Genetic and cytological investigation of Nucleostemin-1 in Drosophila melanogaster

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GENETIC AND CYTOLOGICAL INVESTIGATION OF NUCLEOSTEMIN-1 IN DROSOPHILA MELANOGASTER

A Dissertation
Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In

The Department of Biological Sciences

by
Raphyel Ojomo Rosby
B.S., Louisiana State University, 2005
May 2010
Dedication

This thesis is dedicated to my late aunt Carolyn Ann Evans-Mitchell. She always did her best to encourage all of us, by her words, but more so by her actions.
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# Table of Contents

Dedication ........................................................................................................................................... ii

Acknowledgments ................................................................................................................................. iii

List of Figures ........................................................................................................................................ v

List of Abbreviations ............................................................................................................................. vii

Abstract ................................................................................................................................................ x

Chapter 1. Literature Review .................................................................................................................. 1

Chapter 2. Endogenous *Drosophila melanogaster* NS1 Localization and Characterization of a Nucleostemin-like GTPase ................................................................. 20

Chapter 3. Over-expression of NS1 and Associated Phenotypes ............................................................. 40

Chapter 4. RNAi-Mediated Knockdown of NS1 and Associated Phenotypes Suggest a Role in Large Subunit Ribosome Biosynthesis ................................................................. 48

Chapter 5. Conclusions and Future Direction ....................................................................................... 74

Literature Cited ....................................................................................................................................... 81

Appendix A: Chromosome Maps of Transgenic Stocks and Description ............................................... 89

Appendix B: Permissions ..................................................................................................................... 92

Vita ......................................................................................................................................................... 99
## List of Figures

1.1 Pre-rRNA processing in *S. cerevisiae* ................................................................. 6

1.2 A simplified view of the complex process of ribosome biosynthesis in yeast .............. 8

1.3 Mammalian Nucleostemin comprises nine domains .................................................. 11

1.4 Current NS model for cell cycle regulation ................................................................... 14

2.1 Amino acid sequence comparison of *Drosophila* and human nucleostemin proteins ................................................................. 29

2.2 Western blot using the anti-NS1 .................................................................................. 30

2.3 NS1 was expressed in all *Drosophila* cell types examined ........................................ 32

2.4 Immunolabeling of endogenous NS1 in nurse cells and primary spermatocytes .......... 33

2.5 Immunolabeling of endogenous NS1 in the larval wing disc ........................................ 34

2.6 Immunolabeling of endogenous NS1 in the larval brain cells ....................................... 35

3.1 Exogenous GFP-NS1 and mRFP-fibrillarin localized in different regions of the nucleolus ................................................................................................................. 43

3.2 When over-expressed as a GFP fusion, NS1 localized to nucleoli and salivary gland polytene chromosomes .......................................................... 44

3.3 Gross over-expression of GFP-NS1 caused melanization within the proventriculus .... 45

4.1 Semi-quantitative RT-PCR to monitor loss of NS1 transcript levels ............................. 54

4.2 RNAi-mediated depletion of NS1 transcripts correlated with a loss of NS1 protein ........ 55

4.3 Tissue-specific loss of NS1 caused growth arrest in larval salivary glands ................. 57

4.4 Loss of NS1 caused growth arrest in salivary glands .................................................. 58
4.5  Eye specific expression of NS1 is required for development of the *Drosophila* eye ................................................................. 59

4.6  NS1 is required for normal growth of *Drosophila* ommatidia ......................................................... 60

4.7  Depletion of NS1 affects larval midgut growth and differentiation.................................................. 61

4.8  NS1 is required for normal development of midgut imaginal island cells in third instar larvae ............................................................... 62

4.9  NS1 is required for export of RpL11 ....................................................................................................... 64

4.10  NS1 is required for export of RpL26 ....................................................................................................... 65

4.11  Ultrastructural analysis of wildtype Malpighian tubules ................................................................. 68

4.12  Loss of NS1 in the Malpighian tubules resulted in reduced cytoplasmic ribosomes ............................................................... 69

4.13  Depletion of NS1 leads to a depletion of cytoplasmic ribosomes .................................................... 70
List of Abbreviations

BiFC: Bi-Molecular Fluorescence Complementation
BrdU: Bromodeoxyuridine
cDNA: Complementary DNA
CS2: Chitin Synthase 2
CTP: Cytidine Triphosphate
DAB: 3, 3’-diaminobenzidine
da-GAL4: daughterless-GAL4
DAPI: 4’-6-diamidino-2-phenylindole dihydrochloride
DFC: Dense Fibrillar Component
ey-GAL4: eyeless-GAL4 Driver Line
FC: Fibrillar Center
FCS: Fetal Calf Serum
G1: GTP binding domain 1
G2: GTP binding domain 2
G3: GTP binding domain 3
G4: GTP binding domain 4
G5: GTP binding domain 5
GAL4-UAS: GAL4 Upstream Activation Sequences
GC: Granular Component
GFP: Green Fluorescent Protein
GNL3L: Guanine Nucleotide binding protein-Like 3 (nucleolar)-Like
GTP: Guanine Triphosphate
(H)DM2: (Human) Double Minute 2 Homologue
HNS: Human Nucleostemin

IPTG: Isopropyl β-D-1-thiogalactopyranoside

LB: Luria Bertani

LSU: Large Ribosomal Subunit

MDM2: Murine Double Minute 2 Homologue

MG: Midgut

MICs: Midgut imaginal Island Cells

mRFP: Monomeric Red Fluorescent Protein

mRNA: Messenger Ribonucleic Acid

mTOR: Metazoan Target of Rapamycin

NES: Nuclear Export Signal

NOR: Nucleolar Organizer Region

NPC: Nuclear Pore Complex

NS: Nucleostemin

NS1: Drosophila melanogaster Nucleostemin 1

NS2: Drosophila melanogaster Nucleostemin 2

NS3: Drosophila melanogaster Nucleostemin 3

NS4: Drosophila melanogaster Nucleostemin 4

NST-1: Caenorhabditis elegans Nucleostemin 1

p14ARF: Alternate Reading Frame

PBS: Phosphate Buffered Solution

pBS: pBluescript(-)

PCR: Polymerase Chain Reaction

PMSF: phenylmethylsulfonyl fluoride

PPP2R5A: Protein Phosphatase 2 Regulatory Subunit B (B56)
PTEN: Phosphatase and Tensin homologue
RNPs: Ribonucleoprotein Particles
RpL11: Large Ribosomal Subunit Protein 11
RpL23: Large Ribosomal Subunit Protein 23
RpL26: Large Ribosomal Subunit Protein 26
RpL5: Large Ribosomal Subunit Protein 5
RpS6: Small Ribosomal Subunit Protein 6
rRNA: Ribosomal Ribonucleic Acid
RSL1D1: Ribosomal L1-Domain-Containing 1 Protein
RT: Reverse Transcription
sal-GAL4: Salivary Gland GAL4 Driver line
SDS-page: Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
SFM: Serum Free Medium
siRNA: Small Inhibitory Ribonucleic Acid
snoRNP: Small Nucleolar Ribonucleoprotein Particle
SSU: Small Ribosomal Subunit
TCA: Trichloroacetic Acid
TOR: Target of Rapamycin
UTP: Uridine Triphosphate
XNS: Xenopus Nucleostemin
Abstract

Mammalian nucleostemin (NS) is a nucleolar GTP-binding protein implicated in cell cycle progression, stem cell proliferation, and ribosome assembly. *Drosophila melanogaster* contains a four-member nucleostemin family (NS1-4). Nucleostemin 1 (NS1) is the closest orthologue to human NS; it shares 33% identity and 67% similarity with human NS. We show that NS1 has intrinsic GTPase and ATPase activity, and that it is present within nucleoli of most larval and adult cells. Endogenous NS1 and lightly expressed GFP-NS1 enrich within the nucleolar granular regions as expected, while over-expressed GFP-NS1 localizes throughout the nucleolus and nucleoplasm, and to several transcriptionally active inter-bands of polytene chromosomes. Over-expression caused melanotic tumors and larval and pupal lethality. RNAi depletion of NS1 caused a loss of imaginal (precursor) cells in the larval midgut, and an apparent block in the nucleolar release of large ribosomal subunits in the terminally differentiated larval midgut polyploid cells. Depletion of 60% of NS1 transcripts lead to larval and pupal lethality. Ultra-structural examination of highly differentiated larval Malpighian tubule cells depleted for NS1 showed a loss of cytoplasmic ribosomes with a concomitant appearance of cytoplasmic pre-autophagosomes. We interpret the appearance of these structures as indicators of cell stress response.
Chapter 1
Literature Review

The nucleolus is the most distinctive sub-nuclear compartment. As such, it was first described by Felice Fontana in 1781 (Olson, 2004). Ribosome biosynthesis was defined nearly 200 years later in the 1960’s as the primary and best understood function of the nucleolus (Pederson, 1998). The nucleolus is not enclosed by a membrane like other organelles, but instead consists of interconnected loops of ribosomal DNA (rDNA) and ribosomal precursors (RNA and protein) (Pederson, 1998).

Metazoan nucleoli typically have three distinct sub-compartments, the fibrillar center (FC), the dense fibrillar component (DFC), and the granular component (GC). The aptly named fibrillar center contains the bulk of the ribosomal DNA. The site of rRNA synthesis remains an ongoing controversy, with some researchers insisting transcription occurs in the fibrillar center, and others insisting that it occurs at the FC-DFC border (Hozak et al., 1994; Thiry, 1992). Regardless of the actual site of rRNA synthesis, rRNA is post-transcriptionally processed in the DFC (Olson, 2004). Finally, the most peripheral component of the nucleolus, the granular component, owes its name to its grainy appearance as observed by transmission electron microscopy (He et al., 2008). The “grains’’ are nascent ribosomal subunits. The granular component contains the immature 60S Large Ribosomal Subunits (LSU) and 40S Small Ribosomal Subunits (SSU), where they are processed prior to their export to the cytoplasm.

Ribosomal genes contain transcribed sequences and intergenic spacers, and are located on one or more chromosomes in arrays of head-to-tail tandem repeats called nucleolar organizer regions (NORs). Three of the four rRNA transcripts, 18S, 5.8S and 28S are transcribed by RNA polymerase I in the nucleolus as a single large precursor RNA (47S in mammals, 37S in
Drosophila, and 35S in yeast). The fourth rRNA species, the 5S molecule, is transcribed in the nucleoplasm of metazoans by RNA polymerase III. In yeast, the 5S rRNA genes are linked to the pre-rRNA genes, and are therefore transcribed in the nucleolus (French et al., 2008). Eukaryotes have 80S cytoplasmic ribosomes, each consisting of a small (40S) and large (60S) subunit. Their large subunit is composed of a 5S RNA (120 nucleotides), a 28S RNA (4700 nucleotides), a 5.8S subunit (160 nucleotides) and approximately 49 proteins. The 40S subunit contains the 18S (1900 nucleotide) RNA and approximately 33 proteins (Alberts et al., 2008).

Although rRNA transcription, rRNA processing and ribosome assembly have been clearly established as the major functions of the nucleolus, it has become evident that the nucleolus participates in many other aspects of cell function as well. Thus, the nucleolus has been implicated in the processing or nuclear export of certain other RNAs (Pederson, 1998). In particular, the initial assembly of the signal recognition particle (a cytoplasmic complex consisting of a 7S RNA and six proteins) and the processing of the telomerase RNA involve the transit of these RNAs through the nucleolus. Furthermore, the nucleolus is involved in processing of the small nuclear U6 spliceosomal RNA (Pederson, 1998). Interestingly, these three nucleolus-associated small nuclear RNAs (the signal recognition particle RNA, telomerase RNA, and the U6 snRNA) share the common feature that they are components of catalytic ribonucleoprotein machines (Pederson, 1998). In addition to these well characterized functions, the nucleolus has been more recently implicated in cell cycle progression, ageing, stem cell and cancer cell maintenance, and protein storage (Beekman et al., 2006; Comai, 1999; Guarente, 1997; Ma and Pederson, 2007; Olson et al., 2000; Pederson, 1998; Pederson and Tsai, 2009; Tsai and McKay, 2002).

