interaction between dSNUP and Ketel/Impβ.

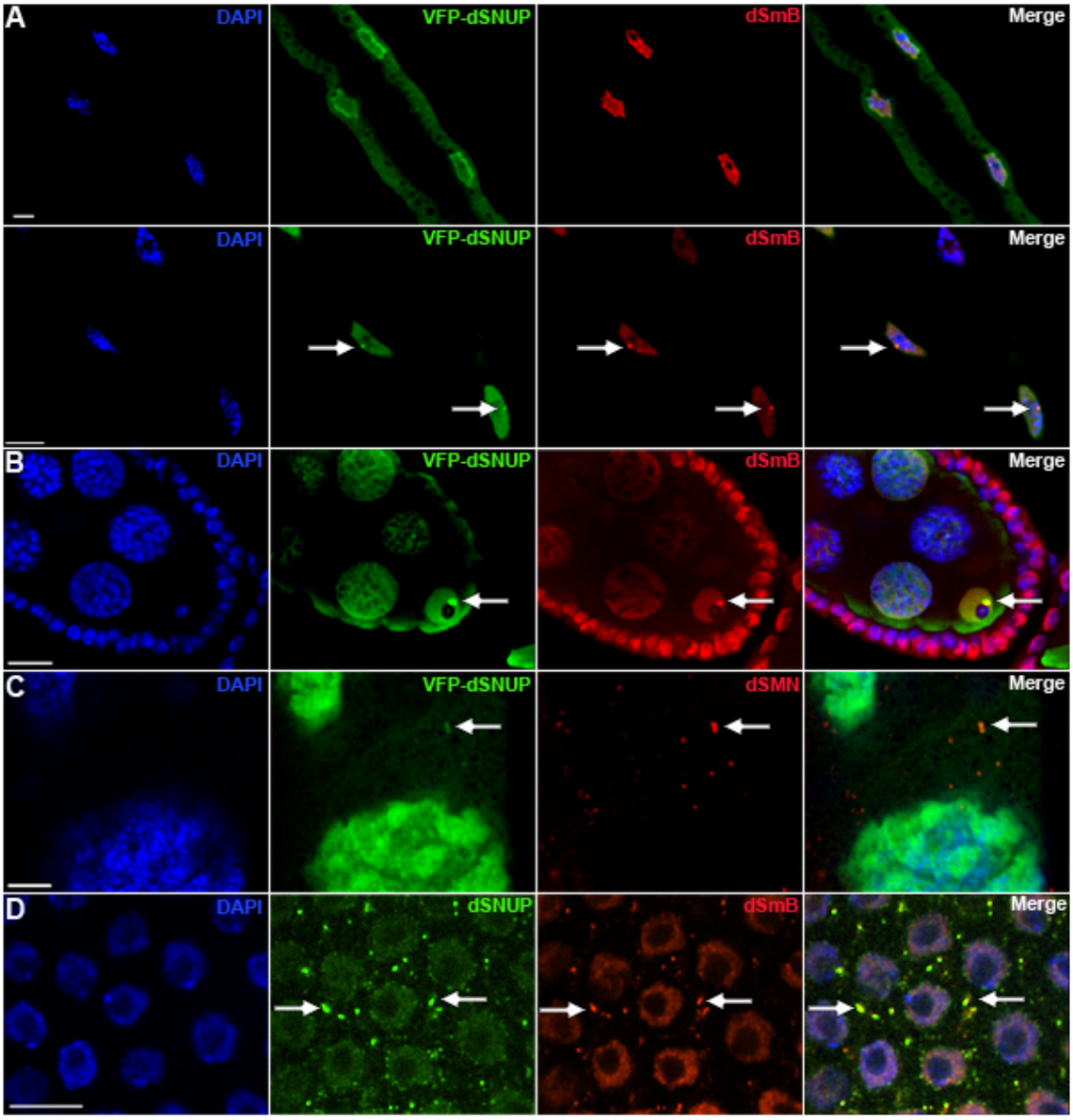


Figure 2.3. Localization of dSNUP. (A) Immunofluorescence with dSmB (Y12) antibody in Malpighian tubules expressing VFP-dSNUP driven by tubulin-Gal4. dSNUP localizes primarily to the nucleus with a relatively pronounced staining of the nuclear periphery and can be found in nuclear foci that are often Cajal bodies (marked by arrows). (B) Immunofluorescence with dSmB (Y12) in egg chambers expressing VFP-dSNUP driven by nanos-Gal4. VFP-dSNUP is enriched in the germinal vesicle (marked by arrow). (C) Immunofluorescence with dSMN antibody in egg chambers expressing VFP-dSNUP driven by nanos Gal4. VFP-dSNUP is enriched in U bodies visualized with dSMN antibody (marked by arrow). (D) Immunofluorescence with dSmB (Y12) and dSNUP Guinea pig antibodies in egg chambers. dSNUP is enriched in U bodies of follicle cells. Image in (D) kindly provided by Zhipeng Lu. Scale bars, 10 μm.

Drosophila snRNP import is importin-β independent

Studies in vertebrates show that SPN1 interacts with Impβ, and that this interaction is mediated via the IBB domain (Huber *et al.,* 1998; Huber *et al.,* 2002; Bhardwaj and Cingolani, 2010). The bipartite IBB of SPN1 is contained within residues 1-65 (Mitrousis *et al*., 2008), and crystal structures reveal that residues 1-16 also contain a nuclear export signal (NES) recognized by the export receptor, Xpo1/Crm1 (Monecke *et al.,* 2009; Dong *et al.,* 2009b). Sequence analysis indicates that dSNUP lacks important residues in the IBB (Figure 2.1B; Huber *et al.,* 2002; Ospina *et al.,* 2005; Mitrousis *et al*., 2008) suggesting that it might not bind to Impβ. Specifically, a highly conserved arginine residue, mutation of which disrupts the interaction of SPN1 with Impβ (Ospina *et al.,* 2005), is not conserved in dSNUP (Figure 2.1B, asterisk). In the absence of an IBB, dSNUP could potentially interact with Ketel/Impβ indirectly through the Sm core (Fischer *et al.,* 1993). Human SPN1 also forms a pre-import snRNP complex with SMN (Narayanan *et al.,* 2002). To enrich for import competent assemblies, cytoplasmic extracts were used to carry out immunoprecipitation and pull down assays. As a positive control for co-immunoprecipitation, we show that, like its human counterpart, dSNUP forms a complex with dSMN (Figure 2.4A). However, consistent with its lack of an apparent IBB domain, dSNUP fails to co-immunoprecipitate Ketel/Impβ (Figure 2.4A).

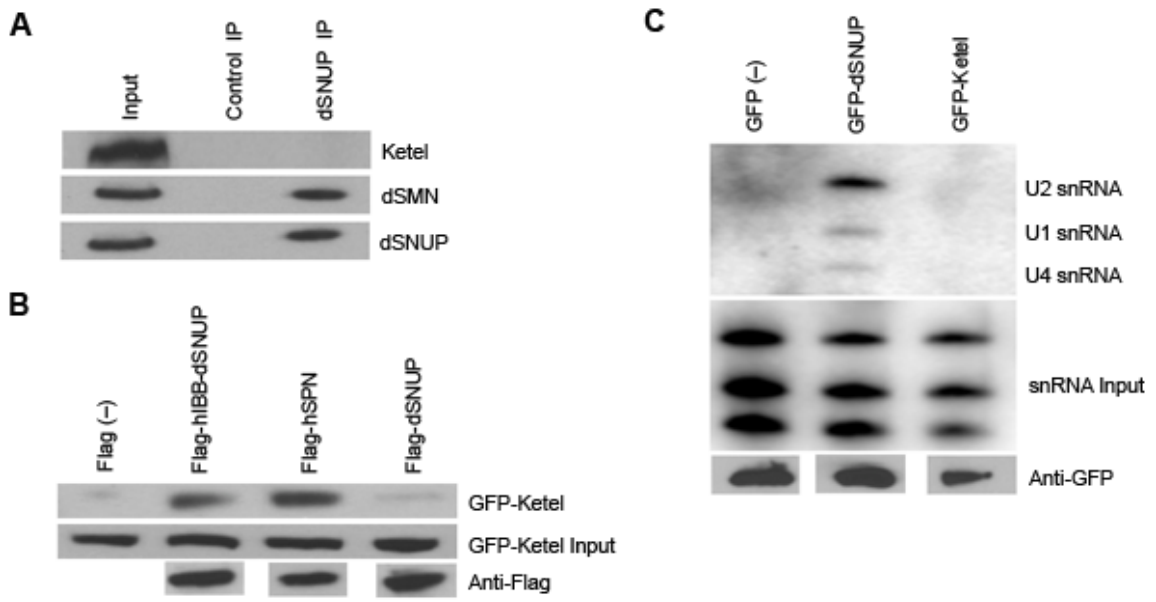


Figure 2.4.Ketel/Impβ does not interact with *Drosophila* snRNPs. (A) Anti-dSNUP Guinea pig IP Western Blot. dSNUP guinea pig antibody does not co-immunoprecipitate Ketel from cytoplasmic S2 cell lysate. (B) Flag conjugated beads IP-Western Blot. Transfected flag tagged proteins -hSPN and –hIBB-dSNUP, but not -dSNUP, co-immunoprecipitate GFP-Ketel in S2 cell cytoplasmic lysate. The amounts of flag-tagged proteins immunoprecipitated are shown with anti-flag (lower panel). (C) GFP IP-Northern Blot. Transfected GFP-dSNUP co-immunoprecipitates snRNAs U2, U1, and U4, but GFP-Ketel does not from S2 cell cytoplasmic lysate. The amounts of GFP-tagged proteins immunoprecipitated are shown with anti-GFP (lower panel).

We also found that Ketel is capable of interacting with an IBB domain by transfecting S2 cells with various Flag-tagged constructs and co-expressing them with GFP-Ketel. As shown in Figure 2.4B, Flag-tagged human SPN1 (Flag-hSPN) or human SPN1 IBB domain fused to the TMG cap-binding domain of dSNUP (Flag-hIBB-dSNUP) co-immunoprecipitate GFP-Ketel, whereas the empty Flag vector (negative control) and Flag-dSNUP do not. Finally, we tested whether Ketel interacts with snRNAs. Immunoprecipitation analysis, followed by northern blotting, showed that while GFP-dSNUP co-immunoprecipitated U1, U2,

and U4 snRNAs (Figure 2.4C), GFP-Ketel failed to do so. Thus, neither the RNA nor the protein components of snRNPs interact with Ketel in *Drosophila*. Taken together with the fact that we were unable to detect Ketel in a complex with dSNUP, these experiments strongly support the interpretation that Ketel does not serve as the snRNP import receptor in *Drosophila* cells.

Moleskin/Importin-7 interacts with snRNPs and Snurportin

The failure of Ketel/Impβ to associate with either dSNUP or with snRNAs suggests the involvement of another import factor. Because splicing is a cell essential function, we reasoned that potential snRNP import receptors must not only be ubiquitously expressed but also should be able to function independent of Impβ. Interestingly, Paraskeva *et al.* (1999) originally showed that epitope-tagged human SPN1 co-purifies with three major proteins: Impβ, the export receptor CRM1, and the transport factor Imp7. The authors went on to show that CRM1 functions as the cytoplasmic recycling factor for SPN1 once it deposits its cargo in the nucleus (Paraskeva *et al.,* 1999). However, the interaction between SPN1 and Imp7 was thought to be indirect due to the fact that Imp7 (formerly RanBP7) was shown to heterodimerize with Impβ (Gorlich *et al.,* 1997). However, Imp7 also binds directly to the nuclear pore complex (Gorlich *et al.,* 1997) and can transport cargoes independently (Jakel and Gorlich, 1998), thus satisfying an important criterion noted above.

The *Drosophila* Imp7 homolog (Moleskin, Msk) is 53% identical to the human protein, and was identified in a dominant suppressor screen for wing blisters caused by the mis-expression of αPS integrin (Baker *et al.,* 2002). All of

the alleles that were discovered in this suppression screen (*msk*^{2}, *msk*^{4}, *msk*^{5}) are late embryonic or larval lethal. It is interesting to note that although *msk* and *ketel* are both essential genes, there are cell types in which Ketel expression is very low (Flybase; Lippai *et al.,* 2000). On the other hand, Msk is ubiquitously expressed (FlyBase), satisfying the other aforementioned criterion. Thus it is possible that Msk/Imp7 plays a more direct role in snRNP import than previously imagined.

To investigate whether Imp7/Msk forms complexes with snRNP biogenesis markers, we carried out co-immunoprecipitation analyses. As shown in Figure 2.5A, anti-dSNUP co-precipitates Msk; dSMN and Ketel are shown as positive and negative controls, respectively. S2 cells transfected with various GFP-tagged constructs also co-precipitated Msk. Figure 2.5B shows that Msk interacts with GFP-dSNUP, -dSMN and -dSmB. GFP-Msk also co-precipitated with U1, U2, and U4 snRNAs as shown in Figure 2.5C. Additionally, RanQ69L (a Ran mutant that is unable to hydrolyze bound GTP; Bischoff *et al*., 1994) disrupts the interaction of flag-dSNUP with Msk (Figure 2.5D). This interaction is also dependent upon RNA, as RNase treatment of cytoplasmic lysate abolishes binding of endogenous Msk to GFP-dSNUP and reduces Msk binding to GFP-dSMN (Figure 2.5E). These results clearly demonstrate that Msk can physically interact with snRNPs, and that Msk interacts with dSNUP in a Ran- and RNA-dependent manner.

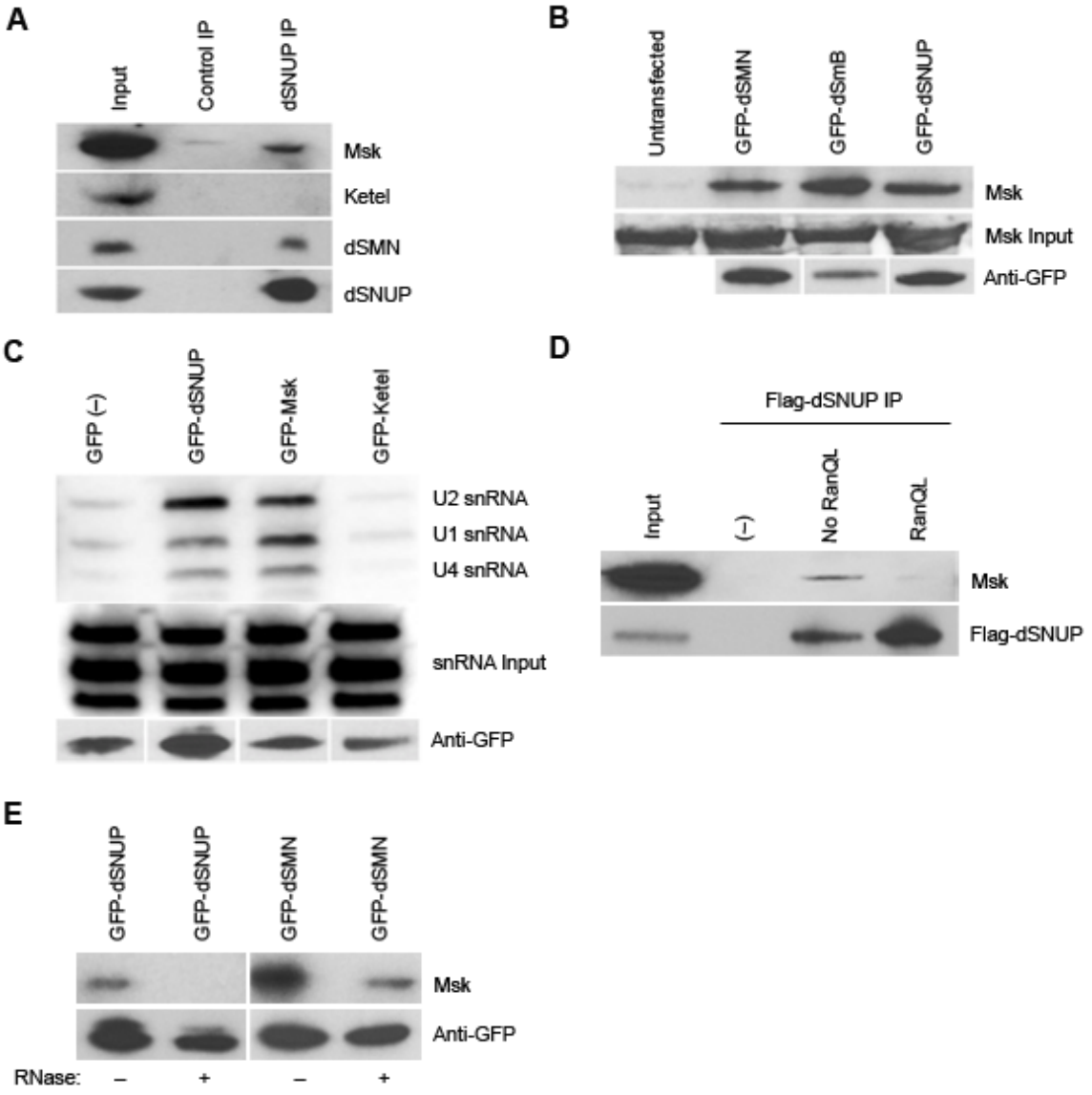


Figure 2.5. Moleskin interacts with *Drosophila* snRNPs. (A) dSNUP Guinea pig IP Western Blot. dSNUP Guinea pig antibody co-immunoprecipitates Msk, but not Ketel from S2 cell cytoplasmic lysate. (B) GFP IP Western Blot. Msk co-immunoprecipitates with transfected GFP-dSMN, GFP-dSmB, and GFP-dSNUP from S2 cell cytoplasmic lysate. (C) Anti-GFP IP Northern Blot. Major U snRNAs U1, U1, and U4 co-immunoprecipitate with GFP-Msk and GFP-dSNUP, but not GFP-Ketel. (D) Anti-flag IP Western Blot. Transfected flag-dSNUP co-immunoprecipitates Msk in the absence of RanQL. This interaction is disrupted by the addition of RanQL. Non-conjugated protein A beads serves as negative control IP (–). (E) RNase anti-GFP IP Western Blot. RNase treatment of cytoplasmic S2 lysate abolishes interaction of transfected GFP-dSNUP and GFP-dSMN with endogenous Msk.

Moleskin/Importin-7 localizes to snRNP-rich structures in the nucleus

As a nucleocytoplasmic transport factor, Msk shuttles from the cytoplasm to the nucleus. As such, previous investigations had shown that the subcellular localization of Msk (a.k.a. DIM-7) is dynamic; in certain cells the protein was primarily found in the cytoplasm whereas in others it was predominantly nuclear (Lorenzen *et al.,* 2001; James *et al.,* 2007). Given that Msk forms complexes with snRNP components and biogenesis factors, we carried out immunofluorescence analyses in *Drosophila* larval and adult tissues. Msk is primarily cytoplasmic within the egg chambers of the ovary (Figure 2.6A), but both nurse and follicle cell nuclei remain largely unstained. However, Msk also shows prominent localization to the nurse cell nuclear periphery and to bright foci within the oocyte germinal vesicle (Figure 2.6A).

In mammalian cells, Cajal bodies are the first detectable sites of nuclear accumulation of newly imported snRNPs (Sleeman *et al.,* 1999). Given the interaction data described above, we suspected that these Msk-positive nuclear foci were Cajal bodies. Indeed, co-staining for Sm proteins and other Cajal body markers demonstrates that the foci correspond to Cajal bodies (Figure 2.6 and data not shown). Note that the oocyte nucleoplasm typically does not stain uniformly with DAPI, only the karyosome is well-stained (Liu *et al*., 2006a). Within the germinal vesicle, Cajal bodies can often be found proximal to the karyosome (Figure 2.6B) although they can also be distally located (Figure 2.6A). Due to their relative prominence within larval Malpighian tubule nuclei, Cajal bodies are perhaps best visualized in this tissue (Liu *et al.,* 2006b).

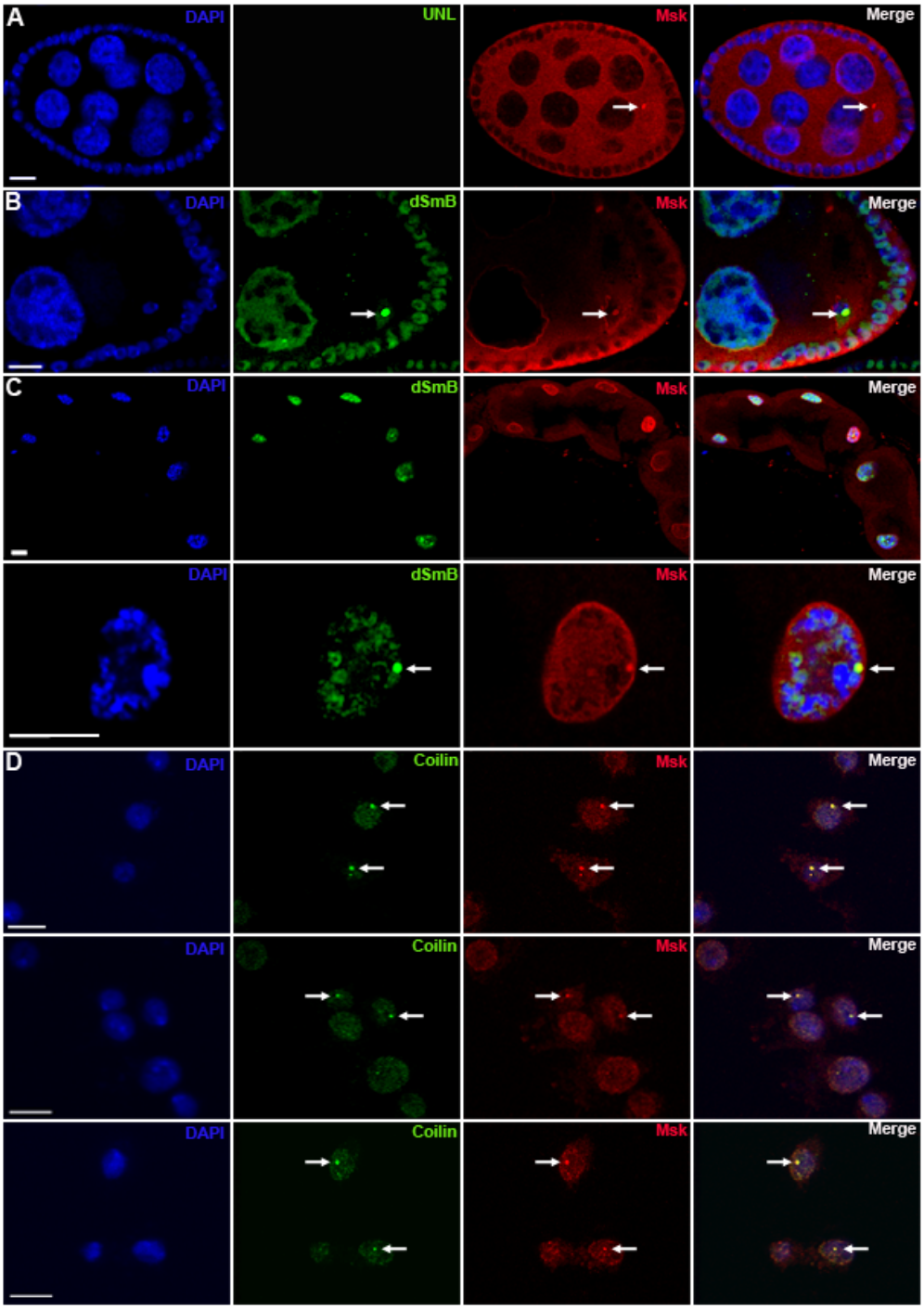


Figure 2.6. Moleskin is enriched in Cajal bodies. (A) Immunofluorescence in egg chambers with Msk antibody. Msk is enriched in the germinal vesicle (marked by arrow). (B) Immunofluorescence in egg chambers with dSmB and Msk antibodies. Msk is enriched in the germinal vesicle (marked by arrow). (C) Immunofluorescence in Malpighian tubules with dSmB and Msk antibodies. Msk is enriched in Cajal bodies of Malpighian tubules. (D) Immunofluorescence in S2 cells with coilin and Msk antibodies. Msk is enriched in Cajal bodies of S2 cells. Scale bars, 10 μm.

In Malpighian tubules (Figure 2.6C), we found that Msk is primarily nucleoplasmic and accumulates in bright nuclear foci. The bright Msk foci colocalize with the snRNP core component, dSmB (Figure 2.6C) or coilin (not shown). In S2 cell cultures, only a fraction of the cells display Cajal bodies. However, whenever we observed the bright nuclear foci that stained with anti-Msk, they invariably also stained positive for coilin, the Cajal body marker protein (Figure 2.6D). These results provide strong support for the notion that Msk is involved in import of Sm-class snRNPs.

Moleskin depletion disrupts snRNP biogenesis and import

RNA interference (RNAi) analysis in S2 cells using dsRNAs targeting Msk revealed that Cajal bodies were disrupted by Msk depletion (data not shown). This finding is consistent with previous results showing that Cajal body homeostasis requires ongoing snRNP biogenesis (Shpargel and Matera, 2005; Lemm *et al.,* 2006). However, because U snRNPs are extremely stable complexes, with half-lives on the order of 3-5 days (Sauterer *et al.,* 1988), this analysis was not terribly informative with regard to snRNP phenotypes. We therefore obtained a presumptive *msk* null mutant from the Bloomington stock center ($msk^{-/-}$), which contains a *piggyback* transposon insertion in exon 1. We confirmed that this allele is indeed a null by western blotting, demonstrating the absence of Msk protein in homozygous mutant larvae (Figure 2.7A). Moleskin null mutants are larval lethals (Lorenzen *et al.,* 2001); a small fraction of mutant larvae survive greater than 10 days, but they do not develop past the second

instar. The extended survival of *msk* mutants suggests that, like Ketel protein (Villanyi *et al.,* 2008), Msk protein also has a long half-life.

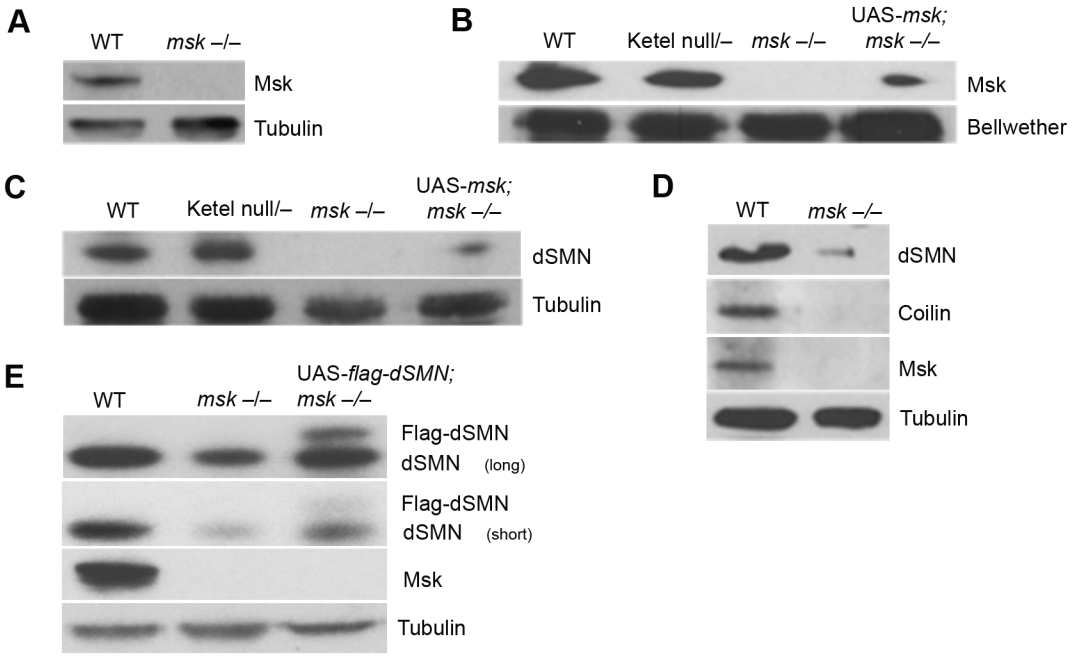


Figure 2.7. Moleskin mutant characterization. (A-C) Western Blot of 2nd instar larvae. (A) *msk*$^{-/-}$ larvae have significantly reduced Msk protein levels. (B) Ketel$^{null/-}$ larvae have WT levels of Msk. UAS-*msk* driven by armadillo-Gal4 in the *msk*$^{-/-}$ background show recovery of Msk protein. (C) *msk*$^{-/-}$ larvae have significantly reduced levels of dSMN, which can be recovered by UAS-*msk* driven by armadillo-Gal4. (D) Western Blot of 1st instar larvae. *msk*$^{-/-}$ larvae have significantly reduced levels of dSMN and coilin by day 1 post egg laying. (E) Western Blot of 2nd instar larvae. UAS-*flag-dSMN* driven by armadillo-Gal4 in the *msk*$^{-/-}$ background show flag-dSMN expression. Long and short exposures (top two panels) of the anti-dSMN blot are shown. As described previously (Shpargel et al., 2009), we note that ectopic expression of epitope-tagged dSMN results in stabilization of endogenous dSMN, due to its preferential incorporation into the SMN complex.