Nucleolar assembly (nucleogenesis) is a very complex and regulated event in the cell
cycle. At the start of mitosis, the nucleolus disassembles. This onset of disassembly coincides with the dissociation of RNA polymerase I from the rDNA genes (Leung et al., 2004) which is likely due to metaphase phosphorylation of Pol I and its transcription factors. In late mitosis, the nucleolus reforms at the nucleolus organizer in a very reproducible temporal manner (Leung et al., 2004). This reformation is characterized by the assembly of ribosomal proteins around the NORs. In *Drosophila melanogaster*, the NORs are located on the X and Y chromosomes (Ritossa and Spiegelman, 1965). In *S. cerevisiae*, the nucleolus organizer is at a single location on chromosome XII (Aris and Blobel, 1988) which is in further contrast to mammals that have several NORs located on several chromosomes. Interestingly, disruption of the interphase nucleolus has been proposed as a common feature in cellular stress responses that normally activate the p53 pathway (Leung et al., 2004).

**Ribosome Biosynthesis**

The process of ribosome biosynthesis is a multi-faceted nuclear event, starting with rRNA transcription, pre-rRNA processing and finally packaging of processed mature rRNAs and ribosomal proteins into ribosomal subunits. In *Drosophila melanogaster*, the 37S pre-rRNA is cleaved into the 18S, 5.8S and 28S rRNAs. The yeast model, however, is the most well established system to study ribosome biosynthesis. Maturation of yeast rRNA and its assembly into ribosomal subunits involves at least 170 accessory proteins comprising endo and exo-ribonucleases, putative ATP-dependent RNA helicases, ‘chaperones’ or ‘assembly factors’ and many small nucleolar ribonucleoprotein particles (snoRNPs) that contain specific guide RNAs (see Figure 1.1) (Fromont-Racine et al., 2003; Zhang et al., 2007). These snoRNPs include the Box C/D and Box H/ACA snoRNAs. The box C/D snoRNAs are characterized by their association with the nucleolar proteins fibrillarin, Nop5/Nop58, and Nop56 (Dunbar et al., 2000). The Box
C/D snoRNAs anneal to specific sites on the pre-rRNA, where they guide site specific methylation of nucleotides in the rRNA (Dunbar et al., 2000). Conversely, the Box H/ACA snoRNPs catalyze site-specific pseudouridylation of the rRNA, and are essential architectural components of vertebrate telomerases (Hamma et al., 2005). H/ACA RNPs comprise four proteins (GAR1, NAP57, NOP10, NHP2) and specific multi-helical snoRNAs (Hamma et al., 2005; Meier, 2005).

During eukaryotic ribosome assembly, the 35S–45S primary transcript is packaged into a 90S ribonucleoprotein particle, together with a subset of assembly factors and ribosomal proteins. Subsequent steps trigger folding, chemical modification in the form of methylation and pseudouridylation, further cleavage of pre-rRNAs and association of additional assembly factors and ribosomal proteins to form 43S and 66S ribosome assembly intermediates (Fromont-Racine et al., 2003). These pre-rRNPs undergo further maturation in the nucleoplasm and then final maturation in the cytoplasm to form functional mature 40S small and 60S large ribosomal subunits, respectively. Large and small ribosomal subunits in the granular components undergo separate maturation steps, but are related in export by the export adapter protein, CRM1. Export of the 40S subunit however, is still uncharacterized in detail (Fromont-Racine et al., 2003). For example, the 20S rRNA in the small ribosomal subunit is cleaved in the cytoplasm to yield the final mature 18S rRNA and the small 5’ fragment of the Internal Transcribed Spacer 1 (ITS1) (see Figure 1.1). The 5’ ITS1 RNA fragment is normally degraded by the cytoplasmic Xrn1 exonuclease, but in yeast strains lacking XRN1, the 5’ ITS1 fragment accumulates in the cytoplasm (Moy and Silver, 1999). Thus, using the cytoplasmic localization of the 5’ ITS1 fragment as an indicator for the export of the small ribosomal subunit, several genes have been identified that are required for small subunit export.
Mutations in Ran-GTPase, Nup82 and Xpo1/Crm1 caused the short 5’ ITS1 to accumulate in the nucleoplasm indicating a failure in small subunit export. Furthermore, mutations in the genes encoding a subset of nucleoporins and the nuclear transport factors Srp1, Kap95, Pse1, Cse1, and Mtr10 cause the 5’ ITS1 to accumulate in the nucleolus, thus affecting SSU assembly (Moy and Silver, 1999).

Conversely, regulation of pre-60S subunit biogenesis and transport is strongly dependent on the function of several putative GTPases in yeast. These include Nog1, Nug2/Nog2, Lsg1/Kre35, and Nug1 (Du et al., 2006; Fuentes et al., 2007; Kallstrom et al., 2003; Reynaud et al., 2005; Saveanu et al., 2001). With the exception of Nog1, all of these proteins belong to a novel family of GTPases called the YawG family of GTPases (Leipe et al., 2002).

Nuclear export of the large ribosomal subunit requires the NES (Nuclear Export Signal) containing adapter protein Nmd3, as well as Xpo1/Crm1, Ran-GTPase, and Nup82. Mutations in Nmd3 lead to a nuclear buildup of large ribosomal subunits (Gadal et al., 2001)(See Figure 1.2).

**GTPases Involved in Ribosome Biosynthesis**

Several small GTPases are required for ribosome biosynthesis. Many of these small GTPases have described roles in rRNA processing, and their mutations lead to aberrant rRNA processing events. In *S. cerevisiae*, there are at least 4 small nucleolar GTPases with described roles in ribosome biosynthesis (Bassler et al., 2006).

Nog1 is a yeast nucleolar GTPase whose function is linked to 60S maturation, and its depletion results in nucleolar accumulation of 60S subunits with a net loss of cytoplasmic 60S subunits (Jensen et al., 2003). Temperature sensitive Nog1 mutants show an accumulation of 35S pre-rRNA intermediates as well as delayed 27S to 25S processing at non-permissive temperatures (Jensen et al., 2003). In Nog1 mutants, the mature 25S rRNA does not accumulate
Figure 1.1 Pre-rRNA processing in *S. cerevisiae*. Panel A. rDNA repeat: Structure of the yeast pre-rRNA 35S containing the mature rRNA, 18S, 5.8S and 25S with external and internal transcribed spacers. Panel B: Pre-rRNA processing requires many coordinated steps to produce the final mature rRNA. (Adapted from Venema and Tollervey, 1999.)*

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to the level of the 18S rRNA which indicates aberrant processing of the large subunit (Kallstrom et al., 2003). Pre-60S complexes purified with Nog1-TAP (Tandem Affinity Purification) are strongly enriched for 27SA2, 27SA3, 27SB, and 7S rRNA intermediates, and somewhat enriched in 25S, 5.8S, and 5S rRNA intermediates which accentuate the direct association of Nog1 with nascent 60S subunits, and its early association in ribosome biosynthesis (Saveanu et al., 2003) (see Figure 1.2). Therefore, it appears that Nog1 associates with the assembling pre-60S particle early and dissociates relatively late, but prior to pre-60S export to the cytoplasm. Northern analysis of Nog1 mutants showed a slight accumulation of the 32S rRNA intermediate and a modest accumulation of 35S precursors, suggesting a delay in the processing at sites A0, A1, and A2 (see Figure 1.2) (Fuentes et al., 2007). Mutations in the conserved residues of the GTP-binding pocket do not affect Nog1’s association with 60S ribosomes, but these mutations disrupt its function in ribosome biosynthesis (Fuentes et al., 2007). Another interesting study of Nog1 shows that metazoan Target of Rapamycin (mTOR) regulates late steps of ribosome maturation in the nucleoplasm via Nog1 in response to nutrients (Honma et al., 2006). mTOR is a kinase that regulates several cellular processes, such as cell cycle, autophagy and ribosome biosynthesis. Hence, mTOR is a master regulator of many functions involved in cell proliferation and growth (Zhang et al., 2006).

The YawG family of GTPases is characterized by the circularly permuted order of their GTP-binding motifs (Bassler et al., 2006). For example, the GTP-binding domains in canonical GTPases like Ras or Ran appear in the order of G1, G2, G3, G4, G5 (Anand et al., 2006), but in Nug1 and other YawG family GTPases, the order of appearance is G4, G5, G1, G2 and G3. Furthermore, this subset of the larger GTPase superfamily is conserved from Archaebacteria to humans (Reynaud et al., 2005).
Figure 1.2 A simplified view of the complex process of ribosome biosynthesis in yeast. The process of ribosome biosynthesis/maturation involves the activity of several GTPases, exo- and endo-nucleases and many non-ribosomal proteins to finally yield a mature ribosomal subunit. Nmd3 is required for export of large ribosomal subunits (adapted from Fromont-Racine et al., 2003). The characterized GTPases that function in ribosome synthesis and transport are highlighted in boxes.*
Nog2 is a YawG family GTPase, but it also localizes to the nucleolus and the nucleoplasm (Saveanu et al., 2001). Like Nog1 mutants, kinetic and steady-state measurements of the levels of pre-rRNAs in Nog2p-depleted cells showed a defect in 5.8S and 25S maturation, and a concomitant increase in the levels of both 27SB5 and 7S5 intermediates (Saveanu et al., 2001). These early processing errors are commonly observed for mutations that impair late steps of 5.8S/25S maturation, that then feed back on early processing steps from A0-A2 (Saveanu et al., 2001). In contrast with the loss of Nog1 which caused nucleolar accumulation of 60S subunits, depletion of Nog2, allowed the pre-60S ribosomal complexes to leave the nucleolus, but they were retained in the nucleoplasm, and not exported to the cytoplasm (Kallstrom et al., 2003). These results suggest that transient and possibly GTP-dependent association of Nog2 with the 60S subunit might trigger late maturation steps (Saveanu et al., 2001). Immunoprecipitation experiments using Nog2 antibodies recovered Nog1, but the inverse was not observed, suggesting that Nog2 had a brief interaction in 60S export or maturation as opposed to Nog1 (Saveanu et al., 2001). Another interesting observation is that Nog2 was enriched in cellular fractions containing nuclear pore complex (NPC) proteins, which further suggested its direct role in late ribosomal maturation or nuclear export (Saveanu et al., 2001).

Nog1 and Lsg1 are nucleolar and cytoplasmic proteins, respectively, and they were never simultaneously associated with the same pre-ribosomal particle (Kallstrom et al., 2003). However, mutations in both proteins showed an accumulation of 60S ribosomal subunits within the nucleolus. Release of the large subunit from the nucleolus into the nucleoplasm likely coincides with the release of the 66S-associated biogenesis factors, yielding a nucleoplasmic pre-60S subunit (Kallstrom et al., 2003). Both Nog1 and Lsg1 co-immunoprecipitated with the export adapter protein, Nmd3. This suggested a sequential association consisting of Nog1 in the
nucleoplasm and Lsg1 in the cytoplasm (Saveanu *et al.*, 2001). Thus, Lsg1 may act to recycle export factors back to the nucleolus (Kallstrom *et al.*, 2003).

Nug1 is yet another protein in the YawG family of GTPases. Nug1 is an essential nucleolus localized GTPase required for nucleolar release of the 60S subunit (Du *et al.*, 2006). The N-terminal basic domain targets Nug1 to the nucleolus, mediates association with pre-60S particles and exhibits non-specific RNA-binding activity (Bassler *et al.*, 2006). Nug1 function was investigated with truncation mutants. Removal of the first 37 amino acids of Nug1 exhibited a slightly reduced growth rate, whereas deletion of the first 100 amino acids or the entire N-terminal domain caused a lethal phenotype. In addition, removal of the last 176 amino acids was also lethal (Bassler *et al.*, 2006). Deletion of the central domain of Nug1 resulted in only a slight growth defect. The central domain of Nug1, which comprises the GTPase fold, is therefore not essential for cell growth and thus may fulfill a redundant role with other GTPases involved in ribosome biogenesis (Bassler *et al.*, 2006). Like Nog1 and Lsg1, Nug1 deficient cells failed to release the 60S subunit from the nucleolus, and therefore failed to export 60S particles from the nucleus (Bassler *et al.*, 2006).

**Mammalian Nucleostemin**

Nucleostemin (NS) is the vertebrate homologue of *S. cerevisiae* Nug1. It should be noted here that expression of a human nucleostemin transgene was unable to rescue growth defects observed in *Nug1* mutants (Bassler *et al.*, 2006). NS was first described in the nucleoli of rat cortical stem cells (Tsai and McKay, 2002). Human NS is 549 amino acids in length, and like Nug1 and Nog2, it is a YawG GTPase family member (Tsai and McKay, 2002; Tsai and Meng, 2009). The structure of NS consists of a N-terminal basic domain (B), followed by a coiled-coil domain (C), five GTP-binding motifs (G5, G4, G1, G2 and G3), an inhibitory region (I) following
the G3 motif and finally a COOH-terminal acidic domain (A) (Tsai and McKay, 2005).

Figure 1.3 Mammalian Nucleostemin comprises nine domains. It has a N-terminal basic domain (B) which spans amino acids 1-47. The basic domain is followed by a coiled-coil domain (C). After the coiled coil domain are the five GTPase binding motifs. Following the fifth GTP binding motif is an inhibitory domain (I) which blocks nucleolar retention of NS when GTP is not bound. Finally the carboxy terminus contains an acidic region (A). (Adapted from Tsai and McKay, 2005.)

Like Nug1, NS shuttles between the nucleolus and the nucleoplasm. NS requires its N-terminal basic domain for transient nucleolar localization. In addition, its GTP binding capacity regulates its long term retention within nucleoli (Tsai and McKay, 2005). The coiled-coil domain appears to regulate protein-protein interactions (Tsai and McKay, 2005). The inhibitory domain is sufficient to block nucleolar retention of NS when GTP is not bound. Furthermore, when the inhibitory domain is deleted, the nucleolar localization was restored in mutants that cannot bind GTP (Tsai and McKay, 2005).

Nucleostemin is up-regulated in several cancer and stem cell lines (Beekman et al., 2006; Hoshi et al., 2007; Qiao et al., 2008; Tsai and McKay, 2002; Tsai and Meng, 2009). Similar up-regulated expression of NS in these two cell types has led to the speculation that NS functions in cell proliferation (Beekman et al., 2006; Politz et al., 2005; Tsai and McKay, 2002; Zhu et al., 2006). Furthermore, the levels of NS drop dramatically just prior to terminal differentiation of stem cells (Tsai and McKay, 2002). After terminal differentiation, nucleostemin is virtually undetectable by Western analysis. Thus, the unique temporal expression pattern has led some researchers to speculate that NS has functions in stem cell
pluripotency as well (Tsai and McKay, 2002). Besides expression data, investigations of nucleostemin’s molecular interactions also suggest a specific role for NS in stem cell pluripotency (Meng et al., 2006; Zhu et al., 2006).

**Mammalian Nucleostemin Interactions**

NS binds several proteins such as p53, MDM2 (the p53 ubiquitin ligase), nucleolar proteins RSL1D1 and B23, and the telomere-specific TRF1 protein (Dai et al., 2008; Ma and Pederson, 2007; Meng et al., 2006; Zhu et al., 2006). By far, the most well characterized interacting partner of NS is p53. In mammals, p53 is a master regulator of cell cycle progression and an initiator of apoptosis.

Stresses such as UV radiation, ionizing radiation, oncogene signaling, hypoxia, blockage of transcription and lack of nucleotides can elicit the activation of p53. p53’s stress responses can vary from cell cycle arrest, to DNA repair, apoptosis and blockage of angiogenesis (Weinberg, 2007). In normal healthy cells, p53 is kept at low levels by a complex regulatory system. One of the primary regulators of p53 stability is MDM2, the ubiquitin ligase that targets p53 for degradation (Weinberg, 2007). In a normal healthy cell, MDM2 is primarily nucleoplasmic, and its ubiquitylation activity prevents p53 stabilization. Conversely, under stressful conditions, the tumor suppressor p14ARF (alternate reading frame) binds MDM2 and sequesters it in the nucleolus, thus stabilizing p53 (Weinberg, 2007). This regulatory system helps to maintain p53 at proper levels under various physiological conditions.

Small interfering RNA (siRNA) knockdown of NS in cultured cells induced p53-dependent cell cycle arrest and apoptosis (Tsai and McKay, 2002). This observation is in perplexing contrast to the scenario in which over-expression of hemagglutinin (HA) tagged NS in cultured cells also caused p53-dependent apoptosis (Tsai and McKay, 2002). Furthermore, when p53 was
stabilized in cultured cells via UV irradiation, NS was down-regulated (Ma and Pederson, 2007). NS knockdown lead to G1 cell cycle arrest in p53-positive cells, but not in cells in which p53 was genetically deficient or depleted by siRNA (Ma and Pederson, 2007). This suggests that NS is tightly regulated, and that too much or too little NS expression may be detrimental to cell viability. A different approach using transgenic mouse models provided more conflicting data: In blastocysts that were deficient for p53 by gene knockout, co-knockout of NS still caused cell cycle arrest and lethality (Beekman et al., 2006), thus suggesting that NS may act independently of p53 (Beekman et al., 2006; Romanova et al., 2009). This is contrary to the cell culture model in which depletion of NS caused p53-dependent cell cycle arrest and apoptosis. The p53-mediated apoptosis when NS was knocked down in cultured cells has not been reproduced in whole animals. This suggests that NS uses an alternative mechanism to regulate cell cycle.

With the wealth of evidence regarding NS interactions with p53 in cell cycle regulation of stem cells or cancer cells (many characterized cancer cells types have a defective p53), it is interesting to note that there is no solid “mechanistic” model that supports a functional interaction between NS and p53. In other words, the p53 link may be a secondary role in the activity of NS. As previously stated, NS is abundant in stem cells and cancer cells, but it appears to be ubiquitously expressed in many mammalian cell types as well (Romanova et al., 2009). It is therefore conceivable that the principal role of NS is in ribosome biosynthesis, and not in direct interaction with p53 to regulate cell cycle progression.

To explain the contrasting observations described above, over-expressed NS might bind and inhibit MDM2 function, thus allowing p53 activation (Dai et al., 2008). Conversely, when NS is knocked down, RpL5, RpL11, and RpL23 are released into the nucleoplasm (Dai et al., 2004). This current model for NS function (Figure 1.4) proposes that nucleolar disruption occurs upon
loss of NS. These ribosomal proteins then bind MDM2 and thereby inhibit its function in
deactivating p53.

![Diagram of cell cycle regulation]

Figure 1.4 Current NS model for cell cycle regulation. Nucleostemin is a highly dynamic
nucleolar protein. Cell culture studies show that over-expression and knockdown of NS
causes p53 activation and subsequent cell cycle arrest and apoptosis. In the current model,
when over-expressed, NS binds MDM2 and inhibits its p53 destabilization activity.
Conversely, when NS is depleted, nucleolar disruption releases RpL11 and RpL5 which in turn
inhibit MDM2 to thus stabilize p53. (Adapted from Dai et al., 2008.)

Besides interacting with p53 and MDM2, NS may also be regulated in part by p14\textsuperscript{ARF}. The
well characterized tumor suppressor protein, p14\textsuperscript{ARF}, shuttles through the nucleolus, which
supports the model of the nucleolus as a storage compartment and regulator of p53 activity
(Olson and Dundr, 2005). In addition, p14\textsuperscript{ARF} localizes in the same compartments of the
nucleolus as does NS. This further suggests that the pro-apoptotic function of p14\textsuperscript{ARF} is in part
regulated by and intimately linked to the nucleolus. p14\textsuperscript{ARF} also regulates sumolation (addition
of a small ubiquitin-like protein) of (H)DM2 [(human) double minute 2 homologue, the
homologue of MDM2] and inhibits its E3 ubiquitin ligase activity, which in turn stabilizes p53
(Tago \textit{et al.}, 2005). NS is also down-regulated when tumor suppressor p14\textsuperscript{ARF} is over-expressed
exogenously (Ma and Pederson, 2007). This scenario serves as a support for the role of p14\textsuperscript{ARF}
as a regulator of NS function.

It is important to note here that \textit{Caenorhabditis elegans} and \textit{Drosophila melanogaster}
lack a described MDM2, p14\textsuperscript{ARF} or nucleolar protein B23 (Jin \textit{et al.}, 2000; Mogila \textit{et al.}, 2006;
Moon et al., 2008). Furthermore, the role and regulation of p53 in *Drosophila* varies from that of mammalian p53; the primary difference is that p53 in *Drosophila* is required for stress-induced apoptosis, but not for cell-cycle arrest (Sogame et al., 2003). In addition, *Drosophila* p53-dependent apoptosis following DNA damage depends on the protein kinase Mnk/Chk2, which phosphorylates p53, but does not change the levels of p53 protein. This observation is in stark contrast to mammalian models in which p53 stability is primarily regulated by MDM2 (Alarcon-Vargas and Ronai, 2002; Brodsky et al., 2004), and the accumulation of p53 is dependent upon phosphorylation by ATM/ATR or Chk2, which blocks p53’s interaction with MDM2.

In (U2OS) human osteosarcoma cells, NS binds nucleolar protein B23 (nucleophosmin) (Ma and Pederson, 2008). B23 is a major nucleolar protein in interphasic vertebrate nucleoli, where it is involved in the assembly of pre-ribosomes (Lindstrom and Zhang, 2008). B23 is a multifunctional nucleolar protein and a member of the nucleoplasmin superfamily of acidic histone chaperones. B23 is essential for normal embryonic development and plays an important role in genomic stability, ribosome biogenesis, and anti-apoptotic signaling. Furthermore, altered protein expression or genomic mutation of B23 is encountered in many different forms of cancer (Lindstrom and Zhang, 2008). With regard to NS, co-immunoprecipitation experiments revealed that NS and B23 co-reside in complexes, and yeast two-hybrid experiments confirmed that they are interacting proteins. The B23-interactive region in NS is the 46 amino acid N-terminal domain. In further support, Bi-Molecular Fluorescence Complementation (BiFC) experiments showed a high degree of co-localization of these two proteins in the granular component of nucleoli (Ma and Pederson, 2008). This interaction with B23 suggests that NS may in fact have a role in ribosome biosynthesis.
In the nucleolus, NS also interacts with the ribosomal L1-domain-containing 1 protein (also called Cellular Senescence-Inhibited Gene) (RSL1D1/CSIG). This protein belongs to the L1p/L10e family, which is defined by its N-terminal ribosomal L1p/L10e consensus sequence (residues 30-260). RSL1D1 colocalizes with NS in the same sub-nucleolar domain, and it also affects the nucleolar distribution of NS (Meng et al., 2006). In addition, RSL1D1 negatively regulates translation of the tumor suppressor, Phosphatase and Tensin homolog (PTEN), thus promoting cell cycle progression. RSL1D1 is abundant in dividing fibroblasts, but expression declines upon replicative senescence, not unlike NS (Ma et al., 2008). Replicative senescence is defined as a state of proliferative arrest accompanying the replicative exhaustion of cultured cells (Ma et al., 2008). The predominant nucleolus localization of RSL1D1 suggests that RSL1D1 acts as a ribosome-associated protein. The hypothesis that RSL1D1 is a ribosome-associated protein is supported by the evidence that RSL1D1 is present in the cell’s polysomal fraction (Ma et al., 2008). Thus, the interaction between NS and RLS1D1 provides insight into a putative role for NS in ribosome biosynthesis.