To determine if there are snRNP specific phenotypes associated with loss of Msk, we carried out immunofluorescence with anti-TMG cap antibodies. Wild-type, Ketel$^{null/-}$, and UAS-*msk* transgenic rescue animals were used as controls.

Moleskin mutants displayed a slight, but reproducible, cytoplasmic TMG accumulation in the Malpighian tubules (Figure 2.8), suggesting a disruption in snRNP import and/or biogenesis. This accumulation was not simply due to the developmental arrest, as Ketel$^{null/-}$ mutants do not display this phenotype, and expression of UAS-*msk* rescues it (Figure 2.8).

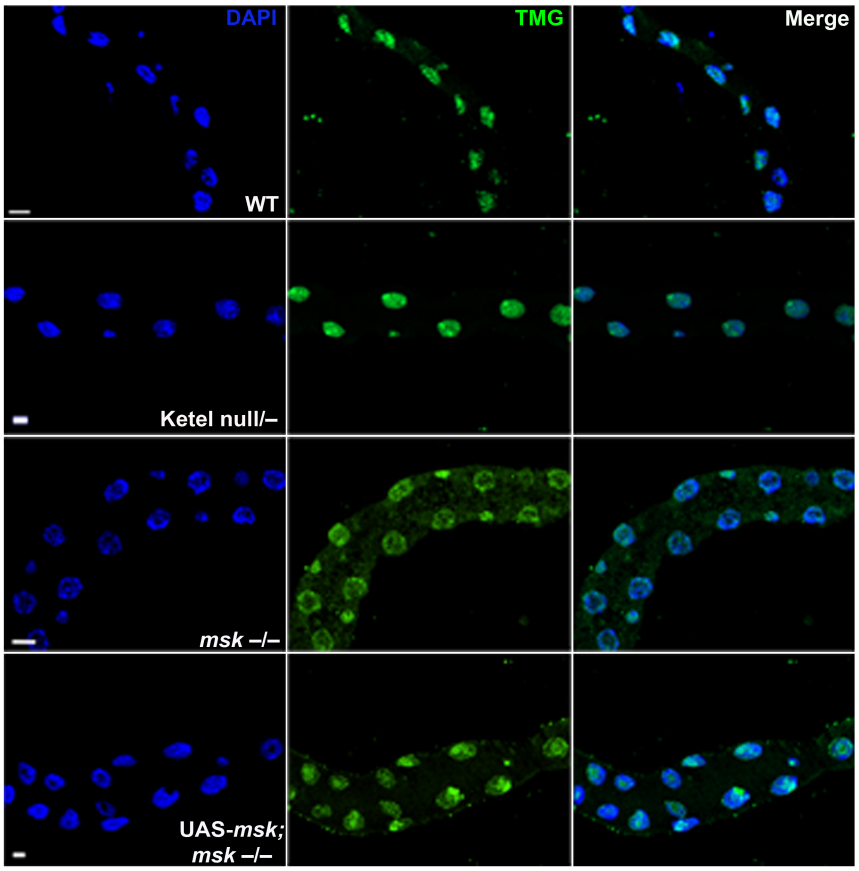


Figure 2.8. Moleskin null mutant Malpighian tubules display TMG cap cytoplasmic accumulation. Immunofluorescence in 2nd instar larvae. Long lived *msk*$^{-/-}$ larvae show cytoplasmic accumulation of TMG in Malpighian tubules while similar long lived larvae Ketel$^{null/-}$ larvae do not. UAS-*msk* driven by armadillo-Gal4 in the *msk*$^{-/-}$ background partially rescues cytoplasmic TMG phenotype. Scale bars, 10 μm.

In an effort to bypass the Msk dependence of this observed snRNP import defect, we generated transgenic flies expressing VFP-dSNUP or VFP-hIBB-dSNUP from UAS promoters. Because we had previously shown that hIBB-dSNUP forms a complex with Ketel (Figure 2.4B), we hypothesized that its expression might rescue snRNP import in Malpighian tubules. We therefore expressed these transgenes in both wild-type and *msk*$^{-/-}$ backgrounds. Using either a ubiquitous tubulin-Gal4 driver or a gut-specific Malpighian tubule driver, we found that expression of VFP-hIBB-dSNUP was dominantly lethal in both backgrounds. It is unlikely that the dominant negative phenotype of the hIBB-dSNUP construct is due to VFP-tagging because expression of the control VFP-dSNUP construct had no such dominant effects and was able to rescue dSNUP RNAi (not shown). The dominant lethality of the hIBB-dSNUP fusion precluded us from testing whether targeting dSNUP to an alternative nuclear import receptor pathway (in this case Ketel) might alleviate the apparent block to snRNP import.

We therefore conducted immunofluorescence with anti-dSMN and anti-coilin antibodies in control and *msk* mutant larvae. Confirming the results noted above for S2 cells, we found that in the Malpighian tubules of *msk* mutants, dSMN and coilin staining was dramatically reduced and Cajal bodies were disrupted (Figure 2.9). Staining for both dSMN and Cajal bodies (anti-coilin) was restored upon expression of (untagged) Msk using a UAS-*msk* transgene (Figure 2.9). As shown in Figure 2.7B, the loss of dSMN is fairly extensive, as it can be detected by western blotting using total larval lysates. Importantly, the expression

of UAS-*msk* transgene partially rescues both Msk and dSMN expression (Figure 2.7B and C), as well as development of the organism beyond larval stages (Lorenzen *et al.,* 2001; this work). Additionally coilin and dSMN reduction is detectable by day one post egg laying (Figure 2.7D). Thus, Msk is required for the stability of dSMN and coilin.

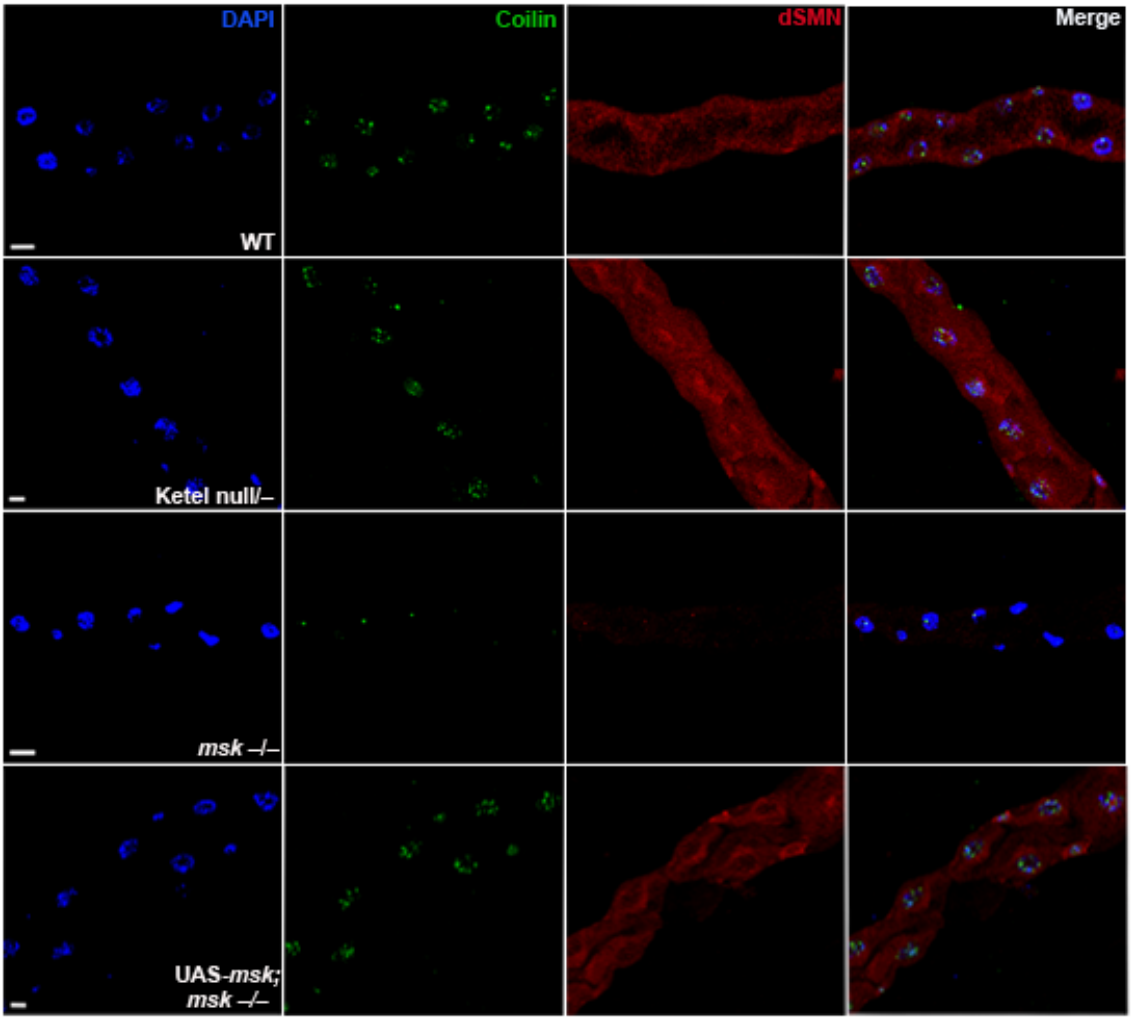


Figure 2.9. Coilin and dSMN are reduced in Moleskin mutant Malpighian tubules. Immunofluorescence in 2nd instar larvae. Long lived *msk*$^{-/-}$ larvae have reduced dSMN and Cajal bodies (anti-coilin) in Malpighian tubules while similar long lived Ketel$^{null/-}$ larvae do not. UAS-*msk* driven by armadillo-Gal4 in the *msk*$^{-/-}$ background show recovery of both coilin and dSMN (UAS-*msk*; *msk*$^{-/-}$). Scale bars, 10 μm.

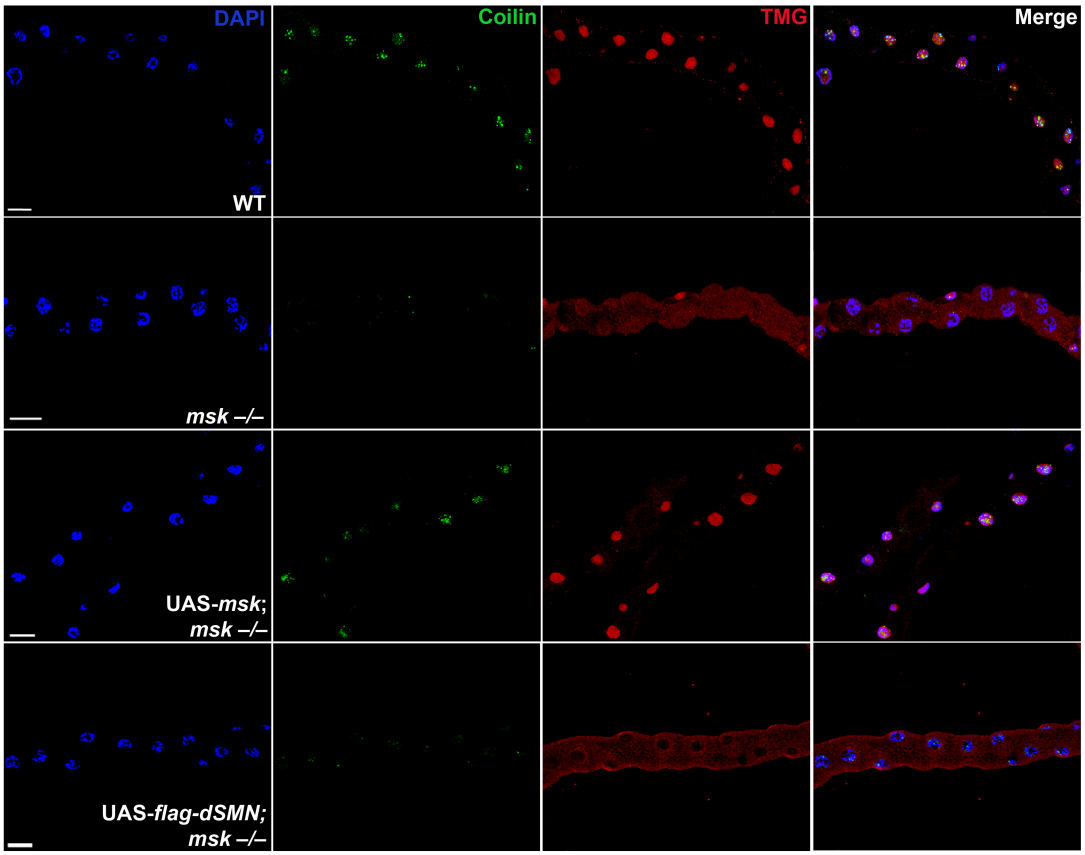


Figure 2.10. Over expression of flag-dSMN does not rescue Coilin and dSMN reduction in Moleskin mutant Malpighian tubules.
Immunofluorescence in 2nd instar larvae. Long lived *msk*$^{-/-}$ larvae have reduced dSMN and Cajal bodies (anti-coilin) in Malpighian tubules compared to WT. UAS-*msk* driven by armadillo-Gal4 in the *msk*$^{-/-}$ background show recovery of both coilin and dSMN (UAS-*msk*; *msk*$^{-/-}$). UAS-*flag-dSMN* driven by armadillo-Gal4 in the *msk*$^{-/-}$ background fails to rescue coilin or dSMN (UAS-*flag-dSMN*; *msk*$^{-/-}$). Scale bars, 20 μm.

SMN plays a crucial role in snRNP biogenesis, and its depletion has been shown to disrupt Cajal bodies in HeLa cells (Shpargel and Matera, 2005). Therefore, the significant reduction of dSMN in *msk* mutant larvae could be responsible for the Cajal body and TMG cap phenotypes. To investigate this possibility, we overexpressed flag-tagged dSMN in the Msk mutant background (Figure 2.7E). Overexpression of flag-dSMN failed to rescue organismal viability,

Cajal body disruption or cytoplasmic TMG cap localization (Figure 2.10). Therefore, Msk function *in vivo* is not limited to the stabilization of dSMN. Taken together with the subcellular localization and biochemical interaction analyses described above, these genetic results provide strong evidence linking Msk to a role in snRNP biogenesis.

DISCUSSION

Vertebrate Imp7 and Impβ form an abundant heterodimeric complex (Gorlich *et al.,* 1997). Because Impβ is entirely sufficient for snRNP import *in vitro* and in *Xenopus* oocytes (Huber *et al.,* 1998; Palacios *et al.,* 1997), it was assumed that the co-purification of Imp7 with SPN1 in HeLa cells was simply an indirect consequence of its interaction with Impβ (Paraskeva *et al.,* 1999). In this study, we show that dSNUP is the *Drosophila* orthologue of human SPN1 and provide convincing evidence that it fails to bind Ketel/Impβ *in vitro* and *in vivo*. Our results strongly favor the interpretation that *Drosophila* snRNP import utilizes the import receptor Msk/Imp7 in place of Ketel/Impβ. Thus, the physical interaction between Imp7/Msk and SPN1/dSNUP is conserved in both humans and *Drosophila*, raising the question of whether Imp7 might play a previously unrecognized role in vertebrate snRNP import.

In mammalian cells, Imp7 has been shown to function as an import receptor for various protein cargoes, independent from its role as an adaptor for Impβ (Jakel *et al.,* 1999; Freedman *et al.,* 2004). Thus, it is possible that Imp7 plays a similar role in the snRNP import pathway in mammals. Previous results from our lab show that SMN can bind directly to Impβ *in vitro*, and that purified

SMN complexes are required for SPN1 independent snRNP import (Narayanan *et al.,* 2004). However, the precise identity of the import adaptor for the Sm-core mediated import pathway is not known. Whether or not the Impβ binding site of SMN is masked while the protein is in the SMN import complex is also unknown. Several possibilities thus exist *in vivo*: Impβ may bind directly to SMN, or indirectly through an unidentified adaptor protein (e.g. Imp7/Msk), or some combination of both scenarios, as they are not mutually exclusive.

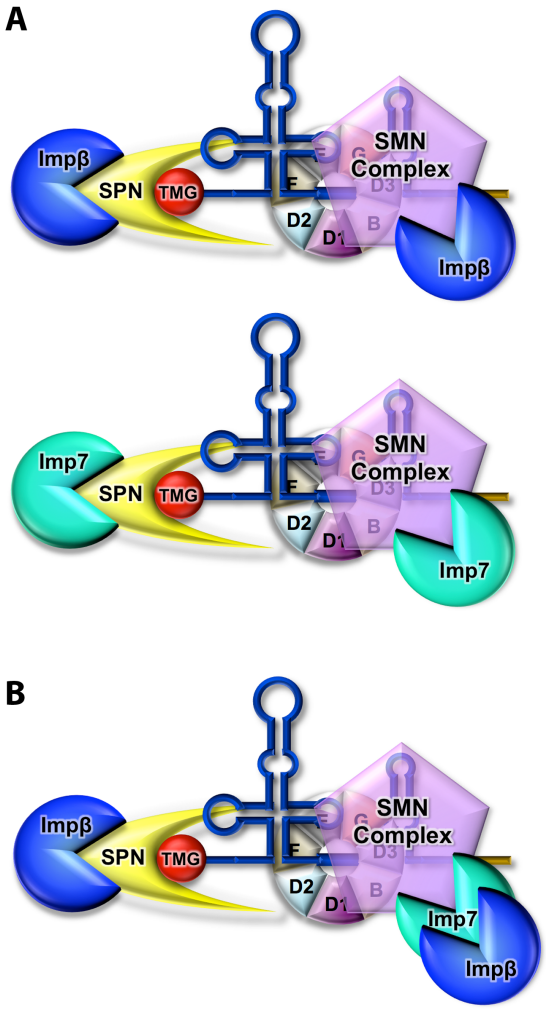
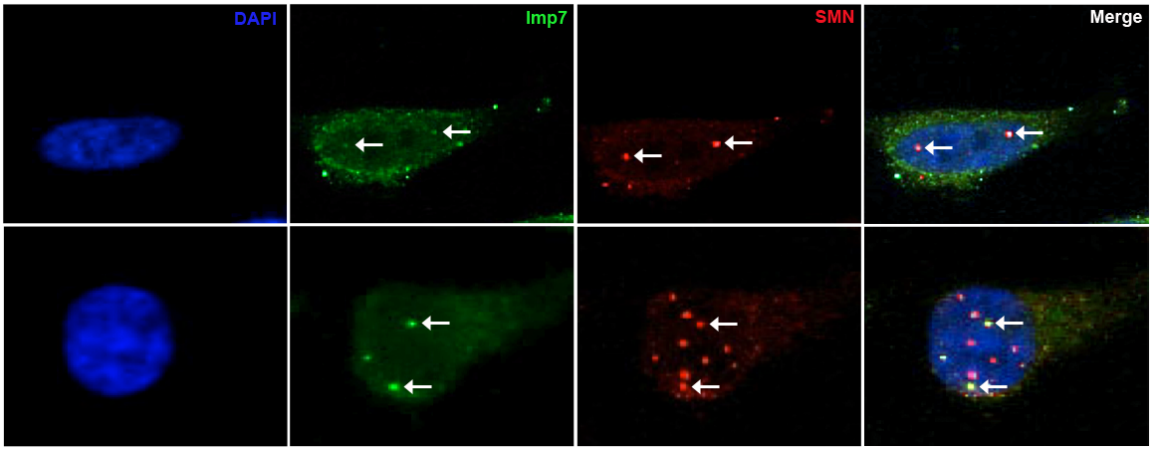


Figure 2.11. Models of Imp7's role in snRNP import. (A) Imp7 and Impβ could function redundantly as an autonomous snRNP import receptors. (B) Alternatively, Imp7 could function as an Sm core snRNP import adaptor for Impβ.

We envision two models by which Imp7 could function in the nuclear import of snRNPs in vertebrates. In one scenario, Imp7 and Impβ could have partially redundant functions, wherein they could each independently function as import receptors in single snRNP import events (Figure 2.11A). Alternatively, Imp7 could serve as an import adaptor for Impβ, functioning together in the same import cycle (Figure 2.11B). Curiously, we find that an unidentified band of the appropriate size co-purifies with the SMN complex in numerous publications (Baccon *et al.,* 2002; Pellizzoni *et al.,* 2002; Yong *et al.,* 2004; Carissimi *et al.,* 2005; 2006a; 2006b). Thus it is possible that Imp7 is the unidentified Sm core import adaptor protein in vertebrates, definitive identification of which remains a subject of considerable interest.

Traditionally, import receptors are thought to be bound immediately by RanGTP in the nucleus; subsequently, the receptors are recycled back into the cytoplasm. However, there is evidence that Imp7 may be a bit different from traditional nuclear import receptors. Unlike Impβ, Imp7 does not require RanGTP for histone H1 nuclear import (Jakel *et al*., 1999). The lower affinity for RanGTP is hypothesized to be a potential advantage. By delaying the dissociation of Imp7 from H1, Jakel *et al.* (1999) suggested that Imp7 could accompany the histone to the chromosome for assembly into chromatin. This same idea could be applied to our surprising finding that Msk/Imp7 localizes to Cajal bodies in both *Drosophila* and human cells (Figures 2.6 and 2.S1, respectively). Hence, Msk/Imp7 might act in a chaperonin-like fashion inside the nucleus, ferrying snRNPs to Cajal bodies for potential interaction with coilin and/or SMN (Narayanan *et al*., 2004;

Ospina *et al*., 2005; Shpargel *et al*., 2005; Liu *et al*., 2000; Tanackovic *et al*., 2005).



Supplemental Figure 2.S1. Human Imp7 is enriched in Cajal Bodies of Mammalian Cells. Immunofluorescence in HeLa cells. Imp7 is enriched in a subset of Cajal bodies (marked by arrows) in HeLa cells. Co-stained with anti-SMN.

Navigating the complex nature of snRNP import mechanisms will require precise molecular dissection of the interactions between snRNPs, their transport receptors and their downstream effectors. Our finding that loss of *msk* function leads (directly or indirectly) to co-depletion of dSMN is particularly significant in this regard. Collectively, our studies provide strong evidence that Ketel/Impβ is not the TMG cap import receptor in *Drosophila* and that Msk/Imp7 is required for ongoing snRNP biogenesis. Furthermore, we provide important food for thought regarding a potential role for Imp7/Msk in mammalian snRNP import. Imp7/Msk may have different binding capacities than Impβ/Ketel in particular tissues or for individual species of U snRNPs. Additional experiments will be needed to clarify these and other important questions. Understanding the role of Imp7/Msk in

snRNP biogenesis in both vertebrate and invertebrate systems should elucidate how the two different nuclear import pathways complement one another.

MATERIALS AND METHODS

DNA Constructs

dSNUP, hIBB-dSNUP, dSMN, dSmB, Msk, and Ketel full-length cDNAs were PCR amplified with appropriate primers flanked by Gateway recombination sequences (Invitrogen, Carlsbad, CA). These products were recombined initially into pDONR221 (Invitrogen) before entry into green fluorescent protein (GFP)-tagged pAGW, flag-tagged pAFW (*Drosophila* Genome Research Center), or *pBI-UASC-mVenus* (gift from Brian McCabe).

Recombinant Protein Expression and S2 Cell Transfections

GST-dSNUP was expressed in BL21-star bacteria (Invitrogen) by 1 mM IPTG induction for 3 h. Lysate was extracted by sonication and passed over glutathione beads. S2 cells were transfected using Cellfectin as directed (Invitrogen), and cells were harvested 4 days after transfection.

Antibodies

A rabbit polyclonal anti-dSNUP antibody (dSNUP) was generated (Pacific Immunology, Ramona, CA) using GST-tagged dSNUP. A guinea pig polyclonal anti-dSNUP antibody was generated (Pocono Rabbit Farm and Laboratory, Canadensis, PA) using dSNUP.

GST (Santa Cruz Biotechnology, Santa Cruz, CA; mouse; 1:1000), GFP (Roche, Indianapolis, IN; mouse; 1:1000 and Abcam, Cambridge, MA; rabbit,

1:1000), dSmB (Y12) anti-sDMA (gift from J. Steitz, Yale, New Haven, CT; anti-mouse; 1:3000), dSMN (Praveen, *et al.*, 2012; affinity purified anti-rabbit; 1:2000), dSNUP (affinity purified rabbit; 1:3000), Msk (gift of L. Perkins; rabbit; 1:2000), Ketel (gift from J.Szabad; anti-rabbit; 1:5000), bellwether (Abcam, Cambridge, MA; mouse, 1:5000), Flag (Sigma-Aldrich, St. Louis, MO; HRP conjugated anti-flag; 1:8000), and tubulin (Sigma-Aldrich, St. Louis, MO; rabbit; 1:10,000) antibodies were used for Western blotting. Secondary antibodies used were goat anti-mouse-, goat anti-Guinea pig-, and goat anti-rabbit, all conjugated with horseradish peroxidase at 1:5000 (Pierce).

Msk (gift of L Perkins; rabbit; 1:1000), Coilin (gift of J. Gall; guinea pig; 1:1000), dSMN (Praveen, *et al.*, 2012; affinity purified rabbit; 1:200), dSNUP (guinea pig; 1:200), dSmB (Y12) anti-sDMA (gift from J. Steitz, Yale, New Haven, CT; mouse monoclonal; 1:200), Imp7 (Sigma-Aldrich, St. Louis, MO; rabbit; 1:250), and hSMN (mouse monoclonal; clone 8, BD Biosciences, 1:250) were used for immunofluorescence. GFP (Abcam, Cambridge, MA; rabbit; 1.5 µl), GFP (mouse; Roche; 1.5 µl), and dSNUP (guinea pig; 10 µl) antibodies, and flag conjugated agarose beads (Sigma-Aldrich, St. Louis, MO; 15 µl per IP) were used for immunoprecipitation in buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT).

Immunoprecipitation

S2 cell cytoplasmic lysate was prepared by resuspending cells in 5X pellet volume of buffer A. Resuspended cells were incubated on ice 30 min. to allow swelling, mixed 10X with a p200 pipette, and incubated an additional 10 min. on

ice before passing through a 27.5 gauge needle 40X. Cells were spun 1 min. 13,000 rpm in microfuge, and the cytoplasmic supernatant treated with protease inhibitor cocktail (Pierce). For RNase experiments, S2 cell cytoplasmic lysate was divided into equal fractions, which were untreated or treated with 1 ug RNase per 5 µg lysate for 1 h. at 37°C. For RanQ69L experiments, bacterially expressed GST-RanQ69L was added to cytoplasmic lysate. Cytoplasmic fractions were incubated with antibody 1 h. (no antibody added for negative control IP) at 4°C before being incubated over night at 4°C with 15 ul protein A beads (Pierce). Bound proteins were washed 5X with 1 mL buffer A.

For IP Northern experiments, bound RNA was directly Phenol/Chloroform extracted off beads, denatured in formamide loading buffer, and run on a 10% polyacrylamide-urea gel (Invitrogen), transferred to a nylon membrane, and probed with ^{32}P-labeled PCR products corresponding to the *D. melanogaster* U1, U2, and U4 snRNAs.

RNAi

dSNUP dsRNAs were transcribed *in vitro* from PCR products flanked with T7 promoters. *Drosophila* S2 cells were placed in SF-900 media and treated with fresh 14 µg/mL double-strand RNA (dsRNA) each day for 4 d. before harvesting. Cytoplasmic extracts were generated 4 d. after transfection. 50 µg of cytoplasmic extract was loaded on a polyacrylamide gel for western blotting analysis to confirm knockdown.

Fly Stocks

Oregon-R was used as the wild-type strain. A Msk null line containing a piggy back insertion in intron 1 of Msk (*msk*$^{-/-}$), Msk^{B185}, w^{1118}; PBac{5HPw^{+}}msk^{B185}/ TM3, Sb^{1} Ser^{1}, and a line containing *msk* with a UAS promoter (UAS-*msk*), w*; P{UAS-*msk*.L}47M1/CyO**,** previously characterized (Lorenzen *et al.*, 2001), were obtained from the Bloomington Stock Center (Bloomington, IN). Armadillo-Gal4 was recombined with *msk*$^{-/-}$ and crossed to UAS-*msk* for rescue or previously characterized UAS-*flag-dSMN* (Chang *et al.,* 2008). Previously characterized Ketel$^{null/-}$ (Villanyi *et al.,* 2008) were a gift from Janos Szabad. [The – symbol stands for a small deficiency (ketel^{rx32}) that removes Ketel and a few of the adjacent loci, while the Ketel null (ketel^{rx13}) is a complete loss of function mutant allele (Erdelyi *et al*., 1997).]

The *dSNUP* and *hIBB-dSNUP* transgenic constructs were cloned into *pBI-UASC-mVenus* (Wang *et al.,* 2012) and sent to BestGene (Chino Hills, CA) for embryo injection using the phiC31 system. Transgenes were integrated at site 86fB (Bischof *et al.,* 2007). Flies bearing a UAS:VFP-*Snup* transgene were crossed to a variety of Gal4 drivers, including, tubulin-Gal4 and nanos-Gal4. The *msk*$^{-/-}$ flies were recombined with either *VFP-dSNUP and VFP-hIBB-dSNUP* transgenic lines and with Gal4 drivers. Timed matings were allowed to proceed for 6 h., and larvae were collected for phenotypic analyses on subsequent days.

Immunofluorescence

Drosophila tissues, HeLa and S2 cells were fixed at room temperature for 10 min. in 3.7% paraformaldehyde in phosphate-buffered saline (PBS: 135 mM

NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2). Tissues/cells were then permeabilized with 1% Triton 100x, blocked in PBST (PBS with 0.1% Triton 100x) containing 5% NGS (blocking solution) and then washed with PBST. The primary antibody, diluted in PBST, was incubated with the samples overnight at 4°C. After being washed with PBST, the secondary antibody, diluted in blocking solution, was incubated with the samples 2 h. at room temperature. The samples were stained with DAPI, washed with PBST and mounted in anti-fade solution (0.233 g DABCO, 800 µl water, 200 µl 1 M Tris-HCl pH 8.0, 9 ml glycerol).

Fluorescence Microscopy

Images were taken with a 40X (numerical aperture [N.A.] 1.25) plan apochromatic objective on a laser-scanning confocal microscope (SP5; Leica, Exton, PA). Contrast and relative intensities of the green (Alexa 488 or Venus tag), red (Alexa 594), and blue (DAPI) images were adjusted with Photoshop (Adobe Systems, Mountain View, CA).

CHAPTER III

Characterization of the physical and functional interactions of Moleskin/Importin-β with snRNP

Overview

Biogenesis of small nuclear ribonucleoproteins (snRNPs) is biphasic. In vertebrates, small nuclear RNAs (snRNAs) are exported to the cytoplasm for assembly into pre-snRNPs. The partially assembled snRNPs are then imported back into the nucleus via the import adaptor, snurportin1 (SPN), and the import receptor importin-β. Previous work from our lab has shown that the *Drosophila* orthologue of SPN, dSNUP, does not contain an importin-β binding (IBB) domain, essential for SPN-mediated snRNP import in humans. Although it has been shown that neither dSNUP nor snRNPs interact with importin-β in flies, the import receptor, Moleskin/importin-7, does bind snRNP protein and RNA components. This chapter takes a closer look at the physical interactions of Moleskin (Msk) with snRNPs, revealing that its interaction with the Sm core may be direct. Moreover, Msk depletion induces cytoplasmic accumulation of transfected GFP-dSmB, suggesting that snRNP import is inhibited in the absence of Msk. These results provide further evidence validating a role for Msk in the snRNP import pathway

INTRODUCTION

Uridine-rich small nuclear RNAs (U snRNAs) are the primary, essential components of the spliceosome, and thus, play a crucial role in splicing (Mattaj et al, 1993); (Tarn & Steitz, 1997). Small nuclear RNAs (snRNAs) of the Sm class are transcribed by RNA polymerase II in the nucleus and exported to the cytoplasm by a complex composed of the phosphorylated adaptor for RNA export (PHAX), the cap binding complex (CBP80/20) and the export receptor, Xpo1/RanGTP (Ohno et al, 2000). Once in the cytoplasm, the survival motor neuron (SMN) complex mediates the assembly of snRNPs by loading seven Sm proteins onto the snRNA (Meister et al, 2002); (Paushkin et al, 2002). The SMN complex serves as a scaffold upon which Sm proteins and the snRNA are assembled and ensures that the correct Sm proteins assemble onto the snRNA. The role SMN plays in snRNP assembly is crucial, because without Sm assembly, snRNPs are incapable of import, and thus, cannot participate in active splicing within the nucleus. After Sm core assembly, the 5'-end methylguanosine cap structure of the snRNA is hypermethylated to form a trimethylguanosine (TMG) cap by trimethylguanosine synthase (Tgs1); (Mouaikel et al, 2002); (Verheggen et al, 2002), and this is thought to be a signal for nuclear import (Palacios et al, 1997); (Mattaj & De Robertis, 1985); (Hamm et al, 1990); (Fischer et al, 1993). The snRNP import adaptor, Snurportin1 (SPN), binds to the TMG cap of the newly assembled snRNP, and importin-β (Impβ) subsequently binds SPN to facilitate nuclear translocation through the NPC (Huber et al, 1998).

U snRNPs do not appear to have a classical NLS. In contrast to classical NLS mediated import, U snRNP import does not require importin-α, but Impβ was shown to be required for U snRNP import (Palacios et al, 1997). U snRNPs have two known NLSs: the TMG cap and the Sm core. SPN is the import adaptor for the TMG cap NLS, and the SMN complex is thought to be the import adaptor for the Sm core NLS (Narayanan et al, 2002). *In vitro* snRNP import studies have demonstrated that U snRNPs possess different import requirements. Somatic cells can utilize either snRNP import pathway for U1, U2, U4, and U5 snRNPs, but the TMG cap NLS is required for U1 and U2 snRNP import in *Xenopus* oocytes (Fischer et al, 1993). Interestingly, when *Xenopus* egg extract is supplied to somatic cells, U1 and U2 import becomes TMG cap dependent, suggesting that soluble cytosolic factors mediate the TMG cap dependence of U1 and U2 import (Marshallsay & Luhrmann, 1994). Identification of these cytosolic factors has evaded detection, but we do know that TMG cap independent import is dependent on the SMN complex (Narayanan et al, 2002).

Irrespective of the requirement for TMG capping, and hence, absence of SPN binding, nuclear import is mediated via Impβ in the vertebrate system. Notably, these observations have all been in *in vitro* systems, and they fail to answer the question as to why we see cell specific differences in snRNP import requirements. An *in vivo* model of snRNP import would enable us to better analyze and validate the data obtained from *in vitro* snRNP import assays. Understanding how these two pathways function in the *Drosophila* model system

(should two pathways exist in fruit fly) will help elucidate the significance of the need for two independent snRNP import mechanisms.

My previous work, shown in Chapter II, has taken the first steps in developing a *Drosophila* model system to study these two snRNP import mechanisms. I had identified and characterized *Drosophila* Snurportin (dSNUP), and in contrast to vertebrates, I found that *Drosophila* Snurportin does not contain an IBB and did not interact with Impβ. Most importantly, my work supports the hypothesis that snRNP import in flies is Impβ independent and led to the identification of a potentially novel snRNP import factor, Moleskin (Msk). I discovered that *msk* null mutants are depleted of the snRNP assembly factor, SMN, and the Cajal body marker, coilin is disrupted. In whole, the work shown in Chapter II provided evidence supporting a role for Msk in snRNP biogenesis, which warrants further investigation.

In this chapter, I more closely examined the functional significance and physical interactions of Msk with snRNPs in S2 cells. Surprisingly, I found that the interaction of Msk with dSNUP is RNA dependent, while its interaction with the Sm core protein, dSmB, is RNA independent. Moreover, I discovered that upon dSNUP and/or Msk knock down, GFP-dSmB accumulates in the cytoplasm of S2 cells, indicating that snRNP import may be disrupted. These results provide additional evidence supporting a role for Msk in snRNP import and further characterize and validate the physical interactions I discovered between Msk and snRNP components. Future experiments that clarify the nature of these

interactions will be needed, but this chapter offers crucial insights that will be helpful in designing such experiments.

RESULTS

Interaction of Msk with snRNP proteins

I reconfirmed that Msk does indeed interact with snRNP protein components. Transfected GFP tagged -NLS, -dSMN and -dSPN co-immunoprecipitated Msk from S2 cell cytoplasmic lysate (Figure 3.1). Additionally, an antibody raised against dSmB (Y12) co-immunoprecipitated Msk (Figure 3.1A), further validating and authenticating the interaction. I also found that not only does GFP-dSNUP co-immunoprecipitate Msk, but that GFP-hSPN does as well, suggesting that the residues needed for this interaction are conserved from *Drosophila* to human (Figure 3.1B).

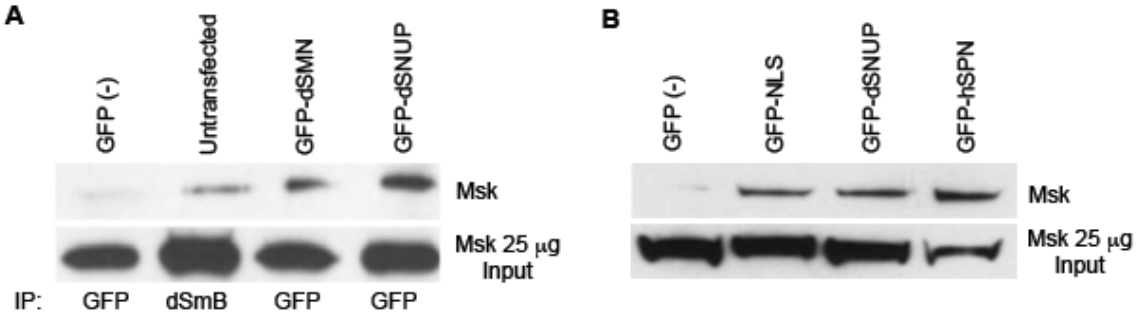


Figure 3.1. Characterization of Msk interaction with snRNP protein components. (A) Anti-dSmB (Y12) and GFP IP-Western. Anti-dSmB or anti-GFP co-immunoprecipitated Msk from untransfected or transfected GFP tagged –dSMN and –dSPN S2 cell cytoplasmic lysate. (B) GFP IP-Western. Transfected GFP tagged –NLS, -dSPN and -hSPN co-immunoprecipitated Msk from S2 cell cytoplasmic lysate.

Interaction of Sm proteins with Msk is RNA independent

My previous results from Chapter II provided evidence that Msk interacts with both the protein and RNA components of snRNPs (see Chapter II, Figure 2.5A-C). To further characterize the nature of these interactions, I conducted RNase immunoprecipitation experiments. SPN is an import adaptor that is known to directly bind to Impβ. If Msk is the snRNP import receptor in flies (replacing the need for Impβ), I envisaged that dSNUP without an IBB should interact with Msk in an RNA independent manner. Intriguingly, I found that the interaction of not only dSNUP, but also dSMN with Msk is RNA dependent (Figure 3.2A and B). In contrast, the interaction of the Sm core protein, dSmB, with Msk is RNA independent (Figure 3.2C). This suggests that dSNUP might be interacting with Msk via Sm proteins bound to RNA. Alternatively, dSNUP may still be interacting directly with Msk in an RNA dependent manner, but that the RNA stabilizes the interaction. Future experiments to distinguish between these two possibilities will need to be carried out and are discussed in Chapter IV.

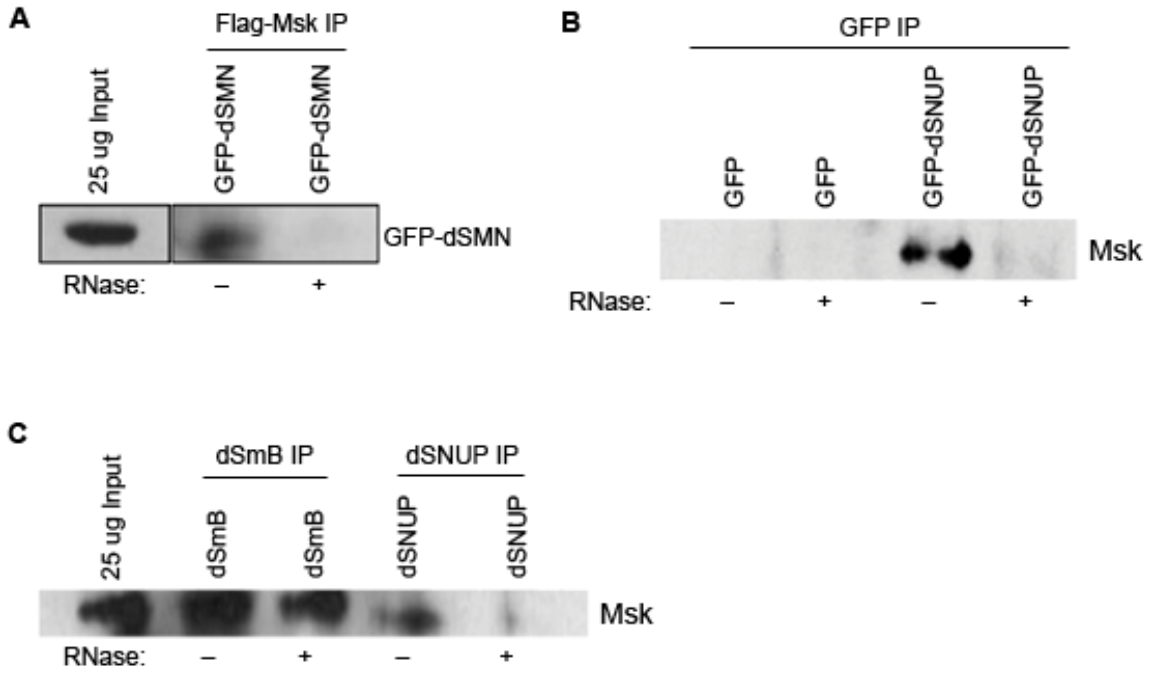


Figure 3.2. Characterization of the RNA dependence of Msk interaction with snRNP components. (A) Flag conjugated beads IP-Western Blot. Flag tagged Msk co-immunoprecipitated GFP-dSMN from transfected S2 cell cytoplasmic lysate. RNase treatment abolished this interaction. (B) GFP IP-Western. Transfected GFP-dSNUP co-immunoprecipitated Msk from S2 cell cytoplasmic lysate. RNase treatment abolished this interaction. (C) Anti-dSmB (Y12) and anti-dSNUP IP-Western Blot. Anti-dSmB and anti-dSNUP (guinea pig) co-immunoprecipitated Msk from S2 cell cytoplasmic lysate. While RNase treatment abolished the interaction of dSNUP with Msk, the interaction of dSmB with Msk was not dependent on RNA.

Snurportin and/or Moleskin knock down affects GFP-dSmB import

Nuclear import of snRNPs in vertebrates essentially follows two alternative pathways: TMG cap dependent and independent import. If there also exists two alternative pathways in flies, snRNPs should continue to be imported even in the absence of dSNUP. Alternatively, if only one import mechanism exists that impinges upon dSNUP and the TMG cap, I would expect snRNP import to be disrupted by knocking down dSNUP protein.

The essentiality of dSNUP for snRNP import was tested by employing dSNUP RNAi in S2 cells followed by transfection of GFP-dSmB after dSNUP knock down. RNAi experiments were designed such that the individual protein products encoded by *dSnup* are not simultaneously affected. Figure 3.3A shows that a dsRNA targeting exon 1 of dSNUP efficiently knocked down GFP-dSNUP. Upon knock down of dSNUP, transfected GFP-dSmB accumulated in the cytoplasm of S2 cells, suggesting a snRNP import defect, as SmB is a core component of the heteroheptameric ring structure of snRNPs (Figure 3.4). In addition to the cytoplasmic GFP-dSmB, I also observed nuclear GFP-dSmB in dSNUP knock down S2 cells. This could indicate that there are two alternative snRNP import pathways, which would allow GFP-dSmB to access the nucleus independently of dSNUP. This remaining nucleoplasmic GFP-dSNUP could also be attributed to residual levels of dSNUP protein that was not efficiently knocked down to levels that would completely perturb snRNP import.

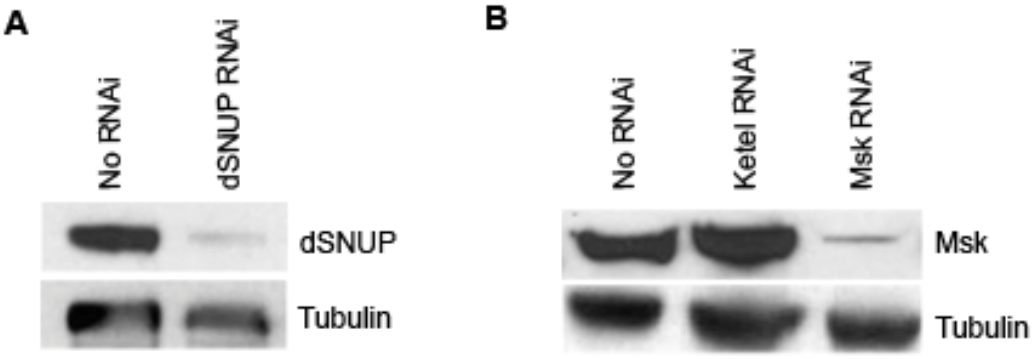


Figure 3.3. Significant knock down in S2 cells treated with dsRNAs targeting Msk or dSNUP. (A) dSNUP Western Blot. S2 cells treated with dsRNA targeting dSNUP for 5 days showed a significant reduction in dSNUP protein levels. (B) Msk Western Blot. S2 cells treated with dsRNA targeting Ketel/Impβ for 5 days showed no reduction in Msk protein levels, while treatment with dsRNA targeting Msk caused a significant reduction in Msk protein levels.

Irrespective of the presence of alternative import pathways in flies or the essentiality of dSNUP in snRNP import, Msk likely plays an important role in snRNP biogenesis as evidenced from my previous work (Chapter II). Assaying for snRNP import after Msk knock down in S2 cells tested the essentiality of Msk for snRNP import. A dsRNA targeting Msk provided robust knock down in S2 cells (Figure 3.3B). I followed the same methods described above for testing the essentiality of dSNUP in snRNP import, and found a similar phenotype in S2 cells treated with dsRNAs targeting Msk. There was a similar level of GFP-dSmB cytoplasmic accumulation after knock down, but a significant pool of remaining nuclear GFP-dSmB (Figure 3.4). Because Msk is a highly abundant protein in S2 cells (significantly more Msk protein than dSNUP in S2 cells), my knock down may not have been efficient enough to disrupt snRNP import entirely. It is also important to note that, even if Msk serves as a snRNP import receptor, there may be a complementary pathway that does not utilize Msk as the import receptor. Alternatively, it is also possible that Msk could function as the Sm core import adaptor or serve a redundant role to another unidentified import receptor (see model from Chapter II, Figure 2.10). Simultaneous dSNUP and Msk knock down produced similar results as seen in the knock down of either individual protein (Figure 3.4).

The cytoplasmic accumulation of GFP-dSmB upon Msk knock down was robust enough that I could detect it by western blotting. S2 cell fractionation of transfected GFP-dSmB was predominantly nucleoplasmic in control S2 cells (no

RNAi), while GFP-dSmB accumulated in the cytoplasm of S2 cells after treatment with dsRNA targeting Msk (Figure 3.5). This result lends additional support to the hypothesis that Msk plays a crucial role in snRNP import and is the likely snRNP import receptor in flies.

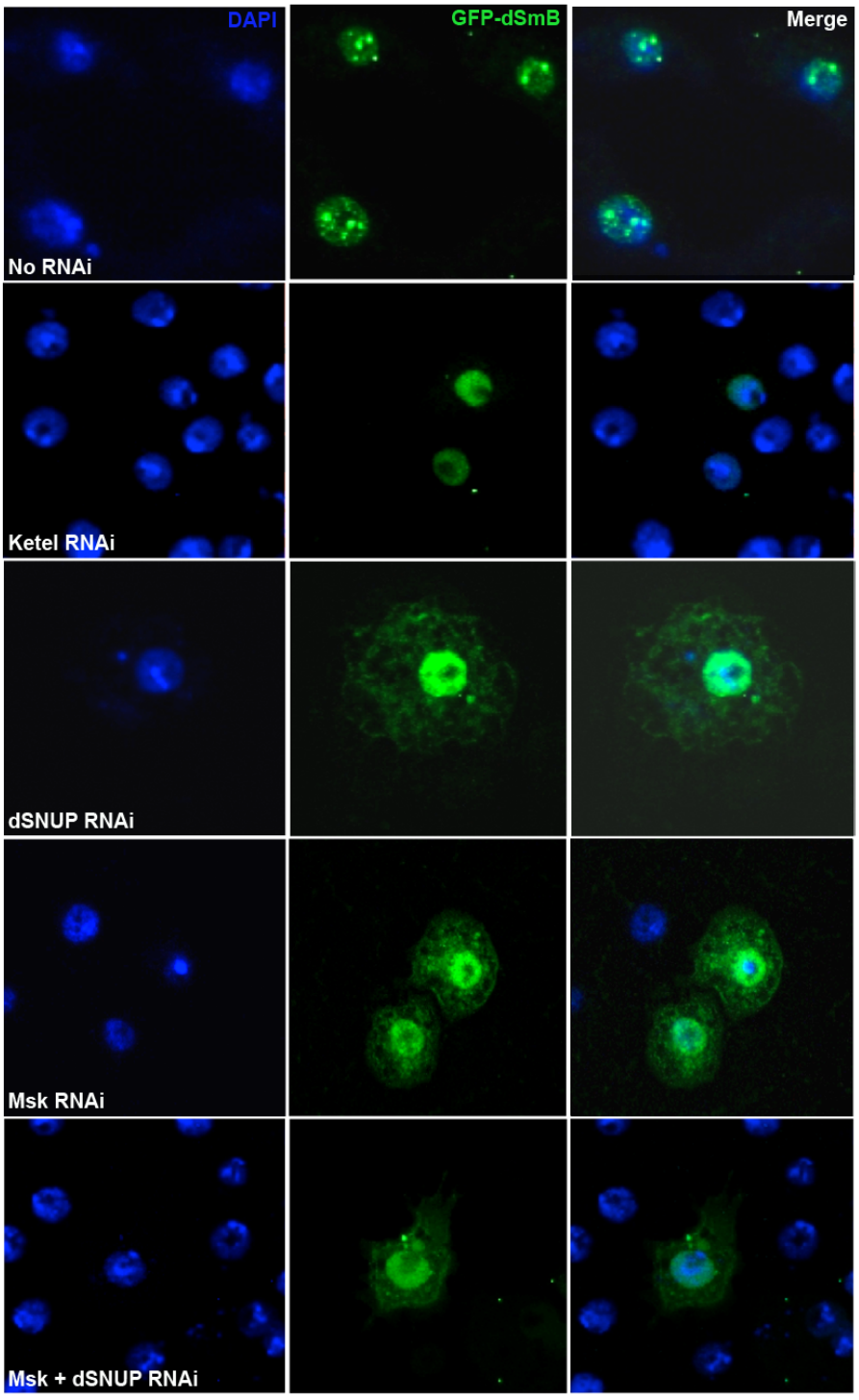


Figure 3.4. GFP-dSmB accumulates in the cytoplasm of dSNUP and/or Msk RNAi treated S2 cells. Transfected GFP-dSmB is nucleoplasmic in control S2 cells (no RNAi) and cells treated with dsRNA targeting Ketel/Impβ. GFP-dSmB accumulates in the cytoplasm of S2 cells upon treatment with dsRNAs targeting dSNUP and/or Msk.

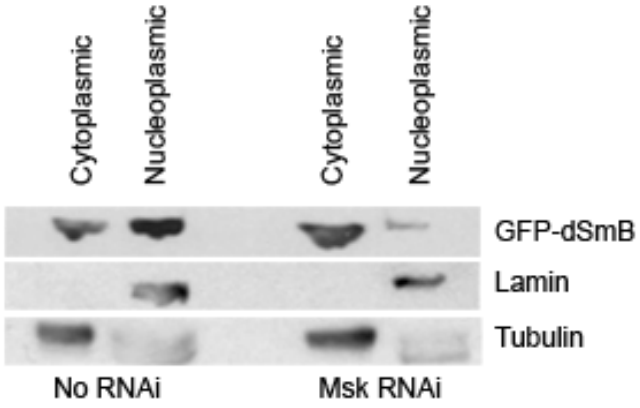


Figure 3.5. S2 cell fractionation following Msk RNAi shows cytoplasmic GFP-dSmB accumulation. Western blot. Transfected GFP-dSmB was predominantly nucleoplasmic in control S2 cells (no RNAi), while GFP-dSmB accumulated in the cytoplasm of S2 cells upon treatment with dsRNA targeting Msk.

DISCUSSION

Previous results from Chapter II strongly favor the interpretation that *Drosophila* snRNP import utilizes the import receptor Msk/Imp7 in place of Ketel/Impβ. Not only is the physical interaction between Imp7/Msk and SPN/dSNUP conserved in both humans and *Drosophila*, but the SPN residues mediating this interaction are very likely conserved from *Drosophila* to human since Msk can interact with hSPN (Figure 3.1B). This result lends further evidence for a previously unrecognized role for Imp7 in vertebrate snRNP import.

I was surprised to find that the interaction of dSNUP with Msk was RNA dependent. This was an unexpected result because my previous work indicated that Msk was a potential snRNP import receptor, so I anticipated that Msk would have similar snRNP binding properties as Impβ, which binds directly to SPN. It is possible that Msk may still bind to dSNUP directly, but that a conformational

change, induced by the binding of dSNUP to the TMG cap, is needed for a stable complex to form and be co-immunoprecipitated.

The RNA independent interaction of Msk with the Sm core protein, dSmB, leaves open some intriguing possibilities. We know that SMN can bind directly to Impβ *in vitro*, and that purified SMN complexes are required for SPN independent snRNP import (Narayanan et al, 2004). However, the precise identity of the import adaptor for the Sm core mediated import pathway is not known. If Msk is found to bind directly to the Sm core, it could be the elusive Sm core import adaptor (Figure 2.10A). Alternatively, Msk may be acting as an autonomous import receptor (Figure 2.10B), but these two scenarios are not mutually exclusive, and this model does not preclude the ability of other importins to provide transport redundancy for snRNP cargo. Future experiments to distinguish between these possibilities will need to be carried out.