In addition to the nucleolar proteins listed above, NS also binds TRF1 (Telomerase Repeat Factor 1) (Zhu et al., 2006). TRF1 is a telomerase associated factor that provides a negative feedback mechanism for telomere length maintenance by blocking the access of telomerase to telomeres to thus elongate them (Zhu et al., 2006). NS in turn negatively regulates TRF1 \textit{in vivo}. This is an interesting observation in that NS has been described as a stem cell maintenance protein, and its association with TRF1 supports a role in telomere maintenance, which is required for stem cell maintenance (Zhu et al., 2006). This observation offers additional support for NS in maintaining the pluripotent capacity of stem cells. NS has also been show to bind human protein phosphatase 2 regulatory subunit B (B56) known as
PPP2R5A (Yang et al., 2005). PPP2R5A belongs to the phosphatase 2A regulatory subunit B family. Phosphatase 2A is one of the four major Serine/Threonine phosphatases, and it plays an important role in negative control of cell growth and division as well as in cell cycle progress (Yang et al., 2005).

Finally, a recent study (Romanova et al., 2009) has established a direct link between NS and ribosome biosynthesis. NS forms a large protein complex (>700 kDa) that co-fractionates with the pre-60S ribosomal subunit in sucrose gradients. This complex contains proteins related to pre-rRNA processing, such as Pes1, DDX21, and EBP2, in addition to several ribosomal proteins (Romanova et al., 2009). The nucleolar retention of DDX21 and EBP2 is dependent on the presence of NS in the nucleolus. Furthermore, the knockdown of NS delays the processing of 32S pre-rRNA into 28S rRNA, and is accompanied by a substantial decrease of protein synthesis as well as the levels of rRNAs and some mRNAs (Romanova et al., 2009). Conversely, when over-expressed, NS significantly promotes the processing of 32S pre-rRNA (Romanova et al., 2009).

**Nucleostemin in Other Species**

The YawG family of GTPases is unusual in having permuted GTP binding domains. Many members this GTPase family have roles in ribosome biosynthesis. Therefore, we expect that this ancient family of GTPases will be well conserved across species.

Recall that Nug1 was required for 60S pre-rRNA processing in yeast (Bassler et al., 2006). Like NS, Nug1 has an amino-terminal basic domain that regulates its localization to the nucleolus. The amino terminal domain of human NS is 46% identical and 70% similar to that of Nug1 (Rosby et al., 2009), suggesting that in addition to localization control, they share the same functional role in the nucleolus.
NST-1, the C. elegans NS homologue was also required for proper ribosome biosynthesis (Kudron and Reinke, 2008). Like mammalian NS, NST-1 is a nucleolar protein. NST-1 mutants consistently had reduced levels of 26S and 18S rRNA. Furthermore mutations in NST-1 caused an inhibition of cell growth, and subsequent cell cycle arrest in the germline stem cells and intestinal cells (Kudron and Reinke, 2008). Specifically, at hatching, wild-type animals had 20 intestinal cells, 14 of which divided at the L1 molt resulting in 34 intestinal nuclei. NST-1 mutants also had 20 intestinal cells at hatching, but these cells did not divide at the L1 molt as seen in wild-type animals (20 versus 31 cells at 16 hours post hatching)(Kudron and Reinke, 2008). Interestingly, when the NST-1 mutation was put into a ced-4 mutant background, which is incapable of apoptosis, the mutant phenotype of reduced cell number persisted. This result suggested that NST-1-mediated lethality and growth defects were probably independent of apoptosis, but due to lack of growth or cell division (Kudron and Reinke, 2008).

XNS (Xenopus laevis NS) was required for cell cycle progression in the neural crest cells. Specifically, there was a greatly reduced mitotic cell count in the neural crest after siRNA injection. This suggested a block in cell cycle progression upon loss of XNS (Romanova et al., 2009).

NS1 is a part of the Drosophila family of NS proteins. This family includes NS1, NS2, NS3 and NS4. To date, the only characterized Drosophila NS family protein is NS3. Unlike mammalian NS which is predominantly nucleolar, NS3 is related to yeast Lsg1, a cytoplasmic protein. NS3 is found primarily in a small cluster of serotonergic neurons, where it regulates growth of the organism through an insulin-like signaling mechanism (Kaplan et al., 2008). NS3, which appears to be required in only these serotonergic neurons, can regulate body size and development through its action in the serotonergic neurons. For instance, loss of NS3 caused
global growth defects, and these growth defects could be rescued by simply restoring NS3 to the 106 serotonergic neurons (Kaplan et al., 2008). NS2 and NS4 have not been characterized in any great detail. NS2 is another nucleolar permuted GTPase. NS4 is apparently dispensable for viability (Kaplan et al., 2008).

This thesis focuses on NS1 in Drosophila melanogaster. Chapter 2 describes the hypothesis that tests whether or not NS1 is expressed in progenitor cells. Chapter 3 describes the phenotypes associated with NS1 over-expression. Chapter 4 then describes the hypothesis that tests if NS1 is required for large subunit export. Chapter 4 also examines sub-cellular phenotypes caused by the loss of NS1.
Chapter 2

Endogenous *Drosophila melanogaster* NS1: Localization and Characterization of a Nucleostemin-like GTPase*

**Introduction**

GTPases exist in every form of life, and they are the oldest enzymes that use nucleotides as co-factors (Reynaud *et al.*, 2005). There are several distinct families of GTPases. NS is a member of the YawG/YlqF family of small GTPases (Tsai and Meng, 2009). This family contains many members that are characterized by their circularly permuted GTP binding domains (Bassler *et al.*, 2006). Some members of this family, such as yeast Nug1 and Nog2 have apparent roles in ribosome biosynthesis and/or export (Bassler *et al.*, 2006; Kallstrom *et al.*, 2003; Saveanu *et al.*, 2003).

Mammalian NS is abundant in stem cells and cancer cells, but virtually undetectable by western blot in terminally differentiated cells (Tsai and McKay, 2002). Therefore, the current model suggests that NS is a putative stem cell maintenance protein, based on its temporal expression pattern. However, it is well documented that several nucleolar proteins that function in ribosome synthesis are up-regulated in rapidly dividing cells, such as p120, B23, Nucleolin, p145 and p40 (Chatterjee *et al.*, 1987a, b; Freeman *et al.*, 1988; Freeman *et al.*, 1986; Korgaonkar *et al.*, 2005; Ochs *et al.*, 1988; Srivastava and Pollard, 1999). This up-regulation appears to be due to an increased demand for ribosomes, rather than for maintenance of pluripotency.

NS homologues can be found throughout a wide range of species, and all known homologues show conservation in their functional and catalytic domains (Kudron and Reinke,

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The sequence homology between homologues suggests that NS is an ancient protein with a conserved role in the cell. In support of this hypothesis, all eukaryotic NS homologues are nucleolar proteins, and most of them have described roles in ribosome synthesis (Bassler et al., 2006; Kudron and Reinke, 2008; Romanova et al., 2009).

This chapter establishes the expression patterns of endogenous NS1 in Drosophila melanogaster and addresses some of the hypotheses associated with mammalian NS. Furthermore, it puts forth the hypothesis that Drosophila NS1 will be up-regulated in rapidly dividing cells, in those cells that are preparing to undergo rapid divisions, and in those cells that have a high metabolic requirement for ribosome biosynthesis.

**Materials and Methods**

**Fly Stocks**

_Drosophila melanogaster_ stocks were maintained at room temperature (22-23°C).

There were two strains used primarily in this study. The _w^118_ strain is effectively wildtype except for eye color; it was used for transformation and as a control fly line for biochemical or microscopic analyses. The second strain used was _GFP-NS1-A1_, which is a transgenic line that expresses GFP-NS1 (see below).

**Protein Purification and Antibody Production**

The full length _Drosophila NS1_ cDNA (AT23067) was amplified by the PCR and ligated into _pET-30a_ (Novagen) between the _HindIII_ and _XhoI_ sites. The forward and reverse primers for amplifying _NS1_ for ligation into _pET-30a_ (Novagen) were 5'-GAGGATGAAGCTTCATGGCTTTTTAAAAAGGTG-3' and 5'-GTGGTTCTCGAGTAGTGTACTATCTACAG-3', respectively. The _HindIII_ and _XhoI_ sites are underlined in the primer sequences. The _pET-30a_ plasmid containing the full length _NS1_ cDNA was transformed into the BL21(DE3) strain of _E._
coli cells (Novagen). Transformed cells were grown in 100 ml of LB broth at 37° C until OD$_{600}$ reached 0.6, at which time expression was induced by adding IPTG (Isopropyl β-D-1-thiogalactopyranoside) to a final concentration of 1 mM. Induction produces a 6xHis-tagged NS1. Upon induction, the temperature was reduced to 26° C for 2-3 hours to allow better expression. Cells were harvested and sonicated in 4 ml of ice cold binding buffer (5 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9) containing 1 mM PMSF (phenylmethylsulfonyl fluoride). The sonicated extracts were centrifuged, and then filtered through a 20 µm filter to remove particulate matter. Filtered extract was added to a charged Ni$^{2+}$ column, and allowed to drain to the top of the column bed. The column was then washed with 10 column volumes of 1X binding buffer. Next, the column was washed with 6 column volumes of 1X wash buffer (60 mM imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9). Finally, bound proteins were eluted by washing the column with 6 column volumes of 1X elute buffer (1M imidazole, 500 mM NaCl and 20 mM Tris-HCl, pH 7.9). Eluted proteins were collected and stored at -80°C. After column purification, 750 µl of 20% TCA was added to 750 µl of the eluted His-NS1 proteins. The solution was then centrifuged for 15 minutes at 4°C. Following centrifugation, the precipitated proteins were washed with 1 ml of 80% acetone. After washing, 300 µl of Laemmli sample buffer and 15 µl of β-mercaptoethanol were added to each tube. Samples were boiled for 10 minutes, after which they were loaded onto a SDS-PAGE mini-gel. Proteins were resolved for approximately 35 minutes at 200 volts. Bands corresponding to the full length His-NS1 were excised and stored in 1.5 ml Eppendorf tubes. Protein bands were sent to Aves Labs (Tigard, OR) for immunization of hens to produce polyclonal antibody (IgY).
Plasmid Constructions and Fly Transformations

The *Drosophila NS1* cDNA (AT23067) was amplified by the PCR (Polymerase Chain Reaction) using 5′-CGACCTCGAGCTCAAGCTTATGGC as the forward primer and 5′-GTCGACGGTACCAGTGTACTACTACAG-3′ as the reverse primer. *HindIII* and *KpnI* sites are underlined in the forward and reverse primers, respectively. The PCR product was cut at the *HindIII* and *KpnI* sites and subsequently ligated into *pEGFP-C3* (Clontech) at corresponding sites. The ATG start codon for *NS1* is italicized in the forward primer. Continuity of the open reading frame between the *GFP* cDNA and the *NS1* cDNA was checked by transfecting HeLa cells with this plasmid; GFP-NS1 localized well to human nucleoli. DNA encoding GFP-tagged NS1 was then removed from *pEGFP-C3* using *NheI* and *KpnI*, and ligated into *pBluescript(-) (pBS-)* at its *SphI* and *KpnI* sites. DNA encoding GFP-NS1 was next removed from *pBS-* using *NotI* and *KpnI*, and ligated into the same sites within *pUAST*, a *Drosophila* P-element transposon-based transformation plasmid (Rorth, 1998; Zhu and Stein, 2004) that contains the selectable *mini-white* gene with its own promoter, along with tandem yeast *GAL4* UASs and the *Drosophila Hsp70* promoter that can drive transgene expression when induced by GAL4 or heat shock, respectively. The final *pUAST* recombinant plasmid and a helper plasmid (*pUCHsΔ2*) encoding transposase were co-injected into homozygous or hemizygous *w^1118 Drosophila* embryos according to established techniques (Rubin and Spradling, 1982). Seven independent insertion lines were recovered that expressed GFP-NS1. Insertions were mapped to the X, 2nd, or 3rd chromosomes using standard segregation analyses with 2nd and 3rd chromosome balancers, *CyO* and *TM3* respectively. The *GFP-NS1-A1* line was used most frequently in these studies; it maps to the 3rd chromosome. Homozygous *GFP-NS1-A1* flies were crossed to flies homozygous for the *daughterless (da)-GAL4* transgene (also on the 3rd chromosome) to induce ectopic
expression of GFP-NS1 in heterozygous progeny (see Drosophila GAL4 system below).

To express a FLAG-tagged version of NS1, the *Drosophila NS1* cDNA (AT23067) was amplified using 5’-CACCGCTTTAAAAAGGTTGAAGACCAAG-3’ as the forward primer and 5’-AATTTATCAATCACATAGTCCTCATCAAATC-3’ as the reverse primer. The amplified cDNA was cloned into *pENTR* using the D-TOPO cloning kit (Invitrogen). We verified that the *pENTR* constructs contained the full length *NS1* cDNA sequence by restriction analysis and PCR. *E. coli* clones containing *pENTR* with the *NS1* cDNA were grown overnight in 2 ml of LB broth. The recombinant *pENTR* plasmids were purified and used to recombine the *NS1* cDNA sequence into *pHFW* using the Gateway LR Clonase Kit (Invitrogen). *pHFW* encodes a FLAG epitope and it contains a heat shock promoter. Recombined clones were identified by restriction analysis. Clones carrying the *NS1* cDNA were grown and prepared for *Drosophila* transformation.

**Cell Culture**

*Drosophila* Schneider-2 embryonic cells (Schneider, 1972) were cultured in standard S2 media supplemented with 10% fetal calf serum, and 1% Pen-Strep (Gibco). Cells were maintained in 25 cm² flasks at room temperature. For cells transfected with *pACNEO*, the medium was supplemented with G418 at a 100 µg/ml concentration.

**Schneider-2 Transformation and Protein Expression**

*Drosophila* S2 cells were cultured in 6 well plates until they were ~75% confluent. Cells were then washed with serum free medium (SFM) twice. After washing the cells, 2 µg of *pHFW* containing the *NS1* DNA and 1 µg of *pACNEO* were diluted in 100 µl of SFM without antibiotics. Next, 9 µl of Cellfectin reagent (Invitrogen) was diluted in 100 µl of SFM without antibiotics. The two mixtures were combined and added to a well of S2 cells and allowed to incubate for 45 minutes at room temperature. After 45 minutes, the transfection mixture was removed and the
cells were washed with 2 ml of SFM. The wash medium was then replaced with 2 ml of SFM containing Pen-Strep for 48 hours. After 48 hours, the SFM was replaced with regular S2 medium with FCS, Pen-Strep, and G418 at 100 µg/ml. To express FLAG-tagged NS1, cells were heat shocked at 37°C for 1 hour, after which they were allowed to recover for 12 hours to overnight before harvesting.

**Western Blots**

Total Schneider-2 cellular proteins were prepared for Western blotting by adding a single 25 cm² flask worth of cells to a 15 ml conical centrifuge tube. The cells were centrifuged for 10 minutes in a clinical centrifuge, and the supernatant was removed by aspiration. The pellet of cells was resuspended in 400 µl of Laemmli sample buffer with 20 µl of β-mercaptoethanol (Amresco, Solon, Ohio), and 5 µl of 100 mM PMSF. The cell suspension was then sonicated (Branson Digital Sonifier Model #250) on ice for 1 minute at 50% amplitude, with cycles of 10 seconds off and on. The protein preparations were then boiled for 10 minutes to denature proteins.

For whole larval or adult protein extracts, 20 third instar larvae or adults were prepared as described above for S2 cells. Standard 10% SDS-polyacrylamide mini-gels were used to resolve proteins from whole larval, adult, or Schneider S2 culture cell lysates. Gels were run for approximately 35 minutes at 200 volts. The resolved proteins were blotted to nitrocellulose for 45 minutes using the Bio-Rad semi-dry system. Blots were blocked for 1 h in 3% nonfat dry milk that had been reconstituted in TTBS (0.9% NaCl w/v, 100 mM Tris pH 7.4, 0.1% Tween 20). Blots were probed for 3 hours with the chicken anti-NS1 polyclonal antibody prepared by Aves Labs at a 1/1000 dilution in blocking solution. Blots were then washed three times for 15 minutes each. The secondary antibody was an affinity purified, peroxidase conjugated goat
anti-chicken IgG (Pierce, Rockford, IL) diluted 1/500 with blocking solution. After probing with the secondary antibody, the blots were washed once for 30 minutes in 200 ml of TTBS. After this final wash, blots were submerged in 100 mM Tris, pH 7.5 containing 3, 3’-diaminobenzidine (DAB) (Pierce, Rockford, IL) at 0.8 mg/ml and cobalt chloride at 0.4 mg/ml. H_2O_2 was added to a final concentration of 0.1%. Upon addition of H_2O_2, the DAB substrate forms a dark visible precipitate produced by the peroxidase, thus indirectly locating the protein (antigen) of interest.

**Immuno-histochemistry**

Preparation of whole mount tissues for immuno-fluorescence microscopy was performed as described by de Cuevas *et al.* (1996). All protocols were performed at 4°C. Tissues were dissected in “B” buffer (852 µL of 200 mM KH_2PO_4, 818 µL of 200 mM KH_2HPO_4, 1.5 ml of 1M KCl, 0.5 ml of 1M NaCl, 66 µl of 1M MgCl_2, and 2.4 ml of 5% paraformaldehyde in a final volume of 20 ml). Tissues were allowed to fix for 20 minutes. The fixed tissues were washed for 30 minutes in 1X PBS containing 0.1% Triton-X 100. After washing, the tissues were blocked in PBS containing 0.1% Triton-X 100 with 5% normal goat serum. After blocking, tissues were probed overnight in the blocking solution containing chicken anti-NS1 (1/250 dilution of a 1:1 glycerol stock). Tissues were washed for 2 hours in 1X PBS containing 0.1% Triton-X 100 with 2% bovine serum albumin. After washing, the tissues were blocked for 30 minutes in 1X PBS containing 0.1% Triton-X 100 with 5% normal goat serum. Next, tissues were probed with Alexa Fluor 488 conjugated goat anti-chicken (Molecular Probes) for 4 hours. In some cases, tissues were counter-stained with 4’-6-diamidino-2-phenylindole dihydrochloride (DAPI, Polysciences, Warrington, PA) at 1.0 µg/ml prior to fluorescence microscopy.
**Fluorescence Microscopy**

Fluorescence microscopy employed a Zeiss Axioskop with a digital camera (SPOT SE) and software for image capture. Editing was performed in SPOT software or Adobe Photoshop.

**Drosophila GAL4 System**

*Flag-NS1* or *GFP-NS1* transgenes were expressed in transgenic fly lines using the GAL4 system. The various transgene constructs contained a *GAL4-UAS* (Upstream Activation Sequences) promoter sequence. To express *GAL4-UAS* driven transgenes, transgenic *Drosophila* lines were used that have the yeast GAL4 transcription factor expressed either ubiquitously such as in the *daughterless-GAL4* line or in a tissue specific manner such as in the *eyeless-GAL4* driver line (Lai et al., 2005). Upon crossing a homozygous transgenic *Flag-NS1* or *GFP-NS1* fly line with one of the *GAL4* “driver” lines, the trans-heterozygous progeny will express the *GFP-NS1* or *Flag-NS1* transgenes in a pattern that is coincidental to *GAL4* expression.

**Results**

**Sequence Comparison of NS and NS1**

The closest orthologue to human NS in *Drosophila* was Nucleostemin-1 (NS1); it shared 33% identity and 67% similarity in protein sequence with human NS (Figure 2.1). The *NS1* gene was located on chromosome 3R in cytological region 89E11.

Like mammalian NS, NS1 had an extensive amino-terminal basic domain. Specifically, the N-terminal basic domain of NS1 was approximately 43% identical and approximately 55% similar to the corresponding region in human NS (Rosby et al., 2009). This high degree of sequence homology (see Figure 2.1) suggested functional conservation. NS1 had a similar permuted order of GTP binding domains, and a conserved coiled-coil domain which like human NS, probably mediates protein-protein interactions. NS1 also had a similar conserved inhibitory domain that
prevents nucleolar retention of mammalian NS when it is not bound to GTP (Rosby et al., 2009; Tsai and McKay, 2005). Finally, the carboxy-terminal acidic region was also conserved between the human and Drosophila NS1 proteins.

Drosophila has a four-member NS family which includes NS1, NS2, NS3 and NS4 (Kaplan et al., 2008). NS2 is another YawG family GTPase that showed differences in functional domains when compared to human NS or Drosophila NS1 (see figure 2.1). NS2 has similar GTP binding motifs, coiled coil and inhibitory domains, but the most significant difference is that NS2 lacked the conserved basic N-terminal residues present in NS and NS1.

Robert Tsai has suggested that Drosophila NS1 is more similar to GNL3L, another nucleolar YawG family GTPase in yeast, than it is to human NS (Tsai and Meng, 2009). It appears that this family of GTPases probably came about due to gene duplication events (Leipe et al., 2002; Tsai and Meng, 2009). This is the simple and most logical explanation for the similarities in structures of YawG GTPases. However, GNL3L has significant differences from NS1 and mammalian NS. The most significant difference is the lack of an extensive amino terminal basic domain in GNL3L. The amino-terminal basic domain is a hallmark of all characterized NS homologues (Kudron and Reinke, 2008; Rosby et al., 2009; Tsai and McKay, 2002).

Anti-NS1 Antibody Characterization

The chicken anti-NS1 antibody recognized endogenous NS1 at approximately 70 kDa on a western blot of S2 cell extracts (Figure 2.2, lane 2). Furthermore, western analysis showed a significant enrichment of NS1 in Schneider S2 cell extract (lane 2) compared to wildtype larval or adult extracts (lane 3). S2 cells are an embryonic cell line derived from 24 hour Drosophila embryos (Schneider, 1972). Like cultured mammalian cells, S2 cells are rapidly dividing cells, and this may explain the relatively high abundance of NS1. Furthermore, like mammalian NS in differentiated cells, the
Figure 2.1  Amino acid sequence comparison of *Drosophila* and human nucleostemin proteins. The similarities between human nucleostemin (HNS) and NS1 were easily identified in the amino-terminal basic domain (light blue underline), the GTP binding motifs (orange underlines), and the inhibitory domain (dotted underline). NS1 was 33% identical and 67% similar to its human homologue. NS2 is another YawG family GTPase in *Drosophila* that showed conservation in the GTP binding motifs, the coiled-coil domain and inhibitory domain. The major difference is that NS2 does not have the conserved N-terminal basic domain (adapted from Rosby et al., 2009).
Figure 2.2 (lane 1) Western blot using the anti-NS1 antibody indicated that endogenous NS1 had an apparent molecular weight (lane 1) of approximately 72 kDa and that it was abundant in Schneider S2 cells (lane 2). The endogenous NS1 protein was undetectable by western blot in adult flies, but over-expressed GFP-NS1 was clearly visible at approximately 96 kDa (lane 3) (adapted from Rosby et al., 2009).
endogenous *Drosophila* protein was undetectable in whole larval or adult extracts (Figure 2.2, lane 3). Thus, the observed expression levels of NS1 in S2 cells provided a strong parallel between the mammalian NS and *Drosophila* NS1 proteins. As a positive control, the anti-NS1 antibody easily recognized exogenously expressed GFP-NS1 as a ~96 kDa protein on the western blot (lane 3) (Rosby *et al*., 2009). The GFP portion of the fusion protein accounts for the additional ~26 kDa.