Flies may very well have two snRNP NLSs as we see in vertebrates, and so it was not terribly surprising that upon dSNUP knock down, I did not observe an absolute disruption in GFP-dSmB import. It was, however, unforeseen that there was not a more pronounced phenotype from Msk versus dSNUP knock down. I would presume that Msk would be involved in both snRNP import pathways (should they exist in flies) if Msk were wholly replacing Impβ function in flies. Therefore, I had predicted that GFP-dSmB import would be severely affected in the absence of Msk because snRNPs would not have an alternate route into the nucleus. Moreover, dual knock down of dSPN and Msk produced no more robust phenotype than either knock down alone. This could indicate that

Msk is only involved in the TMG cap dependent import pathway, since dSNUP knock down would be expected to only inhibit TMG cap mediated import. This interpretation is contrary to the RNase experimental data, which suggested that the interaction of Msk with dSmB is likely direct since it was not dependent on the RNA.

A major caveat of the experimental design (knock down followed by GFP-dSmB transfection) is the fact that GFP-dSmB is likely imported into the nucleus without being bound to snRNAs. Sm proteins, including SmB, have their own NLSs within their C-terminal tails, which have been shown to be imported in *T. brucei* (Girard et al, 2004). Additionally, SmB is involved in other processes outside of snRNP biogenesis (Gonsalvez et al, 2010), and can be found in non-snRNP complexes. For these reasons, dSmB nuclear import, irrespective of snRNP import, is highly probable. Under this presumption, the GFP-dSmB assembled into snRNPs would be the fraction I observe accumulating in the cytoplasm. The remaining nucleoplasmic signal would be GFP-dSmB that is not associated with snRNPs and is freely transported into the nucleoplasm, unperturbed by snRNP import defects.

Understanding the complex nature of snRNP import mechanisms and the interactions between snRNPs and their transport factors will require precise molecular dissection. In sum, this study has illuminated some of the finer details of Moleskin's interaction with snRNPs, and provides additional evidence supporting a role for Msk in the snRNP import pathway. Nonetheless, additional experiments will be needed to clarify the specific molecular details of the physical

interactions of Msk with snRNPs, and the precise role Msk plays in snRNP import.

MATERIALS AND METHODS

DNA Constructs

dSNUP, dSMN, hSPN, and *dSmB* full-length cDNAs and NLS were PCR amplified with appropriate primers flanked by Gateway recombination sequences (Invitrogen, Carlsbad, CA). These products were recombined initially into pDONR221 (Invitrogen) before entry into green fluorescent protein (GFP)-tagged pAGW or flag-tagged pAFW (*Drosophila* Genome Research Center).

Antibodies

A rabbit polyclonal anti-dSNUP antibody (dSNUP) was generated (Pacific Immunology, Ramona, CA) using GST-tagged dSNUP. A guinea pig polyclonal anti-dSNUP antibody (dSNUP2) was generated (Pocono Rabbit Farm and Laboratory, Canadensis, PA) using dSNUP.

GFP (Roche, Indianapolis, IN; anti-mouse; 1:1000 and Abcam, Cambridge, MA; anti-rabbit, 1:1000), dSNUP (affinity purified anti-rabbit; 1:3000), Msk (gift of L. Perkins; anti-rabbit; 1:2000), lamin (Developmental studies hybridoma bank, Iowa City, Iowa, ADC101, anti-mouse, 1:1000), and tubulin (Sigma-Aldrich, St. Louis, MO; anti-mouse; 1:10,000) antibodies were used for western blotting. Secondary antibodies used were goat -anti-mouse-, -anti-guinea pig-, and -anti-rabbit-conjugated horseradish peroxidase at 1:5000 (Pierce).

dSmB (Y12) anti-sDMA (gift from J. Steitz, Yale, New Haven, CT; anti-mouse; 1 µl), GFP (anti-mouse; Roche; 1.5 µl), and dSNUP (anti-guinea pig; 10 µl) antibodies, and flag conjugated agarose beads (Sigma-Aldrich, St. Louis, MO; 15 µl per IP) were used for immunoprecipitation in buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT).

Fractionation and Immunoprecipitation

S2 cell cytoplasmic lysate was prepared by resuspending cells in 5X pellet volume of buffer A. Resuspended cells were incubated on ice 30 min. to allow swelling, mixed 10X with a p200 pipette, and incubated an additional 10 min. on ice before passing through a 27.5 gauge needle 40X. Cells were spun 1 min. 13,000 rpm in microfuge, and the cytoplasmic supernatant treated with protease inhibitor cocktail (Pierce). For RNase experiments, S2 cell cytoplasmic lysate was divided into equal fractions, which were untreated or treated with 1 ug RNase per 5 µg lysate for 1 h. at 37°C. Cytoplasmic fractions were incubated with antibody 2 h. at 4°C before being incubated over night at 4°C with 15 ul protein A beads (Pierce). Bound proteins were washed 5X with 1 mL buffer A.

The nuclear fraction was purified from the pellet remaining after cytoplasmic fractionation. The nuclear pellet was washed several times with phosphate-buffered saline (PBS: 135 mM NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2). The pellet was resuspended in ½ the cell volume of low salt buffer C (20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM NaCl, 0.5 mM DTT) and homogenized with stir bar at slow speeds while slowly adding the same volume of high salt buffer C (20 mM Hepes pH 7.9,

20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The cells were homogenized for 30 min. at 4°C, spun for 5 min. at max speed, and the nuclear fraction harvested from the supernatant.

RNAi

dSNUP and *Msk* dsRNAs were transcribed *in vitro* from PCR products flanked with T7 promoters. *Drosophila* S2 cells were placed in SF-900 media and treated with fresh 14 µg/ml double-strand RNA (dsRNA) each day for 4 days before harvesting. Cells were transfected with GFP-dSmB using Cellfectin as directed (Invitrogen) on day 2. Cytoplasmic extracts were generated 4 d. after transfection. 50 µg of cytoplasmic extract was loaded on a gel for western blotting analysis to confirm knock down.

Immunofluorescence

Drosophila S2 cells were fixed at room temperature for 10 min. in 3.7% paraformaldehyde in PBS. The cells were then washed with PBST (PBS with 0.1% Triton 100x), stained with DAPI, washed with PBST and mounted in antifade solution (0.233 g DABCO, 800 µl water, 200 µl 1 M Tris-HCl pH 8.0, 9 ml glycerol). Images were taken with a 40X (numerical aperture [N.A.] 1.25) plan apochromatic objective on a laser-scanning confocal microscope (SP5; Leica, Exton, PA). Contrast and relative intensities of the green (Alexa 488 or Venus tag), red (Alexa 594), and blue (DAPI) images were adjusted with Photoshop (Adobe Systems, Mountain View).

CHAPTER IV

Discussion and Future Directions

Vertebrate Imp7 and Impβ form an abundant heterodimeric complex, and it was assumed that the interaction of Imp7 with hSPN in HeLa cells (Paraskeva et al, 1999) was merely the interaction of Imp7 with Impβ. On the contrary, my work has shown that the interaction of Imp7/Msk with SPN is conserved in *Drosophila*, suggesting that the interaction seen in humans was not a consequence of Imp7 interacting with Impβ, but with snRNPs. Not only does Msk interact with snRNPs in flies, but there is also evidence that the interaction of Imp7/Msk with SPN and its localization to CBs (Ospina et al, 2005a) are conserved in humans. Most significant of all is the reduction in CB number and dSMN protein in Msk mutant larvae. In sum, these results suggest that the functional significance of Imp7/Msk in snRNP biogenesis may also be conserved.

Addressing the complicated nature of snRNP import pathways

An increasing number of transport receptors are being identified with multifaceted cargo specificities. A combination of different transport factors under certain conditions, in particular cell types, or carrying different U snRNP cargos could provide an explanation for the seemingly complicated snRNP import field.

We know that particular U snRNPs and tissues possess different import requirements. If Imp7 is serving as a partially redundant import receptor, it could explain some of the conflicting snRNP import requirements observed in the literature. Imp7 may have different binding capacities than Impβ in particular tissues or for individual U snRNPs. snRNA structural and length differences could account for the observed disparities in TMG cap dependence, and could also allow for a more diverse range of affinities for various import receptors. This section will discuss these possible scenarios in detail.

Msk/Imp7 import adaptor versus import receptor

Considering the results from my work along with the conservation of Imp7/Msk interaction with SPN suggests that the function of this interaction may be conserved. Since Imp7/Msk has been shown to function as an import receptor/adaptor, it is likely that it serves a similar import role in snRNP biogenesis (Jakel et al, 1999); (Freedman & Yamamoto, 2004). Previous results in our lab show that although SMN can bind directly to Impβ *in vitro*, the SMN complex is required for SPN independent snRNP import. It has not been determined whether the Impβ binding site of SMN is masked while the protein is in the SMN import complex. Several possibilities thus exist *in vivo*: Impβ may bind an unidentified adaptor protein (Imp7/Msk), bind directly to SMN, or bind both simultaneously. I have some preliminary evidence that suggests that the former case is probable.

During the course of experiments conducted to determine if snRNP components bind to Ketel/Impβ, I found that although Ketel does not interact with

snRNPs, it does interact with dSMN. In co-immunoprecipitation assays, I discovered that flag-dSMN was able to interact with GFP-Ketel (Figure A.5C). The fact that Ketel can bind to dSMN, but not to the snRNA or other known snRNP components, suggests that the IBB of dSMN is sequestered when it is bound to snRNPs. If the SMN IBB is conserved between fly and human, this would indicate that the IBB of SMN is not accessible when it is bound to snRNPs. Moreover, the region of SMN that is required for Impβ binding is the same region that binds Sm proteins, adding uncertainty to the hypothesis that SMN is serving as the Sm core import adaptor (Narayanan et al, 2004). Taken together, these findings suggest that there must be some other factor mediating the interaction of Impβ with the Sm core NLS in flies, and possibly in humans.

I had envisioned two models in humans by which Imp7 could function in the nuclear import cycle of snRNPs in vertebrates. Imp7 and Impβ could have partially redundant functions, each independently functioning as import receptors in single snRNP import events (Figure 2.10A). Alternatively, Imp7 could serve as the Sm-core import adaptor for Impβ (Figure 2.10B), but these models are not mutually exclusive. I have several lines of evidence in support of the latter scenario.

Traditional import receptors are immediately bound by RanGTP/Xpo1 and exported to the cytoplasm upon cargo delivery to the nucleus, but I unexpectedly discovered enrichment of Msk/Imp7 in the CBs of both human and fly cells. Although import receptors would be predicted to be immediately recycled upon nuclear import, there is evidence that this is not always the case for import

adaptors. As discussed in Chapter II, Imp7 is predicted to act in a chaperonin-like fashion inside the nucleus, carrying histone H1 to the chromatin for assembly (Jakel et al., 1999). This hypothesis is supported by the fact that Imp7 has a much lower affinity for RanGTP than Impβ (~30 fold lower); (Gorlich et al, 1997). It is important to point out that Imp7 serves as an import adaptor for histone H1, not a receptor.

There is evidence that SPN may be another example of an import adaptor that is not immediately recycled back into the cytoplasm. Previous work from our lab has shown that a mutant SPN (25-27A) that has reduced Xpo1 binding is enriched in CBs following U2 snRNP import in HeLa cells (Ospina et al, 2005a). Although wild type SPN in HeLa cells was not detected in CBs in that assay, the interaction may be too transient to detect because wild type SPN was found to localize to CBs upon leptomycin B (LMB) treatment, which would block the ability of SPN to be exported. It is important to note that only snRNP bound SPN was targeted to CBs, as TMG cap binding mutants were not targeted to CBs. These findings in addition to my discovery that wild type dSNUP is enriched in the CBs of flies, suggests that there is a functional conservation for the association of snRNP bound SPN with CBs.

I also found Imp7/Msk enriched in CBs similar to SPN. This is additional evidence that Imp7/Msk likely binds snRNPs and accompanies snRNPs to CBs, since we know that only snRNP bound SPN can target CBs. Likewise, this finding may suggest that Imp7/Msk could serve as an import adaptor for snRNPs since only adaptors seem to have the capability of resisting immediate export

upon nuclear entry. Further evidence supporting this hypothesis is the RNA independent nature of the interaction of Msk with dSmB, which could mean that Imp7/Msk binds directly to the Sm core. Imp7/Msk may actually be the unidentified Sm core import adaptor in humans. Taking these results together, Msk/Imp7 could serve as an Sm-core import adaptor in both flies and vertebrates. If this hypothesis holds true, and Msk/Imp7 is serving as an import adaptor for the Sm core import pathway, Msk/Imp7 could still function as an autonomous import receptor for the SPN/TMG-cap mediated pathway since it is known to function in either role for other cargos.

RanGTP and snRNP import

Classical NLS cargo import, utilizing Impα/Impβ, is energy and Ran dependent, requiring RanGTP to release Impβ and cargo from the nuclear side of the NPC. Other Impβ import adaptors such as transportin and SPN are able to mediate cargo import in an energy independent manner, and SPN can be imported in the absence of Ran (Huber et al, 2002). Moreover, the transport kinetics of NPC docking to nuclear appearance of SPN-mediated snRNP cargo is faster than Impα mediated transport (Rollenhagen et al, 2003). The differences in adaptor binding could account for the differential Ran requirement for cargo import. Crystal structures show that Impβ is in an open conformation when bound to the snRNP import adaptor SPN, similar to the conformation of Impβ bound to RanGTP. It is thought that by mimicking this conformation, SNP/Impβ does not require RanGTP for release from the nuclear basket of the NPC, but RanGTP is required for the release of the U snRNP from Impβ (Wohlwend et al, 2007).

Therefore, single rounds of snRNP import mediated by SNP/Impβ in HeLa cell *in vitro* import assays would be independent of RanGTP.

While SPN/TMG cap mediated import is Ran and energy independent in somatic cells, the Sm core mediated pathway is Ran and energy dependent (Huber et al, 2002). Like Impβ, the energy dependence of Imp7 mediated import can vary depending on several factors. Imp7 shares a sequence motif similar to the Ran-binding site of Impβ, and is able to bind specifically to the GTP bound form of Ran, albeit at lower affinity than Impβ, but once bound is very stable (Gorlich & Kutay, 1999). Imp7 is known to function in two modes. As an independent import receptor, Imp7 requires RanGTP binding, but when acting as an adaptor with Impβ, RanGTP binding to Imp7 is not required for import (RanGTP must bind Impβ for import to occur via the Imp7/Impβ heterodimer); (Jakel et al, 1999). Therefore, it is reasonable to assume that if Imp7/Msk is acting as an autonomous snRNP import receptor, it should also require RanGTP.

In vitro snRNP import assays supporting the necessity of Impβ for the Sm-core import pathway did not use RanGTP, only ATP was added to the system (Narayanan et al, 2004); (Ospina et al, 2005a). In the absence of exogenous RanGTP, it would only appear that snRNP import requires Impβ if snRNP import via Imp7/Msk is RanGTP dependent. It is possible that Imp7 may serve as an Sm-core import receptor, and Impβ may not be required for snRNP import in somatic cells since RanGTP would be readily available *in vivo*. These HeLa *in vitro* import assays should be repeated with the addition of RanGTP and Imp7, rather than Impβ, to address whether Imp7 can function as an autonomous

snRNP import receptor. I would predict that RanGTP will be necessary for snRNP import to occur independently of Impβ, but these experiments have not been done in somatic cells. Alternatively, Imp7/Msk may be serving as an adaptor for the Sm-core NLS, so import could still require Impβ.

Mutation of the RanGTP binding site of Imp7 (K61D) lowers its affinity to RanGTP by 70-fold, but does not interfere with its ability to bind Impβ (Jakel et al, 1999). Whereas Imp7 K61D cannot support import of ribosomal proteins, since Imp7 acts as an autonomous ribosomal protein receptor, it can import histone H1 as an adaptor. Moreover, RanGTP binding is dispensable for the H1 import adaptor, Imp7, but not the receptor, Impβ. Taking these findings into consideration, if Imp7/Msk is acting as an adaptor for the Sm-core NLS, it may not require RanGTP to import snRNPs, but likely requires Impβ. Fortunately, this crucial RanGTP binding residue (K61) is conserved in flies, so we could easily test the RanGTP dependence of Msk mediated snRNP import *in vivo*. My work has shown that the snRNP specific phenotypes observed in Msk mutants can be rescued with UAS-Msk (Figure 2.7-9), but we could test whether UAS-Msk K61D is also able to rescue. If Msk were merely a snRNP import adaptor in flies, we would expect full rescue of snRNP import since I would not predict RanGTP binding to be essential for import. If rescue is not seen, Msk is likely an import receptor (does not completely negate adaptor role), but there could be issues doing this experiment *in vivo*.

In vitro import assays, from which we have gained most of our knowledge about not only snRNP import, but also Imp7 mediated import, really only address

one round of cargo import. *In vivo* rescue would require the import receptor to be recycled for additional rounds of import. The recycling of Msk K61D back to the cytoplasm would be significantly reduced since it has a 70-fold reduction in RanGTP affinity. Once Msk K61D transports one snRNP into the nucleoplasm, it would be unable to facilitate additional snRNP import events, and hence could inhibit full rescue of the observed snRNP related phenotypes. Although *in vivo* evidence would be preferred, *in vitro* import assays may be our only option to avoid such complications and fully understand the RanGTP dependence of Imp7/Msk snRNP import.

Implications of unintended co-depletions

There is an additional issue that needs to be addressed with regard to previous *in vitro* import assays utilizing depleted lysates. Work from our lab has shown that the in the absence of SPN, snRNPs can be imported via the SMN complex and Impβ in the presence of RanGTP (Narayanan et al, 2004). Although the import of U1 snRNPs in digitonin-permeabilized cells is SMN dependent, this requirement can be overcome with the addition of SPN and Impβ in 5 fold molar excess over U1 snRNPs (Narayanan et al, 2004). These results suggest that SMN is required for the TMG cap independent pathway only (*in vitro)*. Addition of SMN alone from SMN depleted lysates was unable to rescue import, suggesting that there are additional import factors that are co-depleted with SMN. The SMN complex, which is purified with an anti-SMN antibody, is needed to restore U1 snRNP import from SMN depleted lysates. Although the SMN complex failed to contain SPN or Impβ (Narayanan et al, 2004), it is possible that the SMN

complexes used contain Imp7.

Lysates depleted of SMN (using anti-SMN) could have co-depleted Imp7 if the interactions I have discovered in flies are conserved in humans. Moreover, Msk mutants have significantly reduced levels of SMN, suggesting that Msk and SMN protein levels are tightly linked, so depletion of SMN could affect the abundance of Msk also. This potential co-depletion could interfere with the proper interpretation of *in vitro* import assay results. It would be pertinent to identify the Imp7 sized band that co-purifies with the SMN complex since these anti-SMN purified SMN complexes have been used in nearly all of the *in vitro* import experiments to date (Baccon et al, 2002); (Pellizzoni et al, 2002a); (Yong et al, 2004); (Carissimi et al, 2005); (Carissimi et al, 2006a); (Carissimi et al, 2006b). It is highly likely that Imp7 is depleted with SMN if this band is proven to be Imp7, and this would have serious implications for our interpretation of previous experimental results.

There could be similar problems with snRNP import experiments employing Impβ depleted lysates. It is plausible that Impβ depletion using anti-Impβ could co-deplete Imp7 since we know that these two import receptors form an abundant heterodimeric complex (Jakel et al, 1999). Taken together, if Imp7 is sufficiently co-depleted with either SMN or Impβ we will need to re-evaluate all of the previous conclusions drawn from such assays. Future experiments should pay close attention to possible co-depletion issues and address them accordingly. In particular, we should be certain that Imp7 is not adversely affected in these assays if we are to truly believe Impβ is the sole vertebrate

snRNP import receptor.

Tissue specific snRNP import requirements

In contrast to somatic cells, one must be reminded that both the TMG cap and RanGTP are required for *in vitro* snRNP import in *Xenopus* oocytes. Additionally, either Impβ depletion or over expression of the Impβ binding domain of Impα were shown to block U snRNP import both *in vivo* and *in vitro* when using *Xenopus* egg extract (Palacios et al, 1997). These findings provide strong evidence that Impβ is necessary for snRNP import in the germline, but this does not negate the possibility that Imp7/Msk could play a significant role in somatic cell snRNP import. In particular, Imp7/Msk may play a pivotal role in the Sm core mediated import pathway or may require the bipartite U snRNP nuclear import signal. We know that SPN only requires the TMG cap to bind to the snRNA, but SPN also makes contacts with Sm proteins and the stem-loop of U1 snRNA (Kuhn-Holsken et al, 2010), and Sm protein assembly onto the snRNA is likely required for TMG cap formation and subsequent SPN mediated import (Mattaj et al, 1993);(Luhrmann, 1990). Since SPN makes several protein contacts, Imp7/Msk and/or Impβ may also require more than one interacting partner to either bind to or to stabilize snRNP interactions to facilitate efficient snRNP import in *Xenopus* oocytes (Figure 4.1).

Figure 4.1. Model of U snRNP import

When *Xenopus* egg extract is supplied to somatic cells, which do not require the TMG cap for U snRNP import, U1 and U2 import becomes TMG cap dependent, suggesting that unidentified soluble cytosolic factors mediate the TMG cap dependence of U1 and U2 import (Marshallsay & Luhrmann, 1994). Imp7/Msk may be one of these contributing factors since unidentified cytosolic factors are predicted to account for the tissue specific differences. I would argue that Impβ may not be necessary (Imp7 may be sufficient) for the TMG cap independent import pathway *in vivo*, however this pathway does not seem to function autonomously in the germline, since the TMG cap is required for import in *Xenopus* oocytes (Palacios et al, 1997); (Marshallsay & Luhrmann, 1994). There is much evidence that suggests that the Sm core is the essential snRNP NLS (Marshallsay & Luhrmann, 1994), while the TMG cap merely increases the efficiency of snRNP import. If the efficiency of the Sm core NLS is so low that import is undetectable, it could help to explain the discrepancies in TMG cap requirements.

The assembly of the Sm core is mediated by the SMN complex, and regardless of which mechanism, snRNP import is thought to be dependent on the SMN complex *in vivo* (Narayanan et al, 2004). Although SMN complex members are fairly well established, it is possible that one or more proteins of the cytoplasmic SMN complex are different in somatic versus germline tissues. We already know of one SMN complex member that varies depending on the cellular compartment (Unrip is a cytoplasmic specific member), and specific U snRNPs contain unique proteins, so it is not inconceivable to imagine SMN complex variations based on cell type. These differences, if they are found, could help to explain the discrepancies observed in various snRNP import assays. It would be interesting to do a more thorough investigation of SMN complex members in various tissues/cell, notably HeLa cells and *Xenopus* oocytes. Abundant proteins like Imp7/Msk may stick to beads alone, and I have experienced such problems, so there could very well be SMN complex members we have not identified because they were thought to be “non-specific” binding partners, like Imp7/Msk or some other component required for Imp7/Msk binding.

The differences seen in U1 and U2 versus U4 and U5 snRNP import TMG cap dependence could be partially accounted for if Imp7/Msk is capable of binding to only a select few U snRNPs, but not all. The differential TMG cap requirement for U1 and U5 snRNP import could be partially explained by stem-loop structures present in U1, but not other U snRNAs, which could dictate import factor binding (Jarmolowski & Mattaj, 1993). However, my results reveal that Msk, like dSPN, is capable of binding U1, U2, U4, and U5 snRNAs from S2 cells,

suggesting that it plays a role in all U snRNP biogenesis pathways. This finding does not rule out the possibility that Imp7 serves a tissue specific function in vertebrate snRNP import. We know that the TMG cap dependence observed in *Xenopus* oocyte snRNP import is cell type specific (Fischer et al, 1994), so Imp7 may only interact with U snRNAs in specific tissue/cell types.

Redundancies in snRNP import

The existence of multiple snRNP import pathways can be compared to the known import pathways of ribosomal proteins. Ribosomal proteins have a NLS consisting of several basic amino acids that can be recognized by four different transporters (Jakel & Gorlich, 1998), and this redundancy is indicative of the importance of ribosomal import to cellular processes. Ribosomal proteins must be imported into the nucleus so that they can be assembled into ribosomal subunits within the nucleolus and so they rely on redundant nuclear import mechanisms to ensure their functionality. In mammals, the same set of import receptors are responsible for both ribosomal and histone core proteins (Jakel & Gorlich, 1998). Two separate pathways import the core histones and linker histones. Linker histones, like histone H1, require a heterodimer consisting of Impβ and Imp7, while core histone import has redundant import receptors from the Impβ superfamily. Similar to the hypothesis that snRNA structural differences may account for various snRNP import requirements, mechanistic import differences are speculated to be due to structural differences between the two main histone classes. The snRNP import pathway also has redundancies built into the system since snRNP import is essential for splicing, and thus for viability

of the cell, so it would not be surprising to find several snRNP import receptors. My work has shown that in flies Msk binds to U1, U2, U4, and U5 snRNAs (Figure 2.5C and data not shown), suggesting that Imp7/Msk plays a role in all snRNP import events. This role could vary depending on the particular U snRNP (e.g. adaptor versus receptor).

Imp7 could also play a redundant or facilitating role for SMN directed snRNP import by binding to the Sm core NLS. *In vivo* experiments carried out by Girard et. al. (2006) did not detect cytoplasmic snRNP accumulation in SMN-depleted HeLa cells via RNAi. This result could be interpreted in several ways. The snRNA may be unable to associate with the Sm core in the absence of SMN since the SMN complex is known to facilitate Sm core assembly. The snRNA would likely be rapidly degraded in the cytoplasm if left unassembled, and thus you would not detect cytoplasmic snRNAs. This hypothesis is supported by the fact that depletion of Sm proteins in yeast inhibits Sm core assembly and leads to U snRNA degradation (Bordonne & Tarassov, 1996). An alternative explanation as to why snRNPs did not accumulate in the absence of SMN could be that snRNPs are imported into the nucleus by multiple pathways *in vivo,* which may not require SMN and have yet to be identified.

The Sm core proteins contain an NLS represented by a basic rich protuberance, which could theoretically be recognized by other import receptors (e.g. Imp7/Msk) in the absence of SMN. SmD1 and SmD3 additionally have lysine and arginine stretches in their C-terminal tails that show similarities to nuclear import signals for ribosomal proteins (Girard et al, 2004), and ribosomal

proteins are known to be Imp7 import substrates (Jakel & Gorlich, 1998). If Imp7/Msk is present in the system and serves as a functional Sm core import factor, SMN may not be needed for import to occur. RNAi of both SMN and Imp7 may show a more prominent import defect, but it is likely that *in vitro* import assays will need to be employed to determine if Imp7 is playing a role in SMN independent import. It is important to point out that regardless of SMN's potential role in snRNP import, it also plays an important role in snRNP assembly. Phenotypes seen in the absence of SMN may not be directly related to import defects, and this should be kept in mind when conducting such experiments and drawing conclusions.

Imp7/Msk and Cajal bodies

Previous work from Paraskeva et al. (1999) showed that the interaction of Imp7 with SPN is sensitive to RanQ69L (prevents GTP hydrolysis), suggesting that this interaction is indeed RanGTP sensitive. For this reason, cytoplasmic lysates were used for all of the import factor interaction studies in this thesis in order to prevent premature import complex disassembly from high concentrations of nuclear RanGTP. One of the most surprising findings from my work was the discovery that Imp7/Msk was found in the CBs of both vertebrates and flies, indicating that the interaction of Imp7/Msk with snRNPs is inherently less sensitive to RanGTP than typical import receptors (e.g. Impβ). These Imp7/Msk-snRNP interactions may even be detectable in whole cell or nuclear lysates since Imp7/Msk can be found in nuclear subdomains.

This unexpected import factor CB accumulation may be indicative of a novel function for Msk/Imp7. Although there is evidence that Imp7 binds SPN in a RanGTP sensitive manner (Paraskeva et al., 1999), I had not expected to see Imp7 in a nuclear body since traditional import receptors are known to immediately bind RanGTP in the nucleoplasm to expedite their transport back into the cytoplasm. This finding could be attributed to Imp7's ~30-fold reduced RanGTP binding affinity compared to Impβ. This lower affinity could delay the dissociation of Imp7/Msk from its snRNP cargo long enough to allow for chaperoning of snRNPs to CBs. I also found a disruption of CBs in the Msk mutant, which is in agreement with the hypothesis that Msk potentially directs snRNPs to CBs, thus serving as a "nuclear chaperone."