**Endogenous NS1 Expression**

Endogenous NS1 was expressed in many *Drosophila* cell types. Like vertebrate NS, *Drosophila* NS1 appeared to be enriched within the granular component of most observed nucleoli (Rosby *et al*., 2009). Immuno-fluorescence with the anti-NS1 also showed that NS1 was present within the nucleoplasm to a lesser degree. In addition, the nucleoli of the larval midgut imaginal island cells (MICs) were labeled more intensely (Figure 2.3A) with the anti-NS1 compared to the polyploid nuclei in the larval midgut (Rosby *et al*., 2009). These larval MICs are “precursor” cells for the adult midgut; during pupation, the MICs undergo rapid divisions to form the adult intestinal epithelium (Yee and Hynes, 1993). The apparent greater abundance of NS1 in the MICs suggested that NS1 may serve a similar role in progenitor cells as does NS in vertebrate stem cells. Overall, it appeared that NS1 was abundant in rapidly dividing *Drosophila* cells, or in those cells preparing to undergo cell division.

In adult follicle cell nucleoli (Figure 2.3B), the granular component was distinctly labeled by anti-NS1 serum, with slight nucleoplasmic labeling. These follicle cells, however, were no longer dividing, and in fact are terminally differentiated. Furthermore, they undergo apoptosis shortly after the stage of oogenesis shown in Figure 2.3B. Thus *Drosophila* NS1 was not exclusively expressed in rapidly dividing cells, but was also expressed in terminally differentiated cells.
Figure 2.3 NS1 was expressed in all *Drosophila* cell types examined. A. Anti-NS1 labeled the granular component of the larger polyploid midgut cells (red arrow). The midgut imaginal island cells (MICs) showed elevated expression (white arrow). The tissue was counter-stained with DAPI to show nuclear volume. B. Anti-NS1 labeled the peripheral granular component of adult follicle cell nucleoli (dashed arrow). DAPI staining showed nuclear volume. Bars, 50 µm (adapted from Rosby et al., 2009).
Figure 2.4 Immunolabeling of endogenous NS1 in nurse cells and primary spermatocytes. A. The nurse cells showed a relatively high abundance of NS1 in their polyploid nuclei (dashed arrow). Nurse cell nucleoli occupy ~50% of the nuclear volume. The primary function of these nurse cells is to produce ribosomes (Painter and Biese, 1966). Primary spermatocytes had a higher nucleoplasmic abundance of NS1 compared to many other cell types (white arrows). Bars, 25 µm (adapted from Rosby et al., 2009).
Figure 2.5 Immunolabeling of endogenous NS1 in the larval wing disc. A. Wing imaginal disc nucleoli were labeled in the granular component (white arrows) with anti-NS1. B. Phase contrast image of the imaginal disc to show phase-dark nucleoli. Bar, 25 µm.
Figure 2.6 Immunolabeling of endogenous NS1 in larval brain cells. A. Larval brain cell nucleoli were labeled with anti-NS1 (white arrows). B. Phase contrast image of the larval brain to show phase dark nucleoli. Bar, 50 µm.
Indirect immuno-fluorescence of nurse cell nucleoli revealed an abundance of NS1 in their large lobed nucleoli (Figure 2.4A). Nurse cells degenerate during late egg chamber development, and they contribute their contents to the developing oocyte. Finding NS1 in abundance within nurse cell nucleoli was strong evidence that Drosophila NS1 is required for ribosome biosynthesis, primarily because the nurse cells actually give a “jump start” to the embryo and subsequent larval development with respect to maternal ribosome contributions. This is intriguing because nurse cells are terminally differentiated cells, but their primary products are ribosomes (Painter and Bieselee, 1966). These nurse cells produce ~2x10^{10} ribosomes before undergoing apoptosis at developmental stages 12 and 13 (Dapples and King, 1970; King, 1970; McCain et al., 2006).

Primary spermatocytes showed a diffuse nuclear labeling with the NS1 antibody, with nucleoli having slightly more labeling (Figure 2.4B). The difference in the labeling pattern observed in primary spermatocytes suggested that these cells might have a different metabolic requirement for ribosomes, or a different shuttling rate for NS1 between the nucleolus and nucleoplasm.

Anti-NS1 immuno-labeling of larval wing imaginal disc (Figure 2.5A) and larval brains showed that these two tissue types also expressed NS1. Wing discs showed ample labeling of the granular component of the nucleolus, whereas the mitotic larval brain tissue (Figure 2.6A) showed a more diffuse nucleolar morphology.

**Conclusions**

When comparing the primary structure of NS1 with that of human NS, there was an acute similarity in the region of the protein with described functions or interactions. The N-terminal basic domain that is required for nucleolar targeting was highly conserved, especially
the basic residues. In addition, the GTP binding motifs were extremely well conserved which suggested a large amount of functional conservation. With regard to the GTP binding domains, NS1 was a true functional GTPase/ATPase (Rosby et al., 2009). Interestingly, NS1 could hydrolyze ATP and GTP with approximately the same efficiency (Rosby et al., 2009). This observation added more complexity to the developing NS1 model, and possibly another facet for NS1 regulation. The coiled-coil domain was also well conserved in the NS1 and NS sequences. All of these structural and functional similarities strengthen the argument that Drosophila NS1 and human NS serve similar or related molecular functions in the cell.

Robert Tsai suggested that vertebrate nucleostemin functions differently than that of nucleostemin homologues in other model organisms (Tsai and Meng, 2009). However, there is only scant evidence to support this claim. Rescue experiments have been attempted to establish the functional complementation. For example, deletion of Grn1 (the NS homologue in S. pombe) results in a slow-growth phenotype, defects in 35S pre-rRNA processing, and a block in nucleolar export of the 60S LSU. The Grn1-null phenotype could be rescued by human GNL3L, but not by human NS (Du et al., 2006). A similar experiment was performed in C. elegans (Kudron and Reinke, 2008), which showed that murine nucleostemin failed to rescue the nst-1-deficient growth phenotype in C. elegans. The nematode NST-1 was expressed by both proliferating and differentiated cells, and it was required for both larval growth and germline stem cell division (Kudron and Reinke, 2008). There have been described instances in which a foreign transgene was not able to rescue loss of the native orthologue (Yang et al., 2007; Ying et al., 2006). In some cases the lack of rescue is due to the transgenic protein’s inability to associate with host proteins that are sufficiently different.

A recent study has established a link between vertebrate NS and rRNA processing
(Romanova et al., 2009). This new discovery supports the hypothesis that nucleostemin orthologues are ancient GTPases that function in ribosome biosynthesis. Like its mammalian counterpart (Romanova et al., 2009), NS1 is expressed in many cell types. Interestingly, localization was slightly different among different cell types. Cells that were undergoing rapid divisions tended to have more nucleostemin within the nucleoplasm, presumably due to more NS1 shuttling between the nucleoli and nucleoplasm, versus less metabolically active cells where NS remained predominantly nucleolar. NS1 may act differently in different cell types, or in rapidly dividing cells versus terminally differentiated cells. For example down-regulation of NS inhibited differentiation of myoblasts to myotubes (Hirai et al., 2009), as opposed to it being required to maintain pluripotency. In addition, NS1 appeared to have a higher nucleoplasmic localization in rapidly dividing Drosophila cells (see Figure 2.4B) compared to non-dividing cells (see Figure 2.3B).

Like vertebrate NS, Drosophila NS1 was highly expressed in cultured cells. Overall, NS1 appeared to be up-regulated in cells undergoing rapid division, and like many other nucleolar proteins, its relative expression may serve as an indicator of cell growth and cell cycle activity (Sommerville, 1986). Other than germline stem cells, only one Drosophila somatic adult stem cell has been well characterized (Tsai and Meng, 2009). This is the adult intestinal stem cell (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). We could not localize NS1 in these Drosophila adult intestinal stem cells by immunohistochemistry. This suggested that adult Drosophila intestinal stem cells may not divide as rapidly as culture cells, and therefore may not have a high requirement for NS1.

Drosophila is not established as a strong model system for the study of cancer, so the link between NS and cancer will be difficult to establish in the fly. However, due to the
sophisticated genetics and amenable cytology offered by *Drosophila*, investigating *Drosophila* NS1 offers a more tractable approach to determine the role that NS homologues have in the cell. This approach in *Drosophila* was also unique because *Drosophila* does not have several of the putative NS-interacting proteins described in vertebrates such as p14<sup>ARF</sup>, B23 and MDM2 (Jin et al., 2000; Mogila et al., 2006; Moon et al., 2008).

In this chapter, NS1 was shown to be the closest *Drosophila* homologue to vertebrate NS. Immuno-fluorescence showed that like vertebrate NS, *Drosophila* NS1 is a nucleolar protein. In this chapter it was confirmed that NS1 has a structure and expression pattern similar to vertebrate NS. This study provided insight into the conserved role for nucleostemin orthologues among species. Proteins that have high degrees of conservation among a wide variety of species usually perform similar functions. NS1 and other nucleostemin homologues are no exceptions.
Chapter 3  

**Over-expression of NS1 and Associated Phenotypes**

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**Introduction**

Mammalian NS localizes primarily within the granular components of interphase nucleoli (Ma and Pederson, 2008). Co-localization experiments have shown that this in stark contrast to the well characterized pre-rRNA methyltransferase fibrillarin (Politz *et al*., 2005) which localizes to the dense fibrillar component. This contrasting localization pattern between NS and fibrillarin helps define the complex compartmentalization of the nucleolus as well as its diverse functions. Furthermore, mammalian NS resides in sub-regions of the granular component that are apparently devoid of rRNA (Politz *et al*., 2005). This observation further defines nucleolar sub-compartmentalization, and indicates that mammalian NS might have a unique function that is independent of ribosome biosynthesis (Politz *et al*., 2005).

In *S. cerevisiae*, Nug1 (the nucleostemin orthologue) can bind RNA non-specifically when over-expressed. This non-specific binding is mediated via the N-terminal basic domain that is conserved in all characterized NS orthologues (Bassler *et al*., 2006; Kudron and Reinke, 2008; Rosby *et al*., 2009). This non-specific RNA binding suggests that over-expressed Nug1 may be associating with ribonucleoprotein particles (RNPs) in the nucleus (Bassler *et al*., 2006). This possibility conflicts with the claim of Politz that mammalian NS does not interact with ribosomal subunits (Politz *et al*., 2005).

Knockdown or exogenous over-expression of NS causes p53-mediated cell cycle arrest in mammalian cell culture (Tsai and McKay, 2002), but transgenic mouse studies have shown contrasting results. Specifically, mice that over-express NS have normal viability (Romanova *et
al., 2009). This result has been supported by similar findings for exogenous expression of NS homologues in *S. cerevisiae* and *C. elegans*. In fact, over-expression of NS in transgenic mice increased the efficiency of 32S pre-rRNA processing, which also contrasts with studies that claim NS does not have a role in ribosome biosynthesis (Romanova et al., 2009). Similar to other NS homologues, we expect exogenous NS1 to localize to the granular component, and over-expression of will be non-lethal.

**Materials and Methods**

**Plasmid Constructions and Fly Transformations**

The *Drosophila NS1* cDNA (AT23067) was amplified using 5’-CACCGCTTTAAAAAGGTGAAGACCAAG-3’ as the forward primer and 5’-AATTTATCACATAGTCCTCATCAAAATC-3’ as the reverse primer. The amplified cDNA was ligated into *pENTR* using the D-TOPO cloning kit (Invitrogen). We verified that the *pENTR* construct contained the full length *NS1* cDNA sequence with restriction analysis and PCR. Clones containing *pENTR* with the *NS1* cDNA were grown overnight in 2 ml of LB broth.

The *pENTR-NS1* plasmids were extracted and used to transfer the *NS1* cDNA sequences into *pPFW* using the Gateway LR Clonase Kit (Invitrogen). This destination plasmid allows expression of NS1 as a FLAG-tagged protein. Recombined destination clones were identified with restriction analysis. Clones carrying the *NS1* cDNA were grown and prepared for embryo injection. *pPFW* has GAL4 UASs that can drive transgene expression when induced by GAL4. The final *pPFW* recombinant plasmid and a helper plasmid (*pUCHsΔ2*) encoding transposase were co-injected into homozygous or hemizygous *w¹¹¹⁸ Drosophila* embryos according to established techniques (Rubin and Spradling, 1982).

Several independent insertion fly lines were recovered that expressed *FLAG-NS1*. 
Insertions were mapped to the X, 2nd, or 3rd chromosomes using standard segregation analyses. Homozygous *FLAG-NS1* flies were crossed to flies homozygous for the *daughterless (da)-GAL4* transgene (also on the 3rd chromosome) to induce ectopic expression of GFP-NS1 in heterozygous progeny.

To produce mRFP-fibrillarin, the full length fibrillarin cDNA (GM13963) was amplified with the forward primer: 5’-CACCATGGGCAAACCAGGATTCA-3’ and the reverse primer: 5’-GTACAATAGCCTAAACCTAAACCCAGCAGAAACG-3’. The fibrillarin cDNA was ligated into *pENTR* as described for NS1, and then recombined into *pTRW* using the Gateway recombination technique. Like *pUAST*, *pTRW* has a heat shock promoter and GAL4 UASs. Flies transgenic for mRFP-fibrillarin were prepared as described for *FLAG-NS1*. The homozygous *mRFP-fibrillarin* flies were crossed to the homozygous *GFP-NS1* flies described in Chapter 2. Heterozygous third instar *mRFP-fibrillarin/GFP-NS1* larvae were heat shocked for 45 minutes at 37°C. The larvae were allowed to recover on standard fly food for 1.5 hours and then dissected in Brower’s fixative containing 2% formaldehyde.

**Results**

**Over-expressing NS1**

A transgenic fly line was constructed to express mRFP-fibrillarin. Fibrillarin is the rRNA methyltransferase within CD box snoRNPs and is often used as a marker for the dense fibrillar component. Heterozygous *mRFP-fibrillarin/GFP-NS1* larvae were examined by fluorescence microscopy to determine the localization of GFP-NS1 relative to mRFP-fibrillarin. mRFP-fibrillarin localized to the DFC while GFP-NS1 localized to the more peripheral granular regions (Figure 3.1). Further sub-localization of GFP-NS1 within the granular regions was not possible, but the results confirmed a mutually exclusive localization pattern between GFP-NS1 and
mRFP-fibrillarin. This result agreed with the argument that NS1 is the *Drosophila* homologue of mammalian NS, as similar exclusive localizations of fibrillarin and NS have been described by Politz *et al.* 2005.

![Image](image_url)

**Figure 3.1** In *Drosophila* salivary glands, exogenous GFP-NS1 and mRFP-fibrillarin localized within different regions of the nucleolus. A. mRFP-fibrillarin localized within the DFC. B. GFP-NS1 localized within the granular regions. C. An overlay of panels A and B showed a mutually exclusive localization pattern of mRFP-fibrillarin and GFP-NS1. Bar, 25 µm.

Both FLAG-NS1 and GFP-NS1 fusion proteins localized to all sub-regions of the nucleoli when over-expressed. But in addition to nucleolar localization, both NS1 fusion proteins localized within the nucleoplasm to a lesser degree. Interestingly, when over-expressed as a GFP or FLAG fusion protein, NS1 localized to polytene chromosomes within salivary gland nuclei (Figure 3.2). Specifically, the protein localized to many of the actively transcribed interbands as shown by DAPI staining which labels the condensed bands on the chromosomes.

In contrast to observations made in mammalian cell culture systems, but consistent with transgenic mouse studies, *in vivo* over-expression of FLAG-NS1 or GFP-NS1 in transgenic flies was generally non-lethal (Rosby *et al.*, 2009). Lethality was observed in three out of 14 GFP-NS1 transgenic lines. But these three lines had multiple transgene insertions suggesting that lethality was due to the multiple insertions and their possible disruptions. None of the
Figure 3.2 When over-expressed as a GFP fusion, NS1 localized to nucleoli and salivary gland polytene chromosomes (A). GFP-NS1 localized within all regions of the giant salivary gland nucleoli. The fibrillar center had less GFP-NS1 (red arrow). Bar, 50 µm. B. GFP-NS1 localized to the salivary gland polytene chromosomes, specifically to some actively transcribed inter-band regions. These chromosomes were counter-stained with DAPI to show the condensed, non-transcribed banded regions. Bar, 25 µm.

Fly lines that had a single transgene insertion of GFP-NS1 were lethal (Rosby et al., 2009).

The NS1 over-expressing third instar larvae that had multiple transgene insertions often displayed a phenotype of melanotic tumor-like formation in the foregut and midgut. Melanotic tumors are thought to arise from aberrant immune responses in larvae. They are often described as free floating dark colored bodies that reside within the larval body cavity (Watson et al., 1991). However, the tumor-like bodies observed were not free floating bodies as described for “true” melanotic tumors, but rather the bodies observed in NS1 over-expressing larvae were restricted to distinct regions of the intestine, including the proventriculus, gastric caeca and midgut (see Figure 3.3). This is interesting, because in normal wildtype flies, the midgut imaginal island cells were enriched for endogenous NS1, suggesting that severe over-expression of NS1 in these cells lead to disruption of normal nucleolar function and lethality.
Figure 3.3 Gross over-expression of GFP-NS1 caused melanization within the proventriculus and midgut of transgenic animals. Red arrows indicate the site where melanization was observed in the proventriculus (PV) and midgut (MG), arrowheads show the gastic caecae (GaC) of a third instar larva.
Conclusions

*Drosophila* NS1 shares many fundamental characteristics with its yeast and vertebrate orthologues. Like the yeast and vertebrate homologues, exogenous NS1 localized to nucleoli. Like mammalian NS, GFP-NS1 selectively localized in a peripheral granular region that was distinct from the DFC which contained mRFP-fibrillarin. This granular component localization suggested that NS1 might play a role in late ribosome assembly or perhaps a role in the transport of ribosomal subunits from the nucleolus.

Remember that Nug1 in yeast binds to RNA in a non-specific manner. Therefore one could speculate that the chromatin association of NS1 was possibly due to non-specific mRNA binding. This hypothesis is supported in that NS1 has an amino-terminal domain that is quite similar to the amino-terminal domain of Nug1, which is responsible for its binding to RNAs.

Although expression was generally non-lethal, extreme over-expression of GFP-NS1 did induce a melanotic tumor-like phenotype in the midgut. This result suggested that the apoptosis observed (Tsai and McKay, 2002) in mammalian cells when NS is over-expressed may effectively be a phenomenon restricted to cultured cells, and is not necessarily due to NS over-expression in the whole animal. In other words, cultured cells might attain a higher level of exogenous expression of NS that is not found in whole animals. Conversely, melanization of the midgut of larvae that over-express GFP-NS1 may in fact be due to apoptosis or necrosis rather than melanotic tumor formation. This hypothesis is based on the contrasting localization of the observed melanotic tissues compared to that defined for “true” melanotic tumors. True melanotic tumors are usually free floating bodies as opposed to those observed in the *GFP-NS1/+; da-GAL4/+* progeny.

Endogenous NS1 appeared to be more abundant within the midgut imaginal island cells,
and disruption of these cells might be the cause of melanization observed in the midgut.

Tissues that have a higher relative abundance of NS1 may be close to the threshold for maximal NS1 expression. In other words, in cells that were already expressing an abundance of NS1, a small increase in NS1 expression might create cytological problems. For example, strongly over-expressed NS1 could disrupt mRNA processing or translation causing apoptosis or necrosis.

Several proteins such as those in masked maternal RNPs are known to bind RNA in a non-specific and/or highly sequence-specific manner to influence properties such as stability of the RNA or the availability of the RNA for translation in the cytoplasm (Curtis et al., 1995; Swamynathan et al., 2000). Therefore, over-expressing GFP-NS1 might elicit a similar outcome in tissues that normally have a relatively high expression of NS1. Further analysis (e.g. with RNAse treatment of the polytene chromosomes to digest the nascent mRNA and thereby release GFP-NS1) is required to verify this hypothesis.
Chapter 4

RNAi-Mediated Knockdown of NS1 and Associated Phenotypes Suggest a Role in Large Subunit Ribosome Biosynthesis*

Introduction

RNA interference (RNAi) is a technique in which double stranded RNAs are used to target a specific mRNA for degradation (Fire et al., 1998). This method is one of the most widely used tools to knockdown or eliminate expression of a specific gene. Mammalian cell culture studies have shown that knockdown or over-expression of NS causes cell cycle arrest and apoptosis, however transgenic mouse studies have only confirmed cell cycle arrest upon depletion of NS (Ma and Pederson, 2007; Romanova et al., 2009; Tsai and McKay, 2002). On the other hand, over-expression in transgenic mice was not generally lethal.

Knockdown of NS homologues in C. elegans, X. laevis, and S. cerevisiae also resulted in cell cycle arrest. In C. elegans, knockdown or mutation in NST-1 caused global growth arrest and cell cycle arrest in the germline stem cells as well as in intestinal cells (Kudron and Reinke, 2008). In X. laevis, cell cycle arrest occurred in neural plate cells when morpholino oligonucleotides targeting XNS were injected into a single cell of a two-cell stage embryo (Beekman et al., 2006; Romanova et al., 2009). Finally, when Nug1 was knocked out in yeast, growth defects as well as cell cycle arrest occurred. Thus, cell cycle arrest is the single most common phenotype associated with all NS deficiency models to date.

Although mammalian NS was found in regions of the nucleolus that are purportedly devoid of rRNA (Politz et al., 2005), a recent study established that murine NS is required for proper processing of the 32S pre-rRNA intermediate (Romanova et al., 2009). Likewise, NST-1

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in *C. elegans*, and Nug1 in *S. cerevisiae* were required for proper rRNA processing (Kudron and Reinke, 2008). In addition, Nug1 was required for export of the large ribosomal subunit (Du *et al.*, 2006). Therefore, in addition to sequence similarities, all of these inter-species similarities for NS homologues support the hypothesis that nucleostemin does in fact play a role in ribosome biogenesis.

Dai *et al.* (2004) suggested that the loss of NS leads to nucleolar disruption and that this disruption would lead to release of large ribosomal subunit proteins RpL11 and RpL23 to the nucleoplasm (Dai *et al.*, 2004; Dai *et al.*, 2008). There they would subsequently block MDM2 function by binding MDM2, thus allowing p53 to be activated (Dai *et al.*, 2008). However, there have been no described instances of nucleolar disruption associated with the loss of NS (Ma and Pederson, 2007). Furthermore, *Drosophila* and *C. elegans* have no identified *MDM2* gene homologue. Therefore, these organisms likely employ a different mechanism to regulate p53 activity (Brodsky *et al.*, 2004). The role and mechanism of vertebrate NS interactions with several tumor suppressor proteins is poorly understood. Immuno-fluorescence has revealed that there was a marked increase in p53 and MDM2 abundance when NS was knocked down in U2OS (human osteosarcoma cell line) cells (Ma and Pederson, 2007). But a recent study using p53 knockout mice suggested that the role of NS in cell cycle regulation is independent of p53 activation (Beekman *et al.*, 2006). This latter result is in contrast with the earlier cell culture work that showed the onset of p53-dependent apoptosis when NS was either over-expressed or depleted (Tsai and McKay, 2002). Furthermore, the apparent up-regulation of mammalian p53 due to NS loss needs clarification to show that the levels of p53 would be physiologically significant, since up-regulation of a gene does not necessarily lead to induction of its downstream effect.
Mammalian NS co-localized with p14^{ARF} in the nucleolus. Furthermore, over-expression of p14^{ARF} decreased NS levels as observed by immuno-fluorescence (Ma and Pederson, 2007). This result provided an alternative mechanism by which NS levels might be regulated.