Nuclear chaperons are known to bind to correctly folded protein subunits to facilitate macromolecular interactions, particularly between proteins and nucleic acids. The assembly of chromatin from DNA and histones is perhaps the best studied nuclear chaperone mechanism, and Imp7 has already been implicated in mediating the interaction of histone H1 with chromatin (Philpott et al, 2000); (Jakel et al, 1999). Some of the basic principals learned from nuclear chaperones involved in DNA-protein interactions will likely apply to the assembly of nuclear RNPs. Nevertheless, the fact that Imp7/Msk appears to serve a nuclear role in relation to U snRNPs is surprising because snRNPs are thought to be nearly fully assembled upon reaching the nucleoplasm. What role can Imp7/Msk be playing then? Imp7/Msk could facilitate the interaction of snRNPs

with nuclear specific protein components, with CB components, like coilin, or possibly other snRNPs, mediating RNP-RNP interactions.

Coilin is the primary marker of CBs, but studies from coilin knockout mice show that 'residual' nuclear bodies form in the absence of coilin. These 'residual' nuclear bodies contain the numerous machineries typically found in CBs. Coilin is thought to be needed to organize these various RNP assembly factors into a single entity, and is hypothesized to be the 'glue' that holds CBs together (Jady et al, 2003); (Tucker et al, 2001). Besides coilin, ongoing transcription has been shown to play a decisive role in the initiation of CB formation, although the precise mechanism of CB assembly is up for debate. It is possible that the direct interaction of coilin with SMN or some other member of the SMN complex (e.g. Imp7) may provide a targeting signal for newly imported snRNPs to CBs (Tucker and Matera, 2005). Regardless of how snRNPs reach the CB, transport factors (e.g. SPN, Imp7/Msk) must be displaced to participate in additional rounds of snRNP import, and Xpo1 likely plays an active role in this process. Xpo1 is also found to accumulate in CBs (Ospina, 2005b), so it is highly likely that this disassembly takes place at the CB particularly for transport factors that remain bound to snRNPs after nuclear entry. If Xpo1 in the CB displaces Imp7/Msk from snRNPs, it would not be entirely unexpected to find Imp7/Msk there. It will be interesting to determine if Imp7/Msk plays a definitive role in CB formation or if Imp7/Msk CB localization is merely a consequence of an Xpo1 binding requirement that takes place at the CB.

Msk could be found in every coilin positive nuclear body in flies (Figure 2.6), but this was not true in HeLa cells (Figure 2.S1). Only a subset of the SMN positive nuclear bodies contained Imp7. Unlike coilin, which also marks histone locus bodies (HLBs), SMN is a distinct marker of CBs. Why do I see this Imp7/Msk in all fly CBs, but not all human CBs? Imp7 may be in all CBs, but the poor immunofluorescence capabilities of the Imp7 antibody used in the HeLa cell experiment could interfere with my ability to detect it. Alternatively, Imp7 may only accompany particular U snRNPs to the vertebrate CB. There is evidence that SPN targets CBs following U2 but not U1 snRNP import (Ospina et al, 2005a), so this could indicate that Imp7 is serving as an adaptor (like SPN) when it is found in CBs. This interpretation would be flawed if SPN truly targets CBs after both U2 and U1 import, but was not detected in the assay used. In contrast to other snRNAs, U1 is not as abundant in CBs (Ospina et al, 2005a), so U1 may bypass the CB more often than U2 or it may have a more transient interaction with CB components. This could explain why SPN did not appear to target CBs following U1 import. No matter which scenario holds true, it fails to fully explain why Imp7 is found in only a subset of HeLa cell CBs. A possible explanation may be found if we discover different subspecies of CBs that contain distinguishing proteins or U snRNPs that could dictate whether transport factors are found there. Much work will need to be done on CB assembly and dynamics before we will be able to explain these findings.

Determining Msk's direct snRNP binding partners

I found that the interaction of not only dSNUP, but also dSMN with Msk is RNA dependent. In contrast, the interaction of dSmB with Msk is RNA independent (Figure 3.2). This suggests that dSNUP might be interacting with Msk through the Sm proteins bound to RNA. Alternatively, dSNUP may still be interacting directly with Msk in an RNA dependent manner, such that, a conformational change in dSNUP subsequent to snRNA binding may enable it to bind Msk.

To distinguish between these two possibilities, one could test the interaction of Msk with bacterially expressed and purified GST-dSNUP in a TMG cap snRNA bound or unbound form. If the interaction of Msk with snRNPs is through the Sm core or some other snRNP component other than dSNUP, Msk should not interact with purified dSNUP in a TMG cap snRNA bound or unbound form. Msk should interact with purified dSNUP in a TMG cap snRNA bound form, but not unbound form if the direct interaction of Msk with dSNUP is merely stabilized by the snRNA. A negative result from an *in vitro* binding assay would be inconclusive since the direct interaction may be dependent on snRNP assembly to ensure the import of only the assembled snRNPs.

Regardless of the direct binding nature of Msk and dSNUP, dSNUP may be required for Msk binding to snRNPs. One could test whether dSMN and Sm proteins still interact with Msk in the absence of dSNUP by knocking down dSNUP in S2 cells with RNAi. I would not expect to see an effect on dSmB binding since I have been able to show that dSmB and Msk interact in an RNA

independent manner (Figure 3.2), in which case, dSNUP is likely to be released from the snRNP upon RNA degradation.

It would also be of interest to investigate other snRNP proteins to determine whether Msk can directly bind to them. By purifying each snRNP component and doing *in vitro* binding assays, in the absence of RNA, one could determine which proteins are able to bind directly to Msk. The absence of a necessary cytoplasmic factor that helps stabilize the interaction of Sm proteins with Msk could interfere with such experiments; so one must keep in mind that a negative result may not accurately reflect *in vivo* binding activity. Another thing to consider when doing pull down assays with bacterially expressed proteins is the fact that they will not be methylated. Although we know that Sm protein methylation is not required for snRNP biogenesis in flies (Gonsalvez et al, 2006), it could potentially affect the binding affinity of Msk. It would be interesting to determine if the interaction of Msk with dSmB is methylation dependent, since we still do not fully understand the reasons for Sm protein methylation.

Spinal Muscular Atrophy (SMA)

Deletions or mutations of the SMN can lead to a devastating and lethal, neuromuscular disease, Spinal Muscular Atrophy (Lefebvre et al, 1995). SMA is an autosomal recessive, neurodegenerative disease that specifically targets motor neurons (Ogino & Wilson, 2004). Although the exact disease pathology of SMA is unknown, *in vitro* studies on SMA mouse models revealed reductions in snRNP biogenesis capabilities (Gabanella et al, 2007), and loss of SMN nuclear foci correlates with the disease phenotype (Coovert et al, 1997); (Lefebvre et al,

1997). Intriguingly, several SMA patient derived SMN mutants show import defects (Narayanan et al, 2004), indicating that snRNP import could play a role in SMA disease pathology.

A missense mutation, E134K, causes the most severe form of SMA. E134K sits in a region responsible for binding Sm proteins (Buhler et al, 1999); (Selenko et al, 2001), but this point mutation does not appear to affect Sm protein binding. It does however severely impair its ability to bind Impβ (Buhler et al., 1999) and shows SMN import defects. *In* vitro import assays have shown that SMN import requires the presence of Sm snRNPs and that labeled U1 snRNP import requires the SMN complex (Narayanan et al, 2004). Taken together, we know that SMN and U snRNP import are coupled, that U snRNP biogenesis correlates with SMA at the molecular level (Paushkin et al, 2002); (Meister et al, 2002), and that snRNPs must be imported. Therefore, it is possible that snRNP import defects could be implicated in SMA pathology, and thus, it is important that we fully understand snRNP import mechanisms and regulation.

Previous work in our lab has shown that the Sm core pathway requires the entire SMN complex, so it is possible that more than one complex member functions as the import adaptor, or that it may be an unidentified member (e.g. Imp7). An important line of investigation will be to identify the actual import adaptor(s) and identify which members, whether known or novel, are needed for Sm-core mediated snRNP import. *In vitro* import assays using the digitonin-permeablized HeLa cell system will likely be the best way to dissect the pathway and determine which factors are necessary and/or sufficient for snRNP import.

We can add bacterially expressed and purified components to the system to determine exactly what proteins are needed for import of Cy3-labelled snRNPs and compare the results to the snRNP import using the purified SMN complex. Importantly, one should pay close attention to RanGTP in the system, as this could be a major determinant in the functionality of various import factors. It will be interesting to ascertain whether other SMA patient mutations are snRNP import defective, and whether they are dependent on Impβ and can be rescued with Imp7. If Imp7 is found to be a redundant import receptor, these studies could provide crucial insight into the disease pathology of SMA.

snRNP import and disease

One would predict that a mutation that disrupts a cell essential function would have a broad impact on all cell types, but this is not always the case. SMA is a prime example of a disease that is thought to be caused by a disruption to a cell essential function (snRNP biogenesis due to SMN mutations), which gives rise to a motor neuron specific defect. Could Imp7/Msk play a role in the pathology of tissue specific phenotypes, and maybe even SMA? Msk mutants have prominent muscle patterning and detachment phenotypes (Liu & Geisbrecht, 2011), similar to SMN mutant flies (Rajendra et al, 2007). Furthermore, Msk mutant embryos have defects in muscle-tendon cell attachment, and it has been shown that Msk is required in the muscle cell, but not the tendon cell. The muscle attachment defects can be recued by activated MAPK or the secreted epidermal growth factor receptor (Egfr) ligand Vein (Liu & Geisbrecht, 2011). That suggests that Msk signals through Vein-Egfr signaling

pathway for tendon cell differentiation and/or maintenance. Regardless of Msk's role in the Vein-Egfr signaling pathway, it is plausible to imagine Msk/Imp7 playing a role in other tissue specific phenotypes since there are clear examples of such phenotypes in Msk mutants.

We do not fully understand why there are tissue specific phenotypes in diseases like SMA, but we know that mutations in nuclear envelope proteins also give rise to tissue specific pathologies. This is thought to be due to unidentified tissue specific expression patterns that mediate disease pathologies. The nuclear envelope proteome was found to vary greatly between tissues (Korfali et al, 2012), and it is possible that complex tissue specific disease pathologies, like SMA, could be due, in part, to nuclear envelope proteome differences. Differences in the nuclear envelope proteome could have an enormous impact on the ability of certain import receptors to import cargo. Imp7/Msk may have a tissue specific role in snRNP import, and this could help to explain SMA disease pathology since import defects have already been observed in SMA patient derived SMN mutations.

snRNP import regulation

Transport adaptors are known to shuttle continuously between the nucleus and cytoplasm, and so they must be assembled and disassembled from import complexes. We know that RanQ69L, which cannot hydrolyze GTP, destabilizes complexes between either Impβ or Imp7 with SPN (Paraskeva et al, 1999), but snRNP import complex assembly and disassembly are poorly understood. It is hypothesized that an interaction between the N- and C-terminus of SPN might

play an auto-regulatory role (Figure 1.9), but this has not been fully examined. Additional regulation of snRNP biogenesis may be provided by post- translational modifications, which might attenuate inter- and intramolecular interactions. For instance, phosphorylation of SPN or other snRNP components could alter the strength of Imp7 versus Impβ binding. Such modulation may result in a decrease or increase in a molecule's binding affinity, and provide a quick and efficient means of regulating snRNP biogenesis. When designing experiments to uncover such regulatory mechanisms, it will be important to keep in mind that regulatory mechanism may be tightly linked to alterations in snRNP biogenesis demand, so the experimental conditions should be scrutinized.

Evolution of snRNP import mechanisms

Nuclear import is essential in all eukaryotes, and import machineries are required to carry out this conserved function. Despite the conservation of import mechanisms, there are vast differences in the components of the nuclear transport apparatus among eukaryotes. Most pronounced are the difference seen in *Drosophila*. Importins have been frequently lost and gained throughout *Drosophila* evolution, and even relatively recent duplication events can rapidly acquire essential function in *Drosophila*. The import adaptor Impα has been closely examined in *Drosophila,* and Impα homologues are a prime example of such rapid changes in import machineries that maintain their essential function.

Impα lacking an IBB has been shown to readily translocate to the nucleus in the absence of Ran and Impβ in yeast (Miyamoto et al, 2002), suggesting that transport adaptors lacking an IBB may be functional. Similar to this yeast Impα

without an IBB, my research has shown that another import adaptor orthologue, dSNUP, lacks an IBB and yet is nuclear. The inability of dSNUP to bind Ketel/Impβ is likely due to an IBB deletion event unique to arthropods because all other vertebrates and *C. elegans* appear to have an IBB (Table A.1; deletion event is not restricted to *Drosophila*). Despite the deletion of a region thought to be vital for SPN function, dSNUP is expected to be functional since it binds snRNPs readily and can be found in CBs (only snRNP bound SPN targets CBs) (Ospina et al, 2005a).

Imp7/Msk is another import machinery component that appears to have retained functionality. The ability of Imp7/Msk to bind to SPN and localize to CBs is conserved from fly to human (Paraskeva et al, 1999); (Figures 2.5A-B and 2.6). This suggests that the role Imp7/Msk is serving in snRNP biogenesis is important, even if it is found to be a redundant role. It is unlikely that this conservation of function is due to convergent evolution because the nucleotide sequence of human and fly Imp7/Msk are ~57% similar.

Karyopherin-βs (e.g. Imp7, Impβ, etc.) were established early in eukaryote evolution, and the entire Karyopherin-β subfamily likely descended from an ancestral form. Although the ancestral Karyopherin-β remains unknown, Imp7/Msk mediated import is considered to be evolutionarily ancient (O'Reilly et al, 2011), and could conceivably have been the first snRNP import receptor to emerge in evolution. Due to the fact that the IBB in both fly and vertebrate SPN (Narayanan et al, 2002) are not essential for nuclear import, Imp7 might play an important role in Impβ independent SPN and snRNP import.

Imp7 is also known to directly bind to and import ribosomal proteins independently; it shares this characteristic with Impβ, transportin, and RanBP5. All of these proteins recognize a basic region in a ribosomal protein (L23a), which may very well be an archetypal import signal that evolved before import receptors diverged in evolution (Jakel & Gorlich, 1998). The Impα/Impβ heterodimer or Imp7 can import a glucocorticoid receptor NLS fragment as well (Freedman & Yamamoto, 2004). These diverse cargo and import roles indicate that Imp7 may be able to import snRNPs independently of Impβ or Impβ/SPN. Alternatively, Ketel/Impβ may interact with snRNPs through Msk/Imp7, and the interaction may be too weak to detect in flies. Regardless of dSNUP's affinity for Ketel/Impβ, I observed no snRNP defects in Ketel/Impβ mutants. This suggests that Ketel/Impβ does not play a necessary functional role in fruit fly snRNP import. I look forward to future research that examines the underlying complexities of snRNP import that we do not yet understand. It seems that all too often we apply findings from a single experiment, done in one particular cell type, under very specific conditions to our broader understanding of *in vivo* processes that are beyond such limited experimental systems. It is very important that we understand and address such inadequacies, so that we do not misinterpret results, and thus push our understanding backward rather than forward.

Summary and concluding remarks

Fly Snurportin can be viewed as an IBB deleted version of vertebrate SPN. Although the earlier report of Imp7 interacting with SPN (Paraskeva et al, 1999) was essentially ignored, the localization of Imp7 to CBs, and the ability of SPN

ΔIBB to show nuclear localization properties, suggests that Imp7 may serve a role in snRNP import in vertebrates. Moreover, the striking reduction of dSMN protein and disruption to CBs in Imp7/Msk null flies indicates that it could play a more central role in snRNP import than one might first contemplate. Considering Imp7/Msk has never before been implicated as a snRNP import factor, and yet may be a vital component to our understanding of snRNP import mechanisms, points to the substantial gaps in our knowledge that have yet to be filled. It will require much work and attention to detail to fully dissect the intricacies of snRNP biogenesis. This thesis provides a stepping stone to guide future studies pertaining to the function of Imp7/Msk in snRNP biogenesis. The identification of dSNUP is also a first step in the development of an *in vivo* model to study snRNP import in *Drosophila.* Given that we see tissue specific differences in snRNP import requirements, developing an *in vivo* model will hopefully enable experiments that were previously impossible.

APPENDICES

I conducted a number of experiments during my graduate career that I did not have sufficient time to follow up on. Data and preliminary results from these studies are included in these appendices, many of which are further elaborated on in the Chapter IV discussion section. Specific details of reagents generated and utilized in the course of my thesis studies can also be found in this section (e.g. construct sequences, vectors, primers, etc.).

Appendix I

Investigation of Snurportin gene evolution

Question under investigation

Do other insects have a gene fusion between SPN and DNTTIP1, and do they have a SPN IBB? What happens in evolutionarily primitive (before insects) and advanced (after insects) invertebrates and vertebrates? Is this genomic architecture unique to flies, and if not, when do the genes split apart in the evolutionary ladder?

Rationale

dSNUP does not contain an IBB, which is required for SPN/TMG cap mediated snRNP import in vertebrates, so I wanted to know when the IBB may have been lost or gained in evolution. Additionally, *dSnup* has a unique genomic architecture since it appears to also encode a downstream gene, *DNTTIP1*. Therefore, I wanted to know if other species have a bicistronic *Spn* gene.

Materials and Methods

Online databases were used to identify the genomic location of *Spn* and *DNTTIP1* orthologues (FlyBase, WormBase, BeeBase, NCBI). Protein and cDNA sequences were also obtained for each. PROSITE was used to locate predicted IBB domains fpr each SPN orthologue.

Results and Discussion

None of the insect species examined have an identifiable SPN IBB, but unexpectedly *C. elegans* do have an IBB (Table A.1). The IBB of SPN could be ancestral and lost in insects or could have been independently and convergently

recruited in the evolution of SPN in different species. The latter hypothesis implies that an important constraint exists for the use of importin-β as the SPN/TMG cap mediated snRNP import receptor.

Insects are the only species examined that encode the protein coding region of SPN within one exon. In respect to the bicistronic nature of *Snurportin* orthologues, only *Drosophila* has this genomic architecture. This feature is unique to *Drosophila*, and all *Drosophila* species have a bicistronic *Snurportin* gene encoding both SPN and DNTTIP1 orthologues in one transcript (not shown). Additionally, all other species examined, with the exception of *Drosophila* and *A. melifera,* have SPN and DNTTIP1 orthologues encoded on opposite DNA strands. Gene fusions like this one are not entirely uncommon in *Drosophila*, and even *Tgs1*, which hypermethylates the snRNA cap, is a bicistronic gene that also encodes the novel protein DTL (CG31241); (Komonyi et al, 2009). The high frequency of bicistronic *Drosophila* genes may be due to evolutionary pressure to reduce its genome size.

The fact that this gene fusion event is not conserved suggests that it serves no functional purpose in relation to the two genes. Moreover, DNTTIP1 plays no known role in snRNP biogenesis, and does not interact with Sm proteins. While I have shown clear evidence that dSNUP interacts with not only dSmB, but other snRNPs, DNTTIP1 does not pull-down dSmB (data not shown).

Evolution of Dicistronic Nature of *SPN* and *DNTTIP1*

	Orientation		Chromosome			
Species	***SPN***	***DNTTIP1***	***SPN***	***DNTTIP1***	**IBB**	***SPN* Exons**
Homo sapien	minus	plus	15	20	Yes	8
Mus musculus	plus	plus	9	2	Yes	8
Xenopus tropicalis	plus	minus	n/a	n/a	Yes	8
Caenorhabditis elegan	minus	plus	2	3	Yes	6
Anopheles gambiae	plus	minus	2	2	No	1*
Aedes aegypti	plus	minus	n/a	n/a	No	1*
Apis mellifera	plus	plus	n/a	n/a	No	1*
Drosophila melanogaster	plus	plus	3	3	No	1*
*SPN homologous region encoded within 1 exon.						

Table A.1. Snurportin and DNTTIP1 interspecies comparison. Strand orientation (plus/minus) and chromosome origin (chromosome number) for SPN and DNTTIP1 are noted. The presence of an identifiable SPN IBB and the number of exons encoding the SPN homologous protein coding region are also shown (3' and 5' untranslated exons not included).

Appendix II

Characterization of dSNUP antibodies

Question under investigation

Do the dSNUP antibodies made in guinea pig or rabbit work for immunoprecipitation, immunofluorescence, or western blotting?

Rationale

We initially had a dSNUP antibody made in a guinea pig, but previous results from our lab were unclear whether the antibody was able to recognize dSNUP by western (data not shown). I made a dSNUP antibody in rabbit to see if I could produce a better preforming antibody. This appendix details the capabilities of each dSNUP antibody.

Materials and Methods

A rabbit polyclonal anti-dSNUP antibody (dSNUP) was generated (Pacific Immunology, Ramona, CA) using GST-tagged dSNUP. A guinea pig polyclonal anti-dSNUP antibody was generated (Pocono Rabbit Farm and Laboratory, Canadensis, PA) using untagged dSNUP.

dSNUP antibodies (anti-guinea pig; 1:100 or anti-rabbit; 1:2000) were used for western blotting. Lysates were made with RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40, 1 mM EDTA) and then run on a 4-12% polyacrylamide gel (Invitrogen), and subjected to western blotting.

dSNUP antibodies (anti-guinea pig or -rabbit; 10 µl) were used for immunoprecipitation. S2 cell cytoplasmic lysate was prepared by resuspending cells in 5X pellet volume of buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10

mM KCl, 0.5 mM DTT). Resuspended cells were incubated on ice 30 min. to allow swelling, mixed 10X with a p200 pipette, and incubated an additional 10 min. on ice before passing through a 27.5 gauge needle 40X. Cells were spun 1 min. 13,000 rpm in microfuge, and the cytoplasmic supernatant treated with protease inhibitor cocktail (Pierce). S2 cell cytoplasmic fractions were incubated with antibody 2 h. at 4°C before being incubated over night at 4°C with 15 µl protein A beads (Pierce). Bound proteins were washed 5X with 1 mL buffer A.

dSNUP antibodies (anti-guinea pig or -rabbit; 1:200) were used for immunofluorescence. S2 cells were fixed at room temperature for 10 min. in 3.7% paraformaldehyde in phosphate-buffered saline (PBS: 135 mM NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2). Cells were then permeabilized with 1% Triton 100x, blocked in PBST (PBS with 0.1% Triton 100x) containing 5% NGS (blocking solution) and then washed with PBST. The primary antibody, diluted in PBST, was incubated with the samples overnight at 4°C. After being washed with PBST, the secondary antibody, diluted in blocking solution, was incubated with the samples 2 h. at room temperature. The samples were stained with DAPI, washed with PBST, and mounted in antifade solution (0.233 g DABCO, 800 µl water, 200 µl 1 M Tris-HCl pH 8.0, 9 ml glycerol). Images were taken with a 40X (numerical aperture [N.A.] 1.25) plan apochromatic objective on a laser-scanning confocal microscope (SP5; Leica, Exton, PA).

Results and Discussion

I have already provided evidence that the dSNUP guinea pig antibody is capable of immunoprecipitation (Figures 2.2C-E and 2.4A), but the dSNUP rabbit antibody failed to co-immunoprecipitate any of the proteins shown to come down with the dSNUP guinea pig antibody (data not shown). The rabbit antibody works very well for western blotting (Figure 2.2A and B), but the guinea pig antibody does not work well. Although dSNUP guinea pig can specifically recognize overexpressed GFP-dSNUP (Figure A.1), it fails to recognize endogenous dSNUP (Figure A.1).

Both dSNUP antibodies were very poor reagents for immunofluorescence on fly tissues or S2 cells (Figure A.2, A.3 and data not shown). S2 cell immunofluorescence with dSNUP guinea pig antibody did not recapitulate the pattern seen with GFP-dSNUP in S2 cells (Figure A.16). There was no perceptible decrease in anti-dSNUP signal upon dSNUP knockdown via RNAi in S2 cells (Figure A.3 and data not shown), so I suspect that most of the signal observed with the dSNUP antibodies is background. It should be noted that the dSNUP guinea pig antibody did work at one point in egg chambers (Figure 2.3D), but we were unable to reproduce these results several years after these initial experiments in egg chambers. This could be due to our inability to reproduce the proper experimental conditions or it could be that the antibody went “bad.”

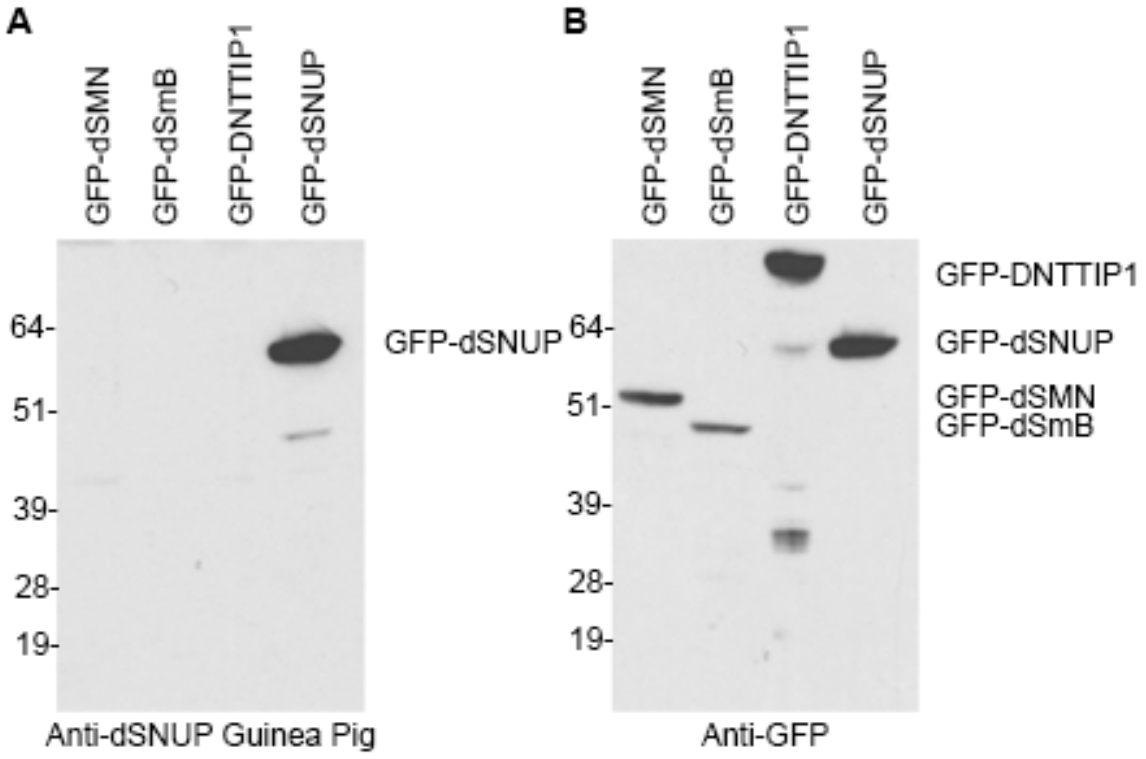


Figure A.1. dSNUP guinea pig antibody recognizes overexpressed GFP-dSNUP but not endogenous. Western blot of whole cell lysate made from GFP-tagged –dSMN, dSmB, -dSNUP, or –DNTTIP1 transfected S2 cells. dSNUP guinea pig antibody recognizes transfected GFP-dSNUP, but not other GFP-tagged proteins.

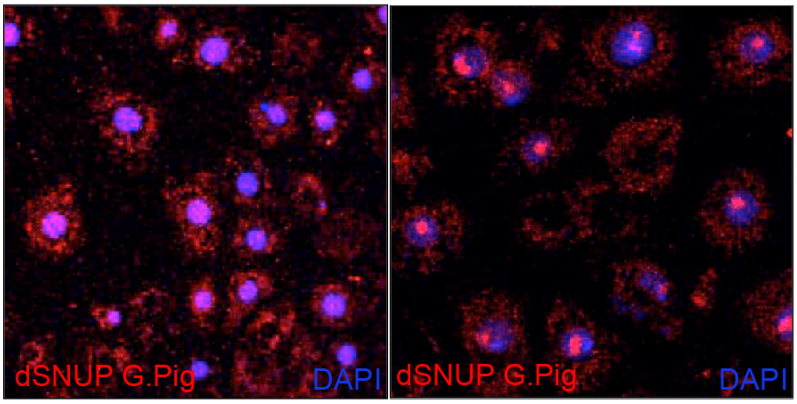


Figure A.2. S2 cell immunofluorescence with dSNUP guinea pig antibody. Immunofluorescence in S2 cells with dSNUP guinea pig antibody does not recapitulate the pattern seen with GFP-dSNUP.

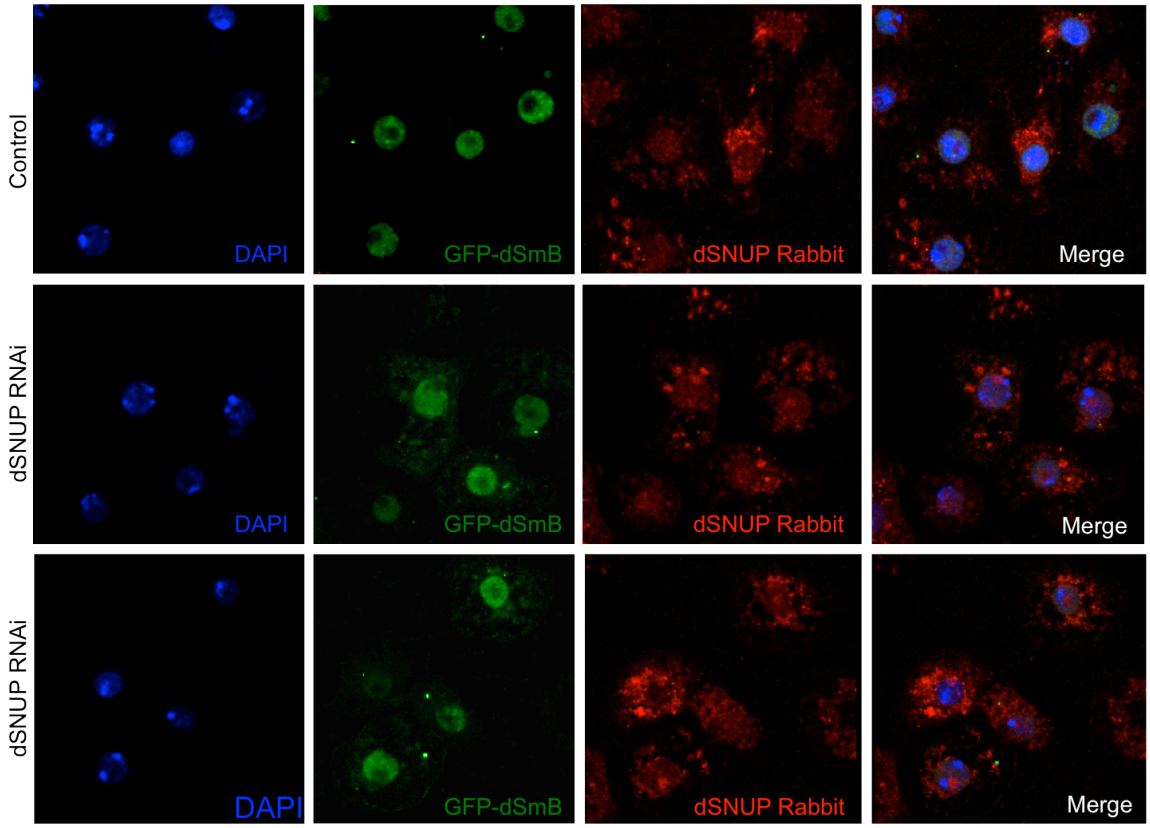


Figure A.3. S2 cell immunofluorescence with dSNUP rabbit antibody. Immunofluorescence in S2 cells with dSNUP rabbit antibody does not recapitulate the pattern seen with GFP-dSNUP. No significant change is observed in dSNUP signal upon dSNUP knockdown via RNAi.

Appendix III

Anti-dSmB co-immunoprecipitates dSNUP

Question under investigation

Does anti-dSmB (Y12) co-immunoprecipitate (IP) dSNUP?

Rationale

Previous results show that anti-dSNUP guinea pig antibody can co-IP dSmB, so I predicted that anti-dSmB should precipitate dSNUP.

Materials and Methods

S2 cell cytoplasmic lysate was prepared (see appendix II) and incubated with dSmB (Y12) anti-sDMA (gift from J. Steitz, Yale, New Haven, CT; anti-mouse; 1 µl) antibody 2 h. at 4°C before being incubated over night at 4°C with 15 µl protein A beads (Pierce). Bound proteins were washed 5X with 1 mL buffer A, eluted with SDS loading buffer, and then run on a 4-12% polyacrylamide gel (Invitrogen), and subjected to western blotting (dSNUP rabbit anti-body 1:2000).

Results and Discussion

As expected, anti-dSmB was able to co-IP dSNUP (Figure A.4). This result confirms the interaction of dSNUP and dSmB, further substantiating that dSNUP is indeed the SPN orthologue since it interacts with several snRNP protein components.

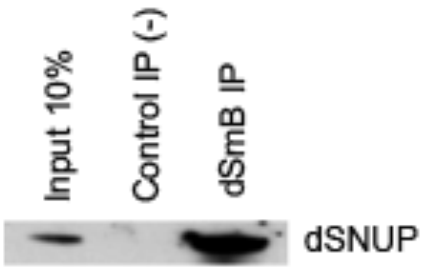


Figure A.4. dSmB antibody co-IPs dSNUP from S2 cytoplasmic lysate. Anti-dSmB (Y12) was used to co-IP dSNUP (anti-rabbit antibody for western blot) from cytoplasmic S2 cell lysate.

Appendix IV

Examination of Impβ/Ketel interacting partners

Question under investigation

Does Impβ/Ketel interact with NLS, dSNUP, dSMN, hIBB-dSNUP, or hSPN?

Rationale

When we discovered that dSNUP did not appear to have the conserved residues of the SPN IBB, we wanted to be sure that dSNUP was not interacting with Impβ/Ketel. In order to test the interaction of Impβ/Ketel with other proteins, we needed a proper positive control that was able to co-IP Impβ/Ketel in addition to our experimental flag-dSNUP construct. Several constructs were tested as potentially positive controls including, flag-NLS, flag-hIBB-dSNUP and flag-hSPN.

Materials and Methods

S2 cytoplasmic extracts were generated 4 d. after transfection (see appendix II for details). Cytoplasmic fractions were incubated with flag conjugated agarose beads (Sigma-Aldrich, St. Louis, MO; 15 µl per IP) for 2 h. at 4°C. Bound proteins were washed 5X with 1 mL buffer A, eluted with SDS loading buffer, and then run on a 4-12% polyacrylamide gel (Invitrogen), and subjected to western blotting (Anti-flag 1:5000; Anti-Ketel 1:5000; Anti-GFP 1:3000).

Results and Discussion

I found that all predicted Impβ/Ketel interacting proteins (flag-NLS, flag-hIBB-dSNUP and flag-hSPN) were able to co-IP GFP-Ketel (Figure A.5A and B). Importantly, endogenous Impβ/Ketel was also precipitated with flag-hIBB-dSNUP and flag-hSPN, but not flag-dSNUP (Figure A.5B). This result corroborates our previous finding that Impβ/Ketel is not interacting with snRNPs, and thus, cannot be serving as the snRNP import receptor in flies.

Surprisingly, I did find that GFP-Ketel comes down with flag-dSMN (Figure A.5C). It is important to note that Impβ/Ketel does not appear to interact with snRNPs, as it does not bind any other snRNP protein or RNA components (Figure 2.4 and Figure A.5). The fact that GFP-dSMN, but not snRNPs interact with Impβ/Ketel suggests that the IBB of dSMN is not accessible in the context of the SMN complex (see discussion section for further elaboration on this subject).

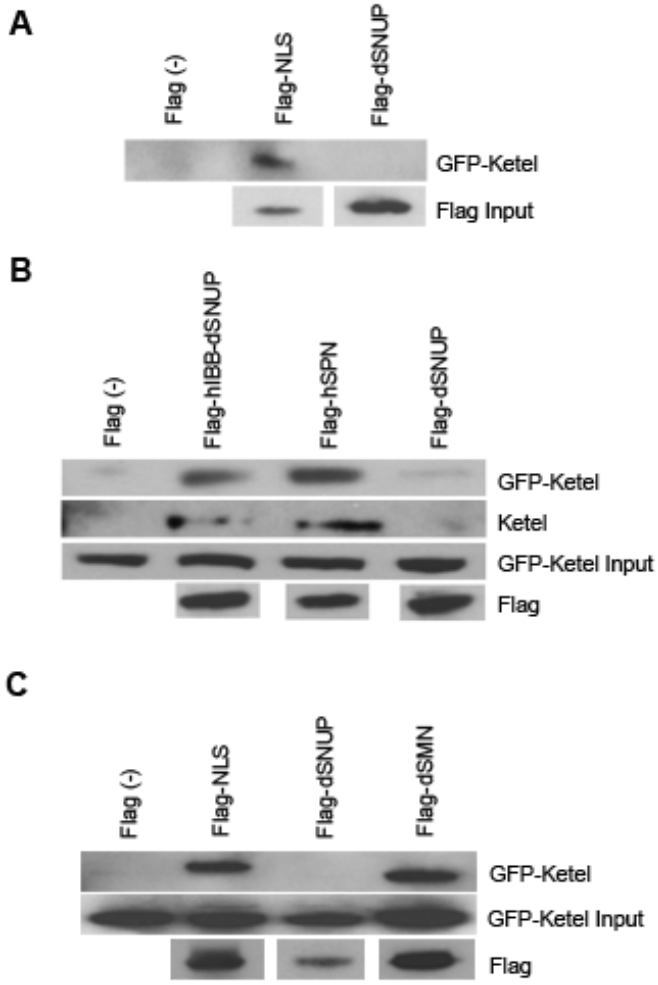


Figure A.5. Impβ/Ketel does not interact with *Drosophila* snRNPs. Flag conjugated beads IP-western blots. (A) Flag tagged –NLS, but not -dSPN co-IP GFP-Ketel from transfected S2 cell cytoplasmic lysate. (B) Flag tagged –hIBB-dSPN and –hSPN, but not -dSPN co-IP endogenous Impβ/Ketel and GFP-Ketel from transfected S2 cell cytoplasmic lysate. (C) GFP-Ketel comes down with flag-tagged –dSMN and –NLS, but not -dSNUP.

Appendix VA

dSNUP *in vivo* RNAi viability assay

Question under investigation

Is *dSnup* an essential gene?

Rationale

All previous snRNP import studies have been done in *in vitro* systems, so we do not know if SPN is an essential gene. Although I was not successful in my attempts to generate a dSNUP mutant, I did have several dSNUP RNAi lines that I could use to test the essentiality of dSNUP for life.

Materials and Methods

Oregon-R was used as the wild-type strain. Lines expressing dsRNA for RNAi of exon 1 dSNUP were obtained from the National Institute of Genetics (Kyoto, Japan; stock numbers 1247R-1 and 1247R-3). A line expressing dsRNA for RNAi of exon 3 dSNUP was obtained from the Vienna Drosophila RNAi Center (Vienna, Austria; stock number 40997). RNAi lines were crossed to a tubulin Gal4 driver line. Flies were allowed to lay embryos for 4 hours. Larvae were collected from plates 24 h. after embryo laying and then transferred and counted on every subsequent day.

Larval lysates were made with RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40, 1 mM EDTA) and then run on a 4-12% polyacrylamide gel (Invitrogen), and subjected to western blotting [dSNUP (affinity purified anti-rabbit; 1:3000) and tubulin (Sigma-Aldrich, St. Louis, MO; anti-rabbit; 1:10,000)].

Results and Discussion

Recapitulating S2 cell dSNUP knockdown results (Figure 2.2A), *in vivo* dsRNAs targeting either exon 1 or exon 3 of dSNUP (dsRNA Ex1 fly or dsRNA Ex3 fly respectively), effectively knock down dSNUP protein levels by western blotting (Figure A.6 A and B).

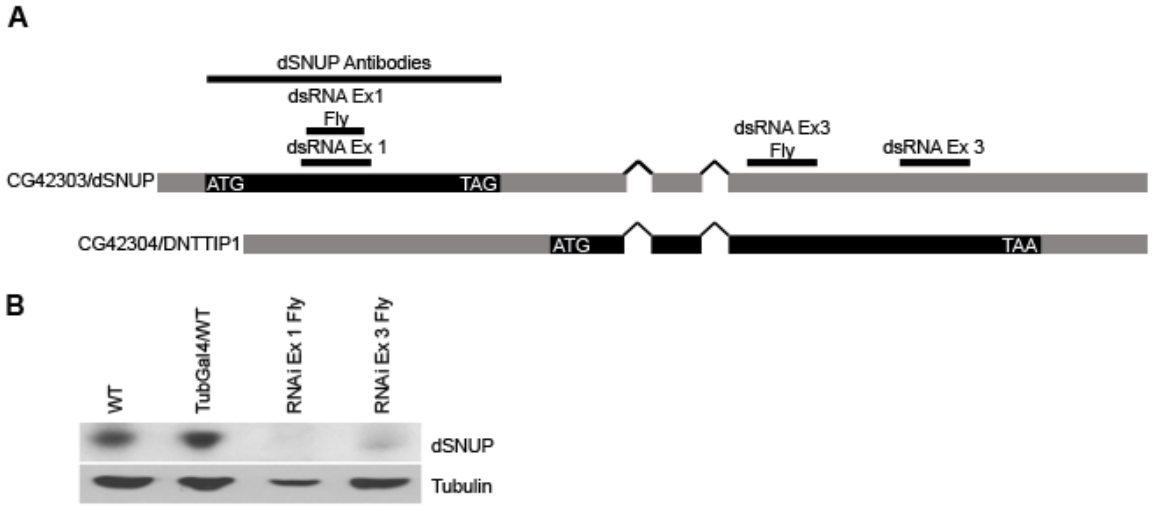


Figure A.6. *In vivo* RNAi of dSNUP. (A) Cartoon of predicted *dSnup* locus transcripts. Translated regions are shown in black and untranslated regions in gray. Black bars indicate dsRNA targets or region of dSPN antibodies. (B) Western blot showing both *in vivo* dSNUP RNAi lines effectively knock down dSNUP protein (rabbit dSNUP antibody).

Under a tubulin Gal4 driver, dsRNA targeting the 3'UTR of dSNUP (Ex3 RNAi) causes lethality at larval stages, and no animals made it to pupation at 22°C (Figure A.7). At 22°C, animals expressing dsRNA targeting exon 1 of dSNUP survived to pupation, but most died in their pupal cases (Figure A.8). Some larval lethality was observed at 29°C for the exon 1 target, and where as ~30% eclosed at 22°C, <1% elcosed at 29°C (Figure A.8 and A.9). This result was expected because Gal4 expression is higher at 29°C, and thus, dSNUP knockdown should be greater at higher temperatures.

The discrepancy between exon 1 and exon 3 targets and the associated developmental stage of lethality may be due to knockdown efficiency differences between each dsRNA. On a similar note, *DNTTIP1*, which is predicted to be encoded by the same transcript as *dSnup*, may be an essential gene. dsRNA targeting exon 3 may be more effective at DNTTIP1 knockdown since it is targeting the protein coding region of *DNTTIP1* rather that the predicted 5'UTR (exon 1 dsRNA target).

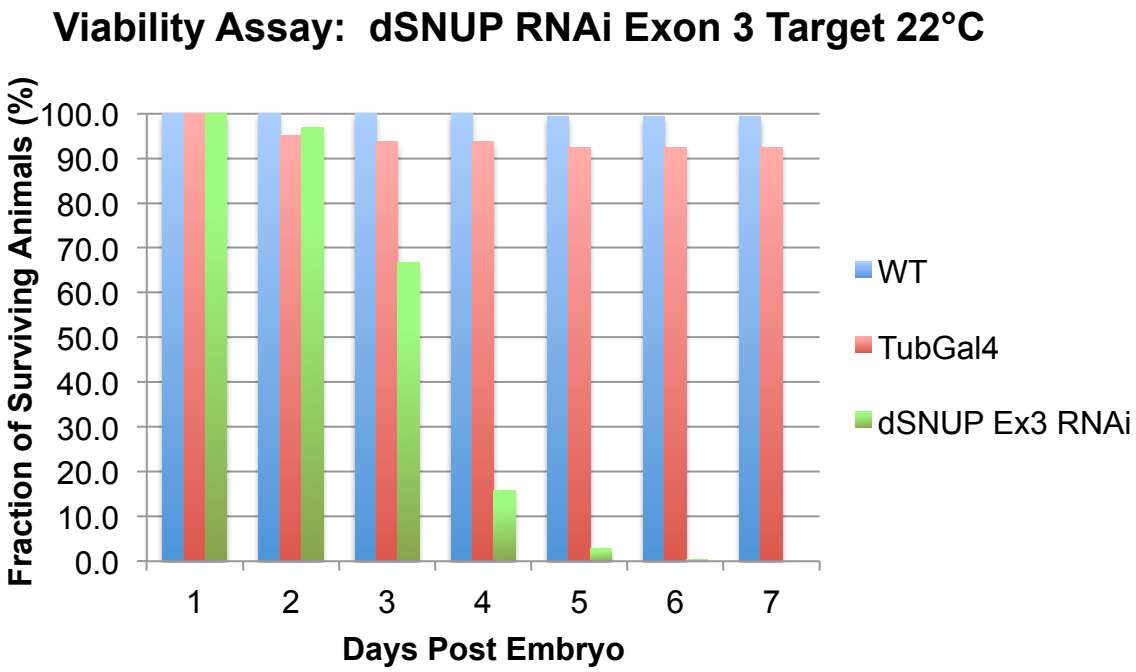


Figure A.7. Viability assay of dSNUP RNAi Ex 3 Target at 22°C. A significant wave of larval death occurs at day 3 and 4 post embryo laying. Most larvae die by day 6. RNAi was driven with tubulin Gal4. n>145.

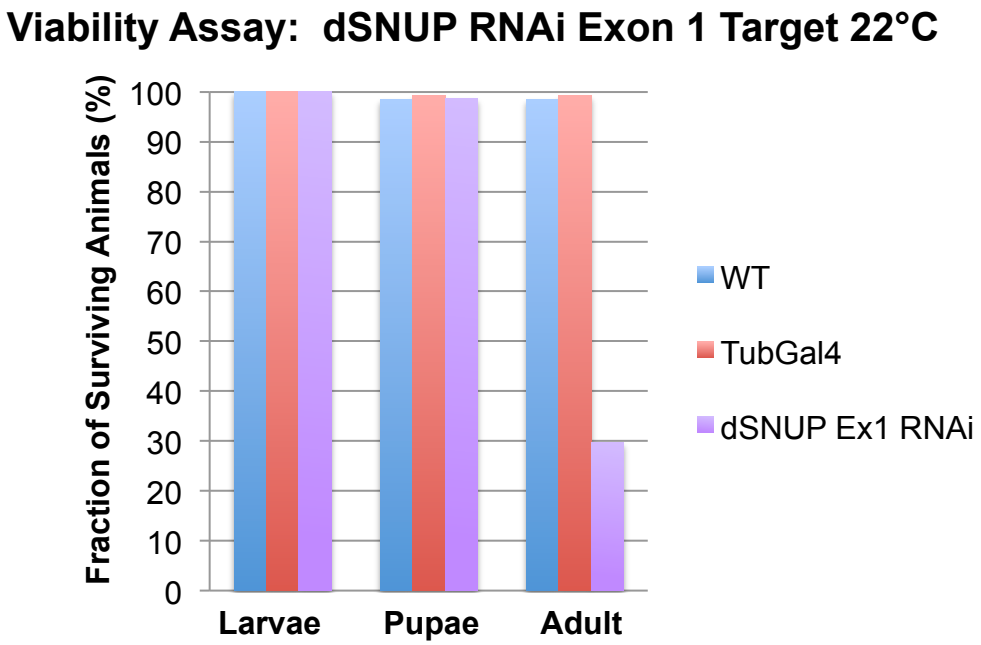


Figure A.8. Viability assay of dSNUP RNAi Ex 1 Target at 22°C. No significant lethality is seen by the ex 1 dSNUP dsRNA before pupation, but many die in their pupal cases. ~30% eclose as adults. RNAi was driven with tubulin Gal4. n>200.

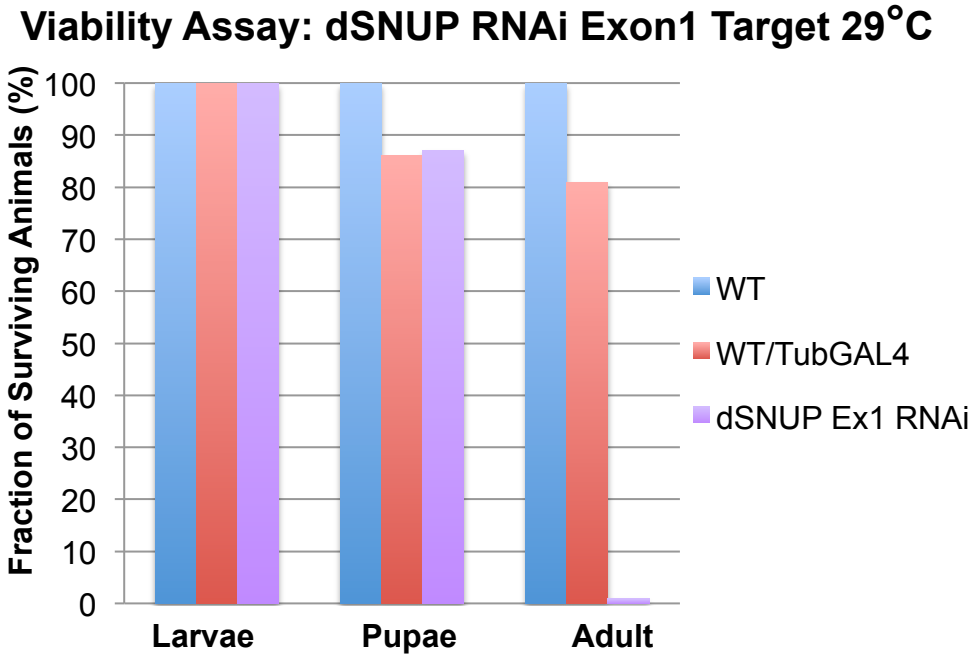


Figure A.9. Viability assay of dSNUP RNAi Ex 1 Target at 29°C. Some lethality is seen before pupation, and nearly all animals expressing ex 1 dSNUP dsRNA die in their pupal cases. RNAi was driven with tubulin Gal4. n>100.

Appendix VB

dSNUP *in vivo* RNAi rescue

Question under investigation

Does expression of VFP-dSNUP rescue lethality of dSNUP RNAi lines targeting either exon 1 or exon 3?

Rationale

Both exon 1 and exon 3 dsRNA dSNUP targets are lethal *in vivo*. The lethality is unlikely to be an off target effect since both dsRNAs produce the same lethal phenotype. The other gene (*DNTTIP1*) that is predicted to be encoded by the *dSnup* transcript is predicted to also be targeted by the dsRNAs. If DNTTIP1 is not essential, I would predict that VFP-dSNUP expression should rescue life.

Materials and Methods

The *dSNUP* transgenic construct was cloned into *pBI-UASC-mVenus* (Wang et al, 2012) and sent to BestGene (Chino Hills, CA) for embryo injection using the phiC31 system. The transgene was integrated at site 86fB (Bischof et al, 2007). The dSNUP Ex3 RNAi flies were recombined with the *VFP-dSNUP* transgenic line. The dSNUP Ex1 RNAi (insertion on Chromosome 2) flies were double balanced and put into the *VFP-dSNUP* background. dsRNA and transgene expression were driven with an actin 5C (Act5C) Gal4 driver. Timed matings were allowed to proceed for 6 h., and larvae (n>110) were collected for phenotypic analyses on subsequent days.

Results and Discussion

Whereas overexpression of VFP-dSNUP had no significant impact on the viability of the dSNUP RNAi line targeting exon 3 (data not shown), the overexpression of VFP-dSNUP significantly rescues the pupal lethality of dsRNA targeting exon 1 of dSNUP. ~98% of the Act5C Gal4 driven dSNUP RNAi animals died in their pupal cases, but upon expression of VFP-dSNUP in the exon 1 knockdown background, only ~9% died before eclosion (Figure A.10). The inability of VFP-dSNUP to rescue exon 3 viability could be attributed to the bicistronic nature of the *dSNUP* gene. The dsRNA targeting exon 3 (3' UTR of dSNUP) also targets the protein coding region of DNTTIP1, and if DNTTIP1 is essential for life, overexpression of VFP-dSNUP alone would fail to rescue. The fact that the exon 1 target can be rescued with VFP-dSNUP fails to rule out the possibility that DNTTIP1 may be essential for life since we do not know if this dsRNA is able to knockdown DNTTIP1.

A possible explanation for why VFP-dSNUP is able to rescue the exon 1 target, but not exon 3, is that VFP-dSNUP would be targeted by the exon 1 dsRNAs, and over expression of VFP-dSNUP could overwhelm the RNAi efficiency. To test this hypothesis, I did western blotting analysis of the larval lysates from Figure A.10. Indeed, I found that endogenous/untagged dSNUP protein levels were significantly up when expressing VFP-dSNUP in the RNAi background compared to RNAi alone (Figure A.11). This suggests that the RNAi mechanism is being inundated by the large quantity of dSNUP mRNA supplied by VFP-dSNUP. It is likely that the RNAi machinery cannot compensate for this

increase in dSNUP mRNA because I saw increased levels of dSNUP protein. It is important to point out that Ex 1 RNAi was able to knockdown both endogenous and VFP-dSNUP to some extent because both VFP-dSNUP and endogenous dSNUP are reduced compared to VFP-dSNUP alone.

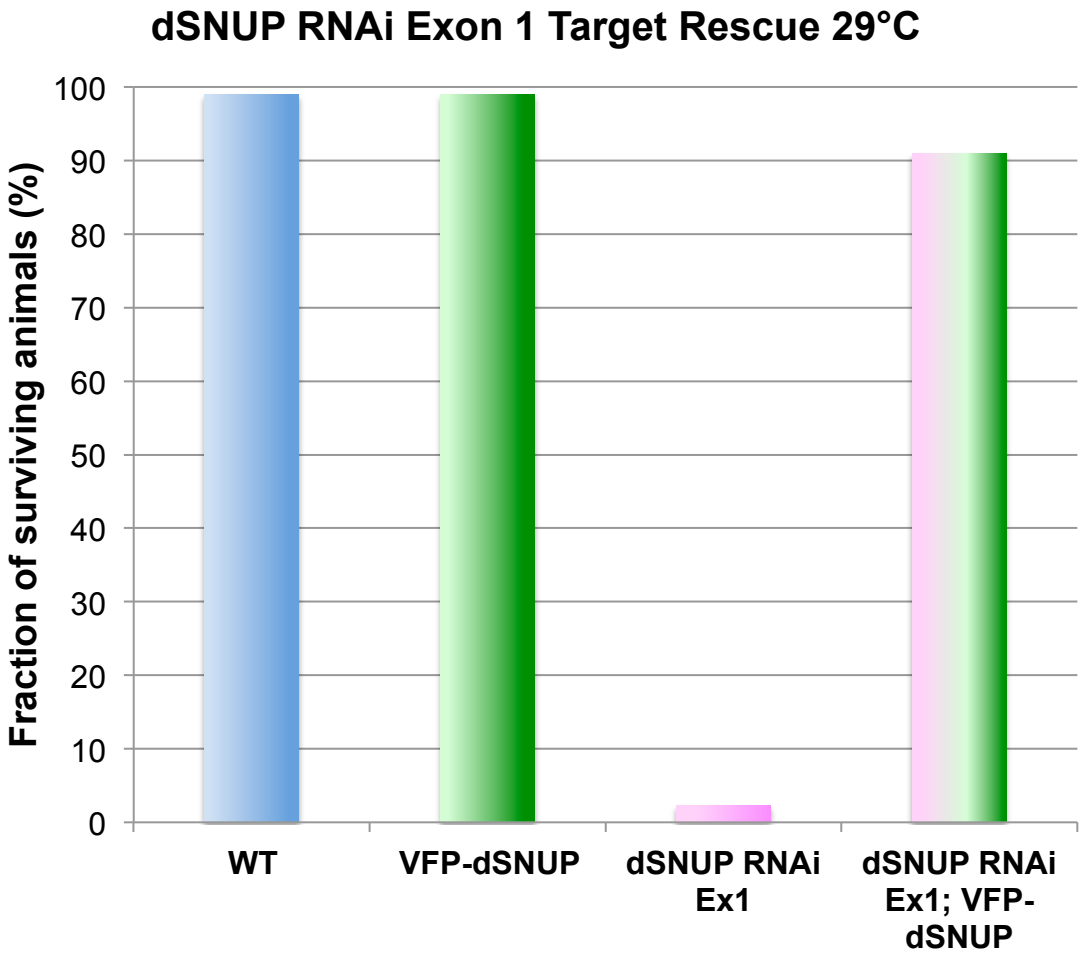


Figure A.10. Viability assay of dSNUP RNAi Ex1 and rescue with VFP-dSNUP. All lines were crossed to Act5C Gal4. Larvae were collected from plates and placed into vials. The number of eclosing adults were counted on subsequent days. Expression of VFP-dSNUP significantly rescued dSNUP RNAi Ex1 lethality. n>110.

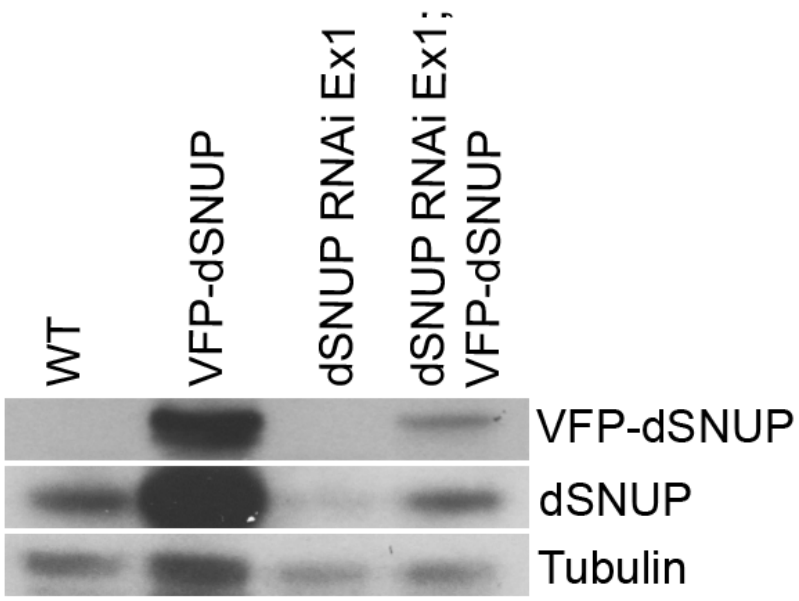


Figure A.11. VFP-dSNUP swamps out dsRNA targeting Exon 1 of dSNUP. An anti-dSNUP (guinea pig) western blot of larval lysates shows that the over expression of VFP-dSNUP (Act5C Gal4) in the dSNUP RNAi background reduces knockdown efficiency of untagged dSNUP compared to dSNUP RNAi without VFP-dSNUP expression.

Appendix VI

RNAi of dSNUP or Msk in egg chambers

Question under investigation

Are snRNA levels affected in the egg chambers upon knock down of dSNUP or Msk?

Rationale

The TMG cap is required for U1 and U2 snRNP import in *Xenopus* oocytes (Fischer et al, 1993). If this holds true in flies, I would predict that egg chambers lacking dSNUP would show reduced levels of U1 and U2 snRNPs because they would not be properly imported and hence, likely degraded. I also wanted to examine Msk knockdown in egg chambers because I would predict that snRNP levels would decrease since I have shown that Msk mutants display snRNP specific defects (Chapter II).

Materials and Methods

Oregon-R was used as the wild-type strain. Lines expressing dsRNA for RNAi of either exon 1 or exon 3 of dSNUP (see appendix V) or dsRNA for RNAi of Msk (Msk ^{33626}; Bloomington, IN; Transgenic RNAi Project, stock number 33626) were obtained. The WT line and RNAi lines were crossed to a germline Gal4 diver, Nanos.

Ovaries were dissected from adult female flies, RNA was Phenol/Chloroform extracted, denatured in formamide loading buffer, and then run on a 10% TBE-urea gel (Invitrogen), transferred to a nylon membrane, and

probed with ^{32}P-labeled PCR products corresponding to *D. melanogaster* snRNAs (U1, U2, U4, and U5) and tRNA:N5 (load control).

Results and Discussion

I was unable to detect any significant change in U snRNA levels from egg chambers in any of the RNAi lines examined. I expected to see a reduction in U1 and U2 snRNAs in dSNUP RNAi lines since we know that in frog oocytes the SPN/TMG cap is needed for U1 and U2 import. Not as surprisingly, there was no reduction in U4 and U5 snRNAs since U4 and U5 are known to have a less stringent SPN/TMG cap requirement. There is more than one U5 isoform, which is developmentally regulated, so the apparent change observed in the larger U5 isoform in dSNUP exon 3 RNAi and Msk RNAi is probably due to egg chamber maturity rather than loss of dSNUP or Msk protein (Figure A.12).

The lack of a discernable snRNA reduction phenotype is likely due to the long half-life of U snRNPs. The half-life of snRNPs is considerably long (3-5 day half-life); (Sauterer et al, 1988). When taking into consideration oogenesis time (~7 days; FlyBase), I would expect approximately half of the snRNA pool to be depleted with 100% knockdown of dSNUP or Msk. Therefore, even if dSNUP and/or Msk knock down has an affect on snRNP biogenesis and/or import, it would be difficult to detect reductions in snRNAs by northern blotting methods since, not only is RNAi not 100% effective, but it is difficult to detect reductions that are <2 fold by northern blotting. More sensitive methods are likely needed to detect the small changes in snRNA levels I would predict from knockdown in the egg chamber. All of the RNAi lines in this experiment were able to efficiently

knock down protein levels when driven with tubulin or actin 5C Gal4 (Figure A.6B and A.11; data not shown for Msk RNAi), so although I did not do a western blot of the egg chambers, I predict Nanos Gal4 was also able to drive expression of the various dsRNAs to promote knockdown.

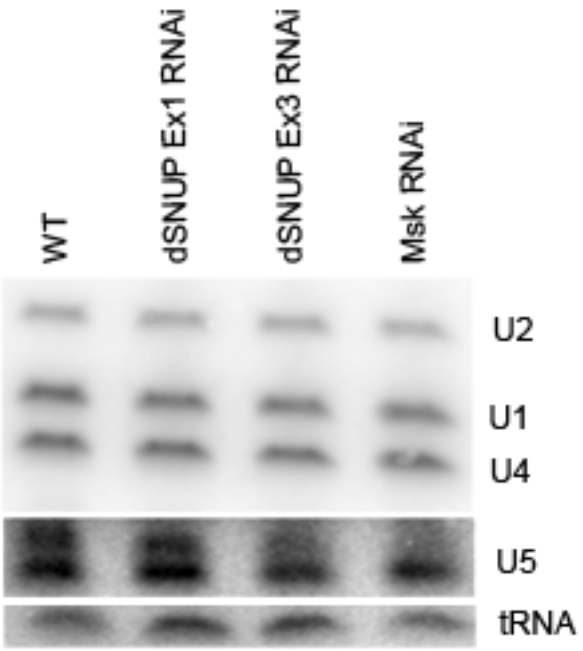


Figure A.12. U snRNA levels in dSNUP and Msk RNAi egg chambers. Total RNA was extracted from the ovaries of Oregon-R (WT) and lines expressing dsRNA against exons 1 or 3 of dSNUP (dSNUP Ex1 RNAi or dSNUP Ex3 RNAi respectively) or dsRNA against Msk (Msk RNAi) crossed to a germline Gal4 diver, Nanos.

Appendix VIIA

Analysis of Msk null mutant viability

Question under investigation

When do Msk null mutant larvae die?

Rationale

I had noticed that Msk null mutant larvae do not make it to pupation, and that a small fraction survive and persist as larvae for up to 20 days post embryo laying. In order to gain a more accurate picture of the percentage of larvae that possess this long-lived larval phenotype, I carried out larval viability assays.

Materials and Methods

Oregon-R was used as the wild-type strain. The Msk null line contains a piggy back insertion in intron 1 of Msk (*msk*$^{-/-}$), Msk ^{B185}, w^{1118}; PBac{5HPw^{+}} msk^{B185}/ TM6 GFP**.** Flies were allowed to lay for 4 hours. Larvae (n>115) were collected from plates 24 hours after embryo laying. Larvae were transferred and counted on every subsequent day.

Results and Discussion

There is a significant wave of larval death that occurs between day 4 and 5 post embryo laying. Larvae that survive until day 6 (~4%) have a long-lived larval phenotype, living significantly past 10 days (Figure A.13). These long-lived larvae persist at the second instar larval stage for up to 20 days, and did not have any identifiable imaginal discs (data not shown). Long-lived larval phenotypes are not unusual in *Drosophila*.

Viability assays of dSMN mutants demonstrated a remarkably similar phenotype, although dSMN mutants have a higher percentage (~30%) of long-lived larvae versus Msk mutants (~15%), and make it to the third instar stage (Praveen et al., 2012). Importantly, dSMN mutants can also live for greater than 20 days as larvae and do not have imaginal discs (Praveen unpublished). Given that Msk null mutants have a significant reduction in dSMN protein, it may be of relevance that both dSMN and Msk mutants have similar long-lived phenotypes. Future studies will need to be carried out to fully understand the connection between dSMN and Msk.

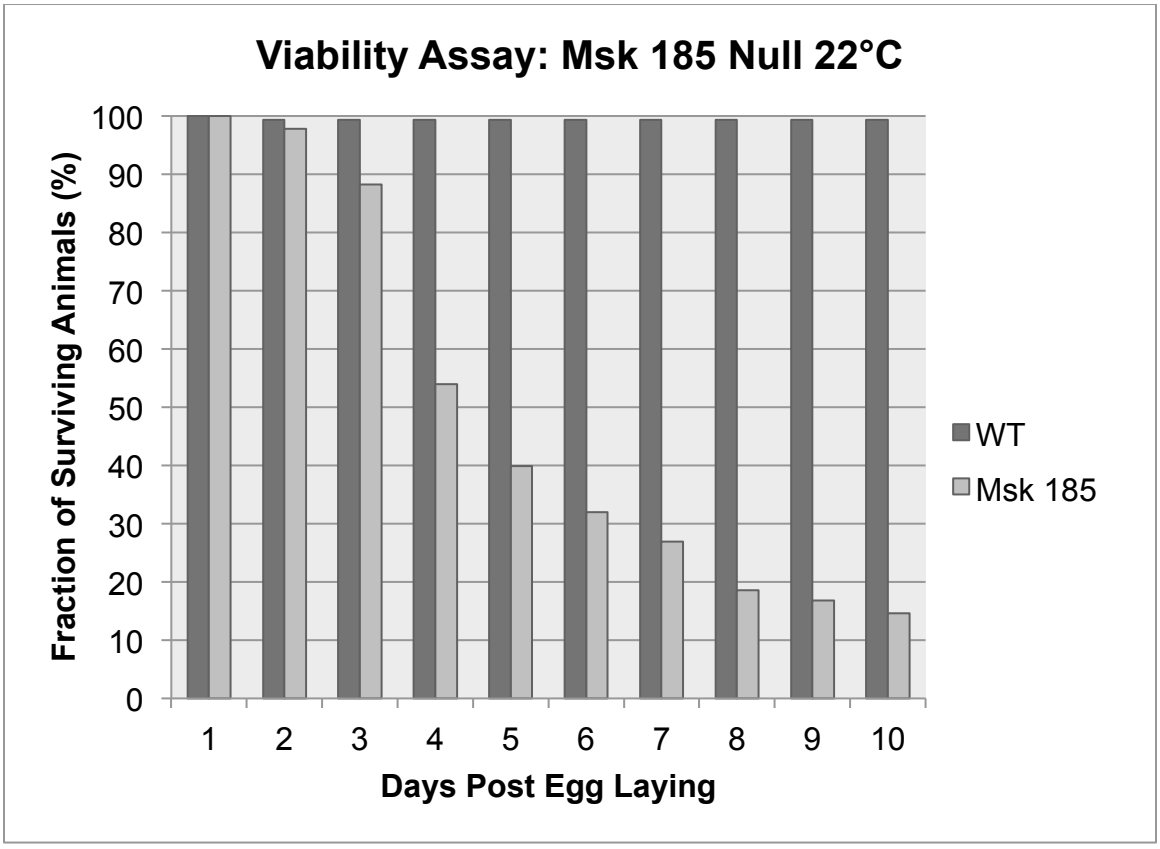


Figure A.13. Viability of Msk^{185} mutant long-lived larvae. A significant wave of larval death occurs at day 4 and 5 post egg laying. Larvae that survive until day 6 (~4%) have a long-lived larval phenotype, living past 10 days as second instar larvae. n>115.

Appendix VIIB

Coilin and dSMN reduction in Msk null mutants

Question under investigation

On what day can I detect a significant reduction of dSMN and/or coilin in Msk null mutants?

Rationale

My previous work from Chapter II showed that there is a dramatic reduction of dSMN (by immunofluorescence and western blotting) in long-lived Msk null mutants, which were analyzed on day 6 post embryo laying. Cajal bodies detected by anti-coilin were also disrupted in these long-lived Msk mutants. I was curious to know how soon after maternal Msk protein loss I could detect a reduction in coilin and dSMN by western blotting.

Materials and Methods

Oregon-R was used as the wild-type strain. The Msk null line contains a piggy back insertion in intron 1 of Msk (*msk*$^{-/-}$), Msk ^{B185}, w^{1118}; PBac{5HPw^{+}} msk^{B185}/ TM6 GFP**.** Larvae were collected on subsequent days, and larval lysates made with RIPA buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1% NP40, 1 mM EDTA) and then run on a 4-12% polyacrylamide gel (Invitrogen), and subjected to western blotting [dSMN (affinity purified anti-rabbit; 1:2000), Msk (gift of L. Perkins; anti-rabbit; 1:2000), Coilin (gift of J. Gall; anti-guinea pig; 1:2000), and tubulin (Sigma-Aldrich, St. Louis, MO; anti-rabbit; 1:10,000)].

Results and Discussion

Remarkably, Msk mutants exhibited significant reductions in both dSMN and coilin by day 1 post embryo laying (Figure A.14). This was unexpected because previous immunofluorescence experiments (not shown) failed to show noticeable phenotypic changes in dSMN and coilin staining at such early stages. It is possible that the tissues examined by immunofluorescence (mainly gut tissues) are not the primary tissues affected by Msk loss of function, and thus whole body analysis would be a better method of looking at loss of snRNP specific proteins in the Msk null mutants.

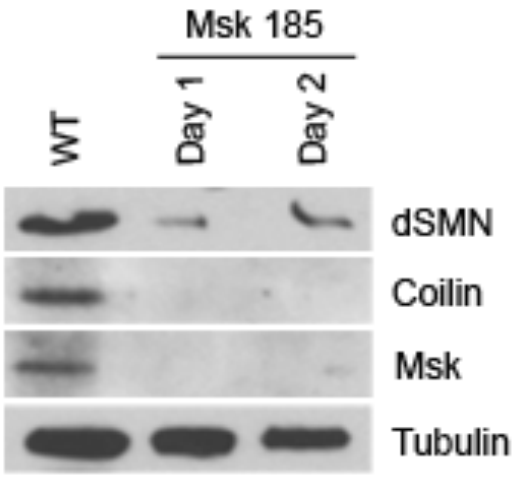


Figure A.14. Msk null mutants display a significant reduction in dSMN and coilin by day 1 post embryo laying. Whole body larval lysates were subjected to western blotting with dSMN and coilin antibodies. Msk and tubulin antibodies were used to detect the loss of maternally contributed Msk or for a load control respectively.

Appendix VIIC

Investigation of U snRNP levels in Msk mutants

Question under investigation

Can I detect U snRNP reductions in whole body Msk null mutant larvae?

Rationale

Msk mutant larvae have dramatically less dSMN by day one post embryo (Figure A.14). I would expect this reduction in dSMN to have a negative impact on U snRNP levels since dSMN is required for efficient snRNP assembly *in* vivo. I therefore carried out northern blotting on whole larval RNA preps to check for possible reductions in snRNAs.

Materials and Methods

Oregon-R was used as the wild-type strain. The Msk null line ($msk^{-/-}$) described in appendix VIIA was recombined with Armadillo-Gal4 and crossed to UAS-*msk* for rescue. Previously characterized $Ketel^{null/-}$ (Villanyi et al, 2008) were a gift from Janos Szabad. [The – symbol stands for a small deficiency ($ketel^{rx32}$) that removes Impβ/Ketel and a few of the adjacent loci, while the Impβ/Ketel null ($ketel^{rx13}$) is a complete loss of function mutant allele (Erdelyi et al, 1997).] Total larval RNA was Phenol/Chloroform extracted, run on a 10% TBE-urea gel (Invitrogen), transferred to a nylon membrane, and probed with ^{32}P-labeled PCR products corresponding to *D. melanogaster* snRNAs (U1, U2, U4, and U5) and tRNA:N5 (load control).

Results and Discussion

Northern blotting of total RNA from Msk mutant larvae revealed an approximate 2-fold reduction in U1 snRNA compared to controls (Figure A.15). UAS-Msk driven by armadillo Gal4 sufficiently rescued U1 snRNA levels, and there was no detectable reduction in Impβ/Ketel mutants. However, I was surprised to only see a reduction in U1 snRNA. Loading of this northern blot was not terribly consistent between samples, and thus, it is difficult to accurately assess changes by eye. Additionally, the long half-life of snRNPs and the fact that northern blotting is not the best method to detect changes in RNA levels could account for the apparent unaffected levels of the other snRNAs. More sensitive methods may be able to detect the reduction in U snRNAs I would predict to see in the Msk mutant larvae.

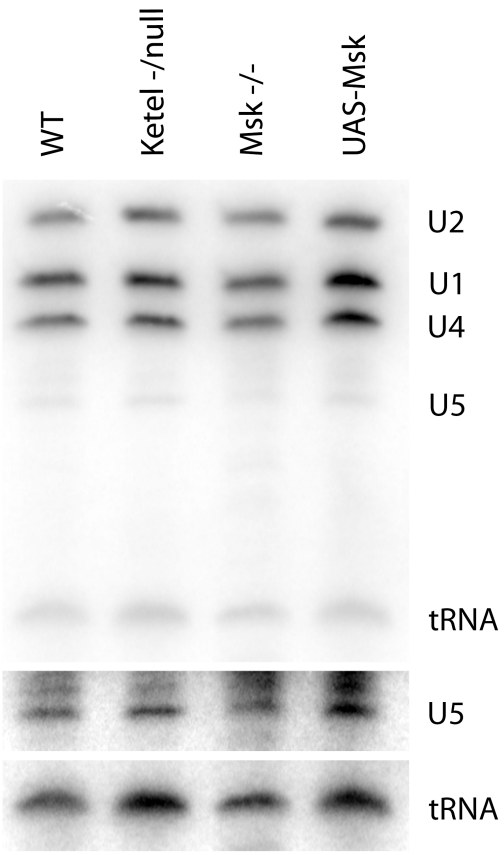


Figure A.15. U1 snRNA is reduced in Msk mutant larvae. Total larval RNA was subjected to northern blotting with ^{32}P-labeled PCR products corresponding to snRNAs (U1, U2, U4, and U5) and tRNA:N5 (load control). Adjustments to brightness/contrast were made in Photoshop for tRNA and U5 snRNA (lower two blots).

Appendix VIIIA

Investigation of dSNUP localization

Question under investigation

What is the localization of GFP-dSNUP in S2 cells?

Rationale

Human cell culture models show that hSPN is predominantly cytoplasmic (Ospina et al, 2005a); (Narayanan et al, 2002), but my *in vivo* expression of VFP-dSNUP revealed a predominantly nucleoplasmic localization pattern. Unfortunately, neither dSNUP antibody is good for immunofluorescence (see appendix II), so to confirm the VFP-dSNUP localization results, I wanted to check the localization pattern GFP-dSNUP in S2 cells.

Materials and Methods

S2 cells were transfected with GFP-dSNUP using Cellfectin as directed (Invitrogen). Untransfected or transfected cells were harvested 4 days after transfection and either fractionated or fixed onto slides.

Cells were fixed at room temperature for 10 min. in 3.7% paraformaldehyde in phosphate-buffered saline (PBS: 135 mM NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2), stained with DAPI for 10 min., washed with PBS and then mounted in anti-fade solution (0.233 g DABCO, 800 µl water, 200 µl 1 M Tris-HCl pH 8.0, 9 ml glycerol). Images were taken with a 40X (numerical aperture [N.A.] 1.25) plan apochromatic objective on a laser-scanning confocal microscope (SP5; Leica, Exton, PA).

S2 cell cytoplasmic lysate was prepared (see appendix II), and the nuclear fraction was purified from the pellet remaining after harvesting the cytoplasmic fraction. The nuclear pellet was washed several times with phosphate-buffered saline (PBS: 135 mM NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2). The pellet was resuspended in ½ the cell volume of low salt buffer C (20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM NaCl, 0.5 mM DTT) and homogenized with stir bar at slow speeds while slowly adding the same volume of high salt buffer C (20 mM Hepes pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). The cells were homogenized for 30 min. at 4°C, spun for 5 min. at max speed, and the nuclear fraction harvested from the supernatant. Fractions were then run on a 4-12% polyacrylamide gel and subjected to western blotting.

GFP (Abcam, Cambridge, MA; anti-rabbit, 1:1000), dSNUP (affinity purified anti-rabbit; 1:3000), lamin (Developmental studies hybridoma bank, Iowa City, Iowa, ADC101, anti-mouse, 1:1000), and tubulin (Sigma-Aldrich, St. Louis, MO; anti-mouse; 1:10,000) antibodies were used for western blotting. Secondary antibodies used were goat -anti-mouse- and -anti-rabbit-conjugated horseradish peroxidase at 1:5000 (Pierce).

Results and Discussion

In vivo localization studies from Chapter II revealed that, unlike its human counter part, VFP-dSNUP was predominantly nuclear. I found that by immunofluorescence, GFP-dSNUP was nucleoplasmic in S2 cells as well (Figure A.16A). Surprisingly however, GFP-dSPN and dSPN were enriched in the cytoplasmic fraction by western blotting (Figure A.16B and C). This apparent

discrepancy in localization could be due to numerous factors involved in lysate preparation. It is possible that dSPN could be leaking from the nucleus since it is not membrane bound like lamin (nuclear fractionation control), but this seems unlikely because GFP-dSmB, which is smaller than GFP-dSNUP, was enriched in the nucleoplasmic fraction using the same fractionation method (Figure 3.5).

Taking into consideration that I loaded based on total protein concentration, the ratio of dSNUP to total protein may be greater in the cytoplasm versus the nucleus. Assuming that the nuclei contain higher total protein concentrations, I would be inaccurately interpreting the results from the fractionation western blot since I loaded relative to total protein. I would need to normalize to a protein control that is known to be equally distributed between the cytoplasm and nucleus to accurately interpret the localization of dSNUP by the fractionation based method. Regardless of such issues, immunofluorescence of GFP-dSNUP in S2 cells and in fly tissues clearly showed that dSNUP was predominantly nucleoplasmic.

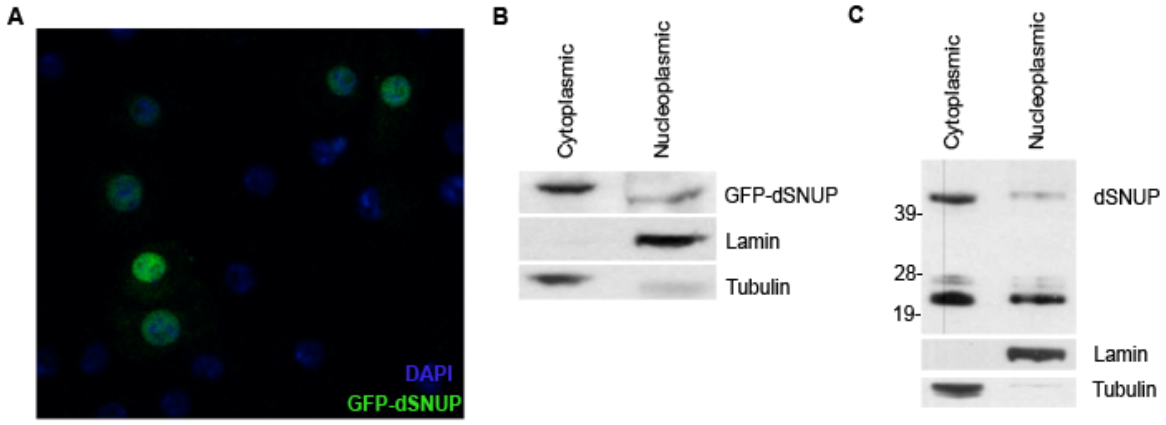


Figure A.16. dSNUP localization in S2 cells. (A) S2 cell immunofluorescence. Transfected GFP-dSNUP is predominantly nucleoplasmic when visualized by IF. (B) Western blot of transfected, fractionated S2 cells. Normalized to total protein, transfected GFP-dSNUP is enriched in the cytoplasmic fraction compared to the nuclear. (C) Western blot of fractionated S2 cells. Normalized to total protein, endogenous dSNUP is enriched in the cytoplasmic fraction compared to the nuclear fraction.

Appendix VIIIB

Investigation of GFP-dSNUP localization after Msk RNAi

Question under investigation

Is dSNUP localization affected by Msk knockdown via RNAi in S2 cells?

Rationale

Previous results have shown that GFP-dSmB localization is affected upon Msk knockdown. GFP-dSmB is a nucleoplasmic protein; accordingly, GFP-dSmB is enriched in the nuclear fraction by western blotting and also by immunofluorescence. GFP-dSNUP has a more puzzling distribution because it is predominantly nucleoplasmic by immunofluorescence, yet enriched in the cytoplasmic fraction by western blotting (Figure A.16). Notwithstanding, a change in the localization pattern by fractionation could have meaning, so I was interested to see if GFP-dSNUP localization was affected by Msk knockdown in S2 cells.

Materials and Methods

Msk dsRNA was transcribed *in vitro* from PCR products flanked with T7 promoters. *Drosophila* S2 cells were placed in SF-900 media and treated with fresh 14 µg/mL double-strand RNA (dsRNA) each day for 4 d. before harvesting. Cells were transfected with GFP-dSNUP using Cellfectin as directed (Invitrogen) on day 2. Cytoplasmic and nuclear extracts were generated 4 d. after transfection and subjected to western blotting (see appendix VIIA for additional details). 50 µg of cytoplasmic extract was loaded on a gel for western blotting analysis to confirm knockdown (Figure 3.3B).

Results and Discussion

Confirming the fractionation result seen in Figure A.16, GFP-dSNUP is enriched in the cytoplasmic fraction of untreated S2 cells (Figure A.17). Upon treatment with dsRNA targeting Msk, GFP-dSNUP appeared to become more nuclear than seen in the control nuclear fraction (Figure A.17). Although it is difficult to make much of this finding due to the discrepancy in localization pattern by various methods, there is a clear change in the nucleocytoplasmic distribution of GFP-dSNUP after Msk knockdown.

We know that in human cells Impβ binding deficient SPN is cytoplasmic like wild type hSPN, but when you inhibit export receptor Xpo1 with leptomycin B (LMB), SPN accumulates in the nucleus (Ospina et al, 2005a). Since the residues needed for Xpo1 and TMG cap binding overlap, hSPN mutants that cannot bind Xpo1 and/or TMG caps have a similar nucleoplasmic pattern (Ospina et al, 2005a); (Dong et al, 2009b). Since dSNUP does not bind to Impβ/Ketel, it is not surprising that by immunofluorescence dSNUP was predominantly nuclear like SPN IBB mutants (Ospina et al, 2005a). Importantly, immunofluorescence could detect some cytoplasmic dSNUP, and this relatively low level of cytoplasmic dSNUP appeared to be enriched by fractionation methods. I can use this perceived problem to my advantage because it allows me to more closely examine changes in the nuclear dSNUP levels by western blotting. A change in cytoplasmic dSNUP is likely of significant importance since dSNUP must first bind to snRNPs in the cytoplasm before being imported.

There are several possible explanations for the increased nuclear dSNUP after Msk knockdown. My previous work has shown that snRNP biogenesis is disrupted in Msk mutant flies. Therefore, I would predict that Msk knockdown would disrupt snRNP biogenesis. In the absence of ongoing snRNP biogenesis, dSNUP would not be needed as an import adaptor and would not need to be exported to the cytoplasm to bind snRNPs. Alternatively, if Msk plays a role in the disassembly of newly imported snRNPs, then dSNUP would remain bound to nucleoplasmic snRNPs, effectively inhibiting dSNUP export since TMG cap binding and Xpo1 binding are mutually exclusive (Ospina et al, 2005a); (Dong et al, 2009b).

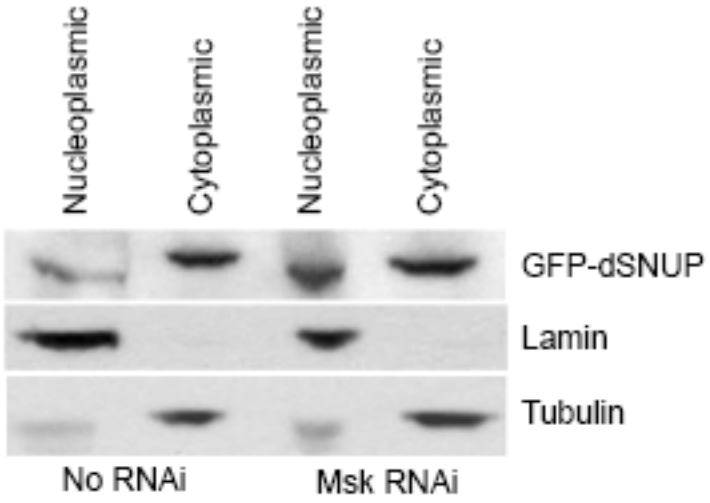


Figure A.17. Transfected GFP-dSNUP nucleoplasmic retention in Msk RNAi S2 cells. S2 cells treated with dsRNA targeting Msk showed nucleoplasmic retention of transfected GFP-dSNUP compared to the predominantly cytoplasmic localization of GFP-dSNUP in control S2 cells (no RNAi).

Appendix IX

A closer look at Msk and coilin localization

Question under investigation

Do I see similar patterns for Msk and coilin staining throughout the cell cycle?

Rationale

We know that coilin has distinct localization patterns, which are dictated by the cell cycle. My previous results have shown that Msk and coilin co-localize in CBs, so I was curious to know if Msk has a similar pattern of localization during the cell cycle.

Materials and Methods

S2 cells were fixed at room temperature for 10 min. in 3.7% paraformaldehyde in phosphate-buffered saline (PBS: 135 mM NaCl, 2.5 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.2), permeabilized with 1% Triton 100x, blocked in PBST (PBS with 0.1% Triton 100x) containing 5% NGS (normal goat serum; blocking solution) and then washed with PBST. The primary antibody, diluted in PBST, was incubated with the samples overnight at 4°C [Msk (gift of L Perkins; anti-rabbit; 1:1000) and Coilin (gift of J. Gall; anti-guinea pig; 1:1000)]. After being washed with PBST, the secondary antibody, diluted in blocking solution, was incubated with the samples 2 h. at room temperature. The samples were stained with DAPI, washed with PBST and mounted in anti-fade solution (0.233 g DABCO, 800 µl water, 200 µl 1 M Tris-HCl pH 8.0, 9 ml glycerol). Images were taken with a 40X (numerical aperture [N.A.] 1.25) plan

apochromatic objective on a laser-scanning confocal microscope (SP5; Leica, Exton, PA).

Results and Discussion

Confirming previous results (Figure 2.6), I found that coilin and Msk colocalize in CBs of S2 cells (Figure A.18 #3), but this was not the only localization pattern seen. Msk and coilin also displayed cytoplasmic distribution (Figure A.18 #1) and perinuclear distribution (Figure A.18 #2) in some cells. The localization of coilin has been well characterized and has been shown to vary depending on the cell cycle.

Coilin distribution during the cell cycle (Liu et al, 2009)

Interphase- throughout nucleoplasm with multiple bright foci

Prophase- prominent foci

Metaphase- foci aligned at metaphase plate (at or near centromeres)

Anaphase- foci disappear-faint signal throughout spindle

Late telophase- only in midbody

Although I did not use distinct cell cycle markers, I was able to see a wide range of coilin distribution patterns in S2 cell culture. Msk had similar localization patterns as coilin. When coilin was throughout the nucleoplasm (presumably during interphase), Msk was also predominantly nucleoplasmic; when coilin was in prominent foci (assumed to be a CB during prophase), Msk was also enriched in the foci. Additionally, when coilin was observed to be cytoplasmic, Msk was also (Figure A.18).

The close association of coilin and Msk is demonstrated not only from their apparent co-localization patterns during the cell cycle, but from my previous work shown in Chapter II. I have shown that Msk mutants have a dramatic reduction in

coilin protein and that their CBs are disrupted (Figure A.14 and Figure 2.9 respectively). These results support a role for Msk in snRNP biogenesis since CBs are tightly linked to on going snRNP biogenesis. Future studies will be needed to fully understand the apparent association between Msk and coilin and why we see such a dramatic impact on coilin protein and CBs in the absence of Msk.

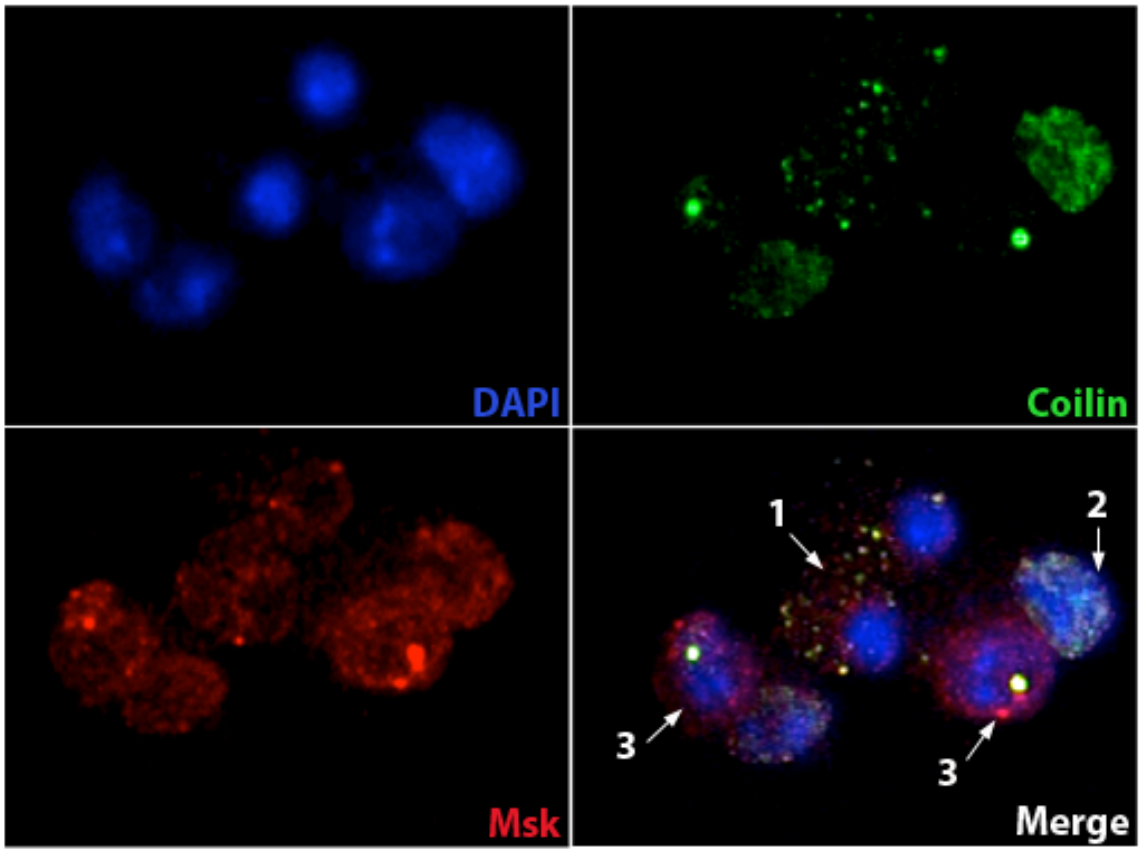


Figure A.18. Variations in the localization of Msk and coilin within an S2 cell culture system. Cytoplasmic Msk and coilin distribution in the middle cell (#1), perinuclear distribution (#2), or one large foci (#3) were observed in addition to multiple nucleoplasmic foci (Figure 2.6).

Appendix X

pBI-UASC-mVenus-(hIBB)-dSNUP characterization and Msk mutant rescue

Question under investigation

Does expression of VFP-hIBB-dSNUP rescue Msk mutant snRNP specific phenotypes?

Rationale

My previous work has shown that Msk mutants display snRNP specific phenotypic defects. Since Msk is known to function as an import adaptor, and fly snRNPs fail to bind to Impβ/Ketel, Msk may be serving as the snRNP import receptor in flies. In an effort to bypass the Msk dependence of the observed Msk mutant snRNP defects, I generated transgenic flies expressing VFP-hIBB-dSNUP from a UAS promoter (see appendix XIA and B for details). Because I had previously shown that hIBB-dSNUP forms a complex with Impβ/Ketel (Figure 2.4B and Figure A.5B), I hypothesized that its expression could rescue snRNP import by utilizing Impβ/Ketel as the SPN/TMG cap dependent snRNP import receptor.

Materials and Methods

Oregon-R was used as the wild-type strain. A Msk null line containing a piggy back insertion in intron 1 of Msk (*msk*$^{-/-}$), Msk ^{B185}, w^{1118}; PBac[5HPw^{+}]msk^{B185}/ TM3, Sb^{1} Ser^{1}, and a line containing *msk* with a UAS promoter (UAS-*msk*), w*; P{UAS-*msk*.L}47M1/CyO**,** previously characterized (Lorenzen et al, 2001), were obtained from the Bloomington Stock Center (Bloomington, IN).

The *dSNUP* and *hIBB-dSNUP* transgenic constructs were cloned into *pBI-UASC-mVenus* (Wang et al, 2012) and sent to BestGene (Chino Hills, CA) for embryo injection using the phiC31 system. The transgenes were integrated at site 86fB (Bischof et al, 2007). The $msk^{-/-}$ flies were recombined with *VFP-dSNUP* or *VFP-hIBB-dSNUP* transgenic lines or with Gal4 drivers (armadillo, daughterless, or gut-specific Malpighian tubule drivers). Timed mattings were allowed to proceed for 6 h., and larvae were collected for phenotypic analyses on subsequent days.

Results

Both VFP-dSNUP and VFP-hIBB-dSNUP protein were expressed in the WT background, but VFP-hIBB-dSNUP protein was significantly lower than VFP-dSNUP (Figure A.19). VFP-hIBB-dSNUP may be unstable and degraded or protein expression may be down regulated due to the fact that VFP-hIBB-dSNUP expression is lethal. Using either a ubiquitous tubulin Gal4 driver or any of the various drivers tested, I found that expression of VFP-hIBB-dSNUP was dominantly lethal in both WT and Msk mutant backgrounds. All drivers tested with the exception of daughterless Gal4 were early first instar lethal.

Daughterless Gal4 driven VFP-hIBB-dSNUP flies in the WT background made it to pupation and died in their pupal cases. Unfortunately, with the daughterless driver in the Msk null background, I did not see reproducible rescue of snRNP defects by immunofluorescence in Malpighian tubules. It is important to note that I also had spotty expression of VFP-hIBB-dSNUP (driven by daughterless Gal4) in Malpighian tubules that I did not see with other Gal4 drivers (data not shown). This poor Malpighian tubules expression may account

for the lack of rescue or it could be that VFP-hIBB-dSNUP bound to Impβ/Ketel is not import competent.

It is unlikely that the dominant negative phenotype of the hIBB-dSNUP construct is due to VFP-tagging because expression of VFP-dSNUP construct had no such dominant effects and was able to rescue dSNUP RNAi (appendix VB). The observed dominant lethality of the hIBB-dSNUP fusion protein prevented me from fully examining whether I could rescue the apparent snRNP import defects seen in Msk null mutants.

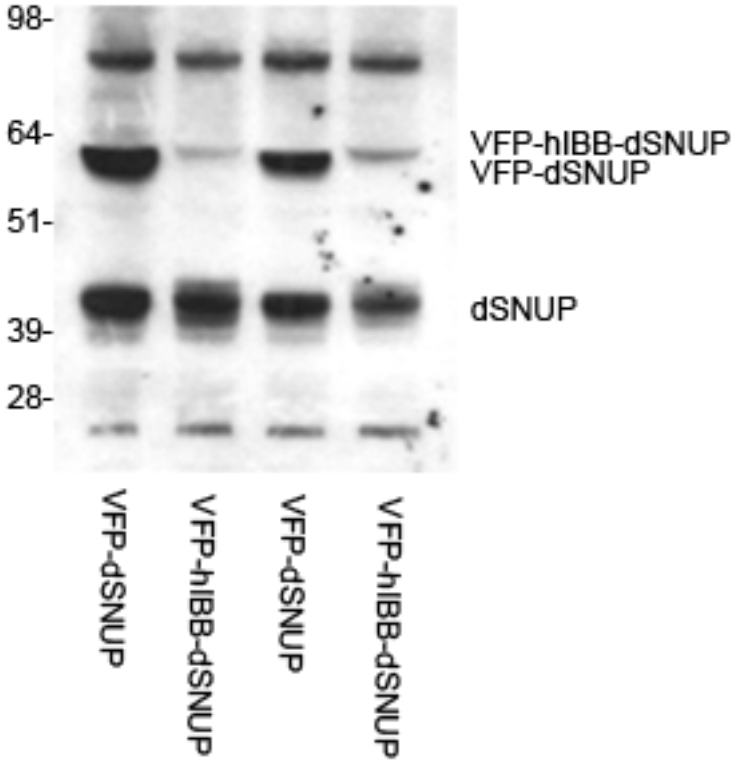


Figure A.19. Expression of VFP-dSNUP and VFP-hIBB-dSNUP *in vivo.* Larval expression of VFP-dSNUP and VFP-hIBB-dSNUP driven by daughterless Gal4. VFP-hIBB-dSNUP protein is much lower than VFP-dSNUP by western blotting.

Appendix XIA

Drosophila transgenic vector map

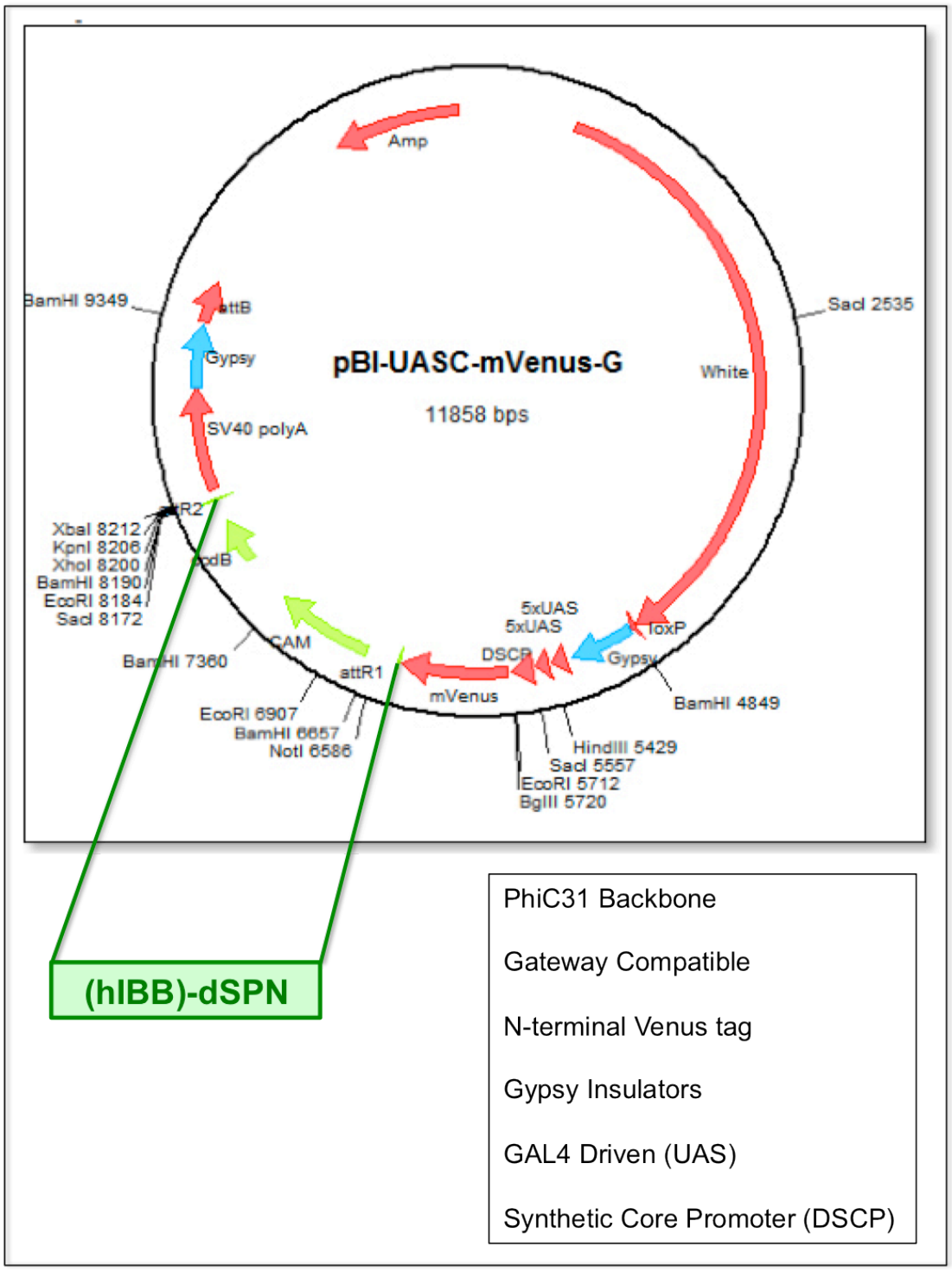


Figure A.20. Vector map of the *Drosophila* pBI-UASC-mVenus-G. This *Drosophila* transgenic vector is based on a PhiC31 compatible backbone with a miniwhite marker and ampicillin resistance (Groth et al, 2004); (Dietzl et al, 2007). There are 10 copies of the Gal4 Upstream Activating Sequence (UAS) to improve expression levels with a *Drosophila* synthetic core promoter (DSCP) to reduce non-specific tissue expression (Pfeiffer et al, 2008), which together is called UASC. Gateway technology facilitates cloning; and attB flanked PCR products (hIBB-dSNUP and dSNUP) were ligated into the attR1/attR2 site. The inserts are flanked by gypsy insulator elements to increase expression levels and reduce insertion site-to-site expression variability (Markstein et al, 2008). Adapted from Wang et al., 2012; http://vectors.mccabelab.org.

Appendix XIB

Sequence details for hIBB-dSNUP

Sequence of dSNUP fused to the IBB of hSPN (hIBB-dSNUP)

hSPN 1-62 aa (bold), **IBB** (bold and underline), R27 required for importin-β/Ketel binding (bold, underline, highlighted)

Fly SPN 63-381 aa

MEELSQALASSFSVSQDLNSTAAPHPRLSQYKSKYSSLEQSERRRRLLELQK SKRLDYVNHARRQQEQDDYRPLQNQEKPVPRKKSGKRSGHQKGIPYRPQLS EWLRHKPDDLNEWLLVPCPVGKRCLVVASKGITKAYSKGGWMFVNFRSSLPG DWQLQKGETILDCVYVEDADTFYVLDAISFGLQEVQECEASFRFYWLRARFEE HDYDKISENNEKKFKLLDHFDFEDPSAVEQALHKYPFFPENKPDLDGFLFYHKE ASYVCRETPLVCWLFPFMMEDVLGLPVNKCYKAPEDYQPSHVLQYMDAFEQK LAEHRRTLKEQKKKVNEQKEDPHTMEAEEDVESDEYDSLKRVLDQQRRLELG EFDMDCAEPPSADGC

Human SPN 1-186 nt

Fly SPN (dSNUP) 187-1140 nt

ATGGAAGAGTTGAGTCAGGCCCTGGCTAGTAGCTTTTCTGTGTCTCAAGAT CTGAACAGCACAGCTGCCCCACACCCCCGCCTATCCCAGTACAAGTCCAA GTACAGTTCCTTGGAGCAGAGTGAGCGCCGCCGGAGGTTACTGGAACTG CAGAAATCCAAGCGGCTGGATTATGTGAACCATGCCCAGCAGGAACAGGA TGACTACCGTCCGCTGCAAAATCAAGAGAAACCGGTGCCAAGAAAGAAAAG CGGGAAACGTTCTGGTCACCAAAAAGGCATCCCGTACAGACCACAACTCTC GGAGTGGCTGCGCCATAAGCCCGACGATCTCAACGAGTGGCTGCTGGTAC CTTGTCCAGTGGGCAAAAGGTGCCTCGTGGTGGCAAGCAAGGGGATCACC AAGGCGTACTCCAAAGGGGGCTGGATGTTCGTGAATTTCCGATCCTCGCTG CCCGGCGACTGGCAGCTCCAAAAGGGTGAAACAATACTAGACTGCGTGTAT GTTGAGGATGCGGACACCTTCTATGTGCTGGATGCCATATCATTTGGGCTA CAGGAAGTGCAGGAGTGCGAGGCGTCCTTTCGTTTCTATTGGTTGCGCGCC CGATTCGAGGAGCACGATTATGACAAGATTAGCGAGAACAACGAAAAGAAA TTTAAGCTCCTGGATCACTTCGACTTCGAGGACCCCTCCGCAGTAGAACAG GCTCTGCATAAGTATCCTTTTTTCCCGGAGAACAAGCCAGACCTAGATGGCT TCCTATTTTATCATAAAGAGGCTAGTTATGTGTGTCGGGAAACTCCCTTAGTA TGCTGGCTGTTTCCATTTATGATGGAGGATGTCCTTGGCTTGCCCGTTAATA AGTGCTATAAGGCCCCGGAGGATTACCAACCCAGCCATGTTTTGCAATATAT GGATGCATTTGAACAAAAGCTGGCAGAGCACAGAAGGACTCTTAAAGAGCA GAAAAAGAAAGTGAACGAGCAGAAGGAGGATCCACACACCATGGAGGCGG AAGAGGATGTCGAAAGCGATGAGTACGATAGCTTAAAGCGAGTACTGGATC AACAGCGACGTCTGGAGCTAGGTGAATTCGACATGGACTGTGCGGAGCCG CCATCAGCTGATGGCTGCTAG

Appendix XIC

dSNUP, Ketel, and Msk dsRNA target sequences

T7 sequence shown in italics

dSNUP forward/reverse primer region shown in bold

RNAi Ex1 dSNUP Target Sequence (317 nt):

GGCTACAGGAAGTGCAGGAGTGCGAGGCGTCCTTTCGTTTCTATTGGTTG
CGCGCCCGATTCGAGGAGCACGATTATGACAAGATTAGCGAGAACAACGAA
AAGAAATTTAAGCTCCTGGATCACTTCGACTTCGAGGACCCCTCCGCAGTA
GAACAGGCTCTGCATAAGTATCCTTTTTTCCCGGAGAACAAGCCAGACCTA
GATGGCTTCCTATTTTATCATAAAGAGGCTAGTTATGTGTGTCGGGAAACTC
CCTTAGTATGCTGGCTGTTTCCATTTATGATGGAGGATGTC**CTTGGCTTGCC
CGTTAATAAG**

Location: 467-783 nt of CG32997 CDS (Exon 1)

RNAi Ex1 dSNUP Forward Primer (T7) (61 nt):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***GGCTACAGGAA
GTGCAGGAGT**

RNAi Ex1 dSNUP Reverse Primer (T7) (61 nt):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***CTTATTAACGG
GCAAGCCAAG**

RNAi Ex3 dSNUP Target Sequence (331 nt):

CCAAAACCCTGCGGCTCTCCTGCTTTCTCAACCGGACTTGCAACCAGTTCA
ACCTCTTCAGCAGCAGCAGCAACAGGAACAAACACAAACGCAAAACCAACA
ACAACCGCAGCATCGCTCCACGGTTACTCCATTGGTGGGCGGCACTCTGCC
CACTCCTGTGCGCCGGCAGATATTCTGGAACACCGCGCAGATTTCGACTAC
CACCAAGTTCGTGCTGGATGTGCAGGCTAACCTATCGTTTGGCTTTGGCAC
CGACGGCAAGGAACGATTAGCCAGCAAGCATCCAGAATTGATACGCTACCT
GCCG**GATGGCGAGGACAGGGAGTGG**

Location: 1749-2079 nt of CG32297 CDS (Exon 3)

RNAi Ex 3 dSNUP Forward Primer (T7) (61 nt):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***CCAAAACCCTG
CGGCTCTCCT**

RNAi Ex3 dSNUP CG32297 Reverse Primer (T7) (61 nt):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***CCACTCCCTGT CCTCGCCATC**

RNAi Ketel Target Sequence (317 nt):

CTGGTGAACACGGCCAACAGTGCGGTGGCGCGAATGGCAGCCGGTCTCC
AGCTGAAGAACCACCTGACCAGCAAGGACGAGAAGGTCAGCCAACAGTAC
CAGGATCGCTGGCATCAGTTTCCCAGCGAGATCCGCGAGTTGATCAAGAAT
AACATCCTGGCTGCTTTGGGTACCGAGAACACCCGACCCTCCTGCGCCGCC
CAGTGCGTGGCCTATGTGGCCGTGATTGAGCTGCCGATAAACCGCTGGCC
CATGCTCATCCAGACACTGGTGAACAAGGTGGTCAGCGAAGGATCC**AGCG AGATGCATCGCGAGTCG**

Location: 148-465 nt of Ketel CDS

RNAi Ketel Forward Primer (T7):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***CTGGTGAACAC GGCCAACAGT**

RNAi Ketel Reverse Primer (T7):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***CGACTCGCGAT GCATCTCGCT**

RNAi Msk Target Sequence (393 nt):

TCGACATTATGCCTGCTCTGCATAACTATGTGACGGTCGACACGCCCGCTT
TCCTCTCCAATCCCAACAGGCTGTTGGCGATTCTCGACATGTGCAAAACGAT
GCTTACTAGTAGCCCTGGCGAGGATCCCGAGTGCCATGCCGCCAAACTGAT
GGAAGTGATTATCTTGCAGTGCAAGGGTCAAATCGACTCAGTGATACATATG
TTCGTGGAGCTGGCTCTGTCCCGGTTAACACGTGAAGTTCAATCCTCAGAG
CTGCGCACTATGTGCCTGCAAGTGGTAATCGCGGCACTCTACTATAATCCC
CAGTTGCTGCTGTCCATTCTGGACAAAATGTCCCAGCAAAACAACGACTCTA
TCAGCGCGCACT**TTATCAAGCAGTGGCTTCACG**

Location: 2069-2461 nt of Msk CDS

RNAi Msk Forward Primer (T7):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***TCGACATTATG CCTGCTCTGC**

RNAi Msk Reverse Primer (T7):

*GAATTCGAGCTCGGATCGATTAATACGACTCACTATAGGG***CGTGAAGCCAC TGCTTGATAA**

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