The strongest link between all characterized NS orthologues is that they appeared to have a role in large subunit biosynthesis. The yeast, C. elegans and mammalian NS orthologues all have described roles in rRNA processing. This suggests that when NS was knocked down, there was a net loss of ribosome function (Du et al., 2006; Kudron and Reinke, 2008; Romanova et al., 2009; Rosby et al., 2009). Interestingly, of all the NS models that have been studied, S. cerevisiae, C. elegans, and D. melanogaster lack MDM2 and p14^{ARF}. This suggests that these simpler model systems may provide a clearer understanding of the role and function of nucleostemin within the nucleolus.

This chapter describes the RNAi-mediated knockdown of NS1, and the associated phenotypes. This chapter also compares NS1 knockdown results with the previously published results for other NS homologues (Beekman et al., 2006; Du et al., 2006; Kudron and Reinke, 2008; Ma and Pederson, 2007; Romanova et al., 2009). The predominant phenotypes such as cell cycle arrest and growth arrest are expected, but this chapter shows that NS1 in Drosophila is required for large ribosome subunit export like Nug1 in yeast.

**Materials and Methods**

**Fly Stocks**

*Drosophila melanogaster* stocks were maintained at room temperature (22-23°C). For tissue specific RNAi expression, the eye specific ey-GAL4 driver (Bloomington stock number 5588), or the salivary gland specific sal-GAL4 (Bloomington stock number 1967) was used. The Daughterless-GAL4 (da-GAL4) driver line was used for ubiquitous transgene expression. The
strain is effectively wildtype except for eye color; it was used for transformation and as a control fly line for biochemical or microscopic analyses.

Plasmid and Vector Construction

The transformation plasmid \( pUASp\)-Nba-CS2-BgX was used to generate transgenic fly lines that express RNAi directed against the \( NS1 \) transcripts. This plasmid contains yeast GAL4 UASs to promote expression, but it also contains a Chitin Synthase-2 intron which serves to stabilize hairpin RNAs (Rorth, 1998; Zhu and Stein, 2004). The first 450 bases of the \textit{Drosophila} NS1 cDNA (AT23067) were amplified using 5'-' ATAAGGATCCAGTAGATCTATGGCTTTAAAAAG -3' as the forward primer and 5'-' GTATCCATGGTAGTCTAGACACCTTGCGGAATTCTTG -3' as the reverse primer. This 450 base sequence was ligated on one side of the CS2 intron between \textit{Knl} and \textit{BamH}i, and on the other side between \textit{Xba}I and \textit{Bgl}II in reverse orientation. The first insert was ligated such that the anti-sense transcript would be produced to prevent translation of a dominant negative truncation product.

Ribosomal proteins, RpL11, RpL26, and RpS6 were amplified using the following primer sets. The \textit{RpL11} cDNA (LD17235) was amplified using 5'-' CACCATGGCGGCGGTTACCAAGGAAGATT-3’ as the forward primer and 5’- CACCATGGCGGCGGTTACCAAGGAAGATT-3’ as the forward primer and 5’- GAGCTCTCATCTCTTTGTTCAAGATGATACC-3’ as the reverse primer. The RpL26 cDNA (RE17611) was amplified using 5’-AATTACACCATGAAACAGAACCAGTTC-3’ as the forward, and 5’-GATTTACGCGGTCTCCATGGCTGGG-3’ as the reverse. The RpS6 cDNA (UT01917) was amplified using 5’-AATTACACCATGAAACAGAACCAGTTC-3’ as the forward, and 5’- TGTTTACTTCTTGCTGGAGACAG-3’ as the reverse. All cDNA sequences were cloned into \textit{pENTR} using the Directional TOPO Cloning Kit (Invitrogen). After verifying individual \textit{pENTR} clones for each cDNA, the \textit{RpL11} cDNA was recombined into \textit{pTGW} which contains a GFP
(Green Fluorescent Protein) tag, and the \( R\text{p}L26 \) cDNA and the \( R\text{p}S6 \) cDNAs were separately recombined with \( p\text{TR}W \) which contains a mRFP (monomeric Red Fluorescent Protein) tag (Carnegie Gateway Collection). The recombination reactions were performed using the LR Clonase Kit (Invitrogen). Verified recombinant destination plasmids were then purified and used for \textit{Drosophila} transformation.

**Semi-Quantitative RT-PCR**

RT samples were subjected to semi-quantitative PCR analysis. This assay used 30 PCR cycles. PCR products were then resolved on a 0.7% agarose gel. Gels were stained with ethidium bromide, and intensities were quantified using ImageQuant TL software. Background subtraction was performed manually by subtracting the fluorescence of a square lacking DNA which had the same area as the band of interest. This square was 1 cm directly below the band of interest. These experiments were done in triplicate. After quantification, mean and standard deviations were calculated for each group of data.

For NS1 amplification, the primers were 5’-CTCAACGATTGGAACACGGGCAAATGAC-3’ as the forward primer and 5’-TCACGCGGCTGTCGAATCTCTACTAA-3’ as the reverse primer. As standardization controls, \( \beta\text{-tubulin} \), and \( R\text{p}L32 \) transcripts were analyzed as described for NS1 transcripts. The primers for amplifying \( \beta\text{-tubulin} \) were 5’-TGCCTGCAGGCTTCCAATGGAC-3’ as the forward primer and 5’-TGGATCGACAGGGTGGCGTTGTA-3’ as the reverse primer. The primers for amplifying \( R\text{p}L32 \) were 5’-GTTGGATCGACAGGCTTCCAATGGAC-3’ as the forward primer and 5’-TTCCAAATGGACAGGTTCCAATGGAC-3’ as the reverse primer.

**RNA Purification**

Twenty wildtype larvae and 20 \textit{RNAi-NS1/Da-GAL4} trans-heterozygous third instar larvae were isolated and frozen at -80°C in Eppendorf tubes. Larvae were homogenized and
RNA was prepared using the Chargeswitch Total RNA extraction kit (Invitrogen). Extracted RNA preparations were treated with DNase to eliminate possible contamination with genomic DNA. Afterwards, 5 µg of the RNA sample was reverse transcribed (RT) using the Superscript III Reverse Transcriptase kit (Invitrogen) with oligo(dT) as the primer.

**Area Calculation**

Total pixel number of digital images was calculated using the Adobe Photoshop CS3 histogram function. Images were outlined and pixel number was calculated. These pixel numbers were then compared to provide relative sizes for ommatidia and salivary glands.

**Results**

**RNAi Mediated Knockdown of NS1: Quantification and Establishment of RNAi Efficiency**

Several transgenic RNAi-NS1 fly lines were prepared. Of these lines, RNAi-NS1-A1 and RNAi-NS1-D1 were used for this study. The RNAi-NS1-A1/+; da-GAL4/+ trans-heterozygous progeny showed lethality to a lesser degree and developed ectopic bristles and eye malformations. In quantifying RNAi efficiency, the homozygous RNAi-NS1-D1 line, proved to be the strongest expressing transgenic RNAi-NS1 fly line. This line was crossed to the homozygous da-GAL4 line, which is an ubiquitous GAL4 expressing fly line. The RNAi-NS1-D1/da-GAL4 heterozygous progeny expressed RNAi ubiquitously and showed the most severe phenotype (approximately 97% larval and pupal lethality) (Rosby et al., 2009). The effective NS1 transcript level was approximately 40 percent of that seen in the wildtype controls (see Figure 4.1).

The original goal was to use the NS1 antibody to perform western blot analysis against endogenous NS1. However, like mammalian NS, NS1 appeared to be relatively low in overall abundance in larvae and adult flies. Therefore, we were not able to successfully perform western blots, and therefore chose to employ semi-quantitative RT-PCR to quantify the
Figure 4.1 Semi-quantitative RT-PCR to monitor loss of NS1 transcript levels. There was approximately 60% loss of NS1 transcript level in the RNAi-NS1-D1/da-GAL4 heterozygous progeny larvae compared to wildtype larvae. The two control transcripts, β-tubulin and RpL32 had similar levels in wildtype and RNAi-NS1-D1/da-GAL4 progeny, which suggests that the knockdown was specific for NS1. The difference in NS1 transcript levels between RNAi-NS1-D1/da-GAL4 and WT was statistically significant (p<.0005).

knockdown efficiency. When we looked at the RT-PCR results for two control transcripts, β-tubulin and RpL32, wildtype and RNAi-NS1-D1/da-GAL4 transcript levels were equivalent between the control and RNAi flies, which showed that the RNAi was specific and effective in depleting NS1 transcript levels.

In addition to RT-PCR, immunohistochemistry showed a loss of the NS1 protein (Figure 4.2). Tissues that expressed the RNAi construct had reduced antibody labeling compared to wildtype tissues. Furthermore no disruption of nucleolar morphology was evident in the RNAi-NS1-D1/da-GAL4 progeny which confirmed similar observations for mammalian
Figure 4.2 RNAi-mediated depletion of NS1 transcripts correlated with a loss of NS1 protein. (A) Anti-NS1 showed nucleolar labeling in the proventiculus of wildtype third instar larvae. (B) DAPI staining of cells in (A) showed nuclear volume in the wildtype proventriculus. (C) RNAi-NS1-D1/+; da-GAL4/+ third instar larvae showed reduced labeling by the anti-NS1 antibody. Bars, 200 µm.
cell culture systems (Ma and Pederson, 2007).

**NS1 Is Essential**

Several of those polyploid tissues that expressed RNAi in a da-GAL4 expression pattern appeared under-developed with an accompanying endomitotic arrest phenotype. A similar phenotype has been described for p21 and cyclin E mutants in *Drosophila* (Edgar and Orr-Weaver, 2001). For instance, salivary gland polytene chromosomes at the third instar stage appeared condensed and under-replicated (Figure 4.3B, Figure 4.4B). The overall size of salivary glands that expressed RNAi directed against NS1 was reduced by approximately 75%. This was one of the most extreme growth arrest phenotypes observed. This observation suggested that NS1 was essential for growth of terminally differentiated cells that have exited the cell cycle. In those under-developed salivary glands, the overall cell number was unaffected by the loss of NS1. Since salivary glands arise prior to embryonic stage 9 (Andrew et al., 2000), a maternal NS1 contribution probably sufficed for the initial mitoses and generation of these cells, but as NS1 protein levels dropped, the cells might have failed to grow. The growth arrest of only the salivary glands using the salivary gland specific GAL4 driver did not affect larval viability. However, the affected pupae weakly adhered to the walls of the culture vials, which suggested that the small salivary glands could not produce adequate glue proteins (Crowley et al., 1983). This in turn suggested a loss of ribosome biosynthesis or maturation.

When eye/head specific knockdown was induced by using the *ey-GAL4* driver line, progeny were often pupal lethal, but the few adult flies that did eclose had underdeveloped eyes and heads (Figure 4.5B). This phenotype was defined by the lack of, or reduced size of one or both eyes. The flies also had ectopic bristle formation in the eye which mimicked a cell competition phenotype (Moreno, 2008). Cell competition takes place if a tissue consists of two
Figure 4.3 Tissue-specific loss of NS1 caused growth arrest in larval salivary glands. (A) Wildtype larval salivary glands were approximately 60% larger than those expressing RNAi (quantified in Adobe Photoshop) (B). This growth arrest was apparent in smaller cell size and in chromatin that appeared under-replicated. The ring glands developed normally (arrows). The cell number was unaffected, which suggested that they underwent normal mitoses early in embryogenesis, when maternal NS1 was still present. Bars, 500 µm.
Figure 4.4 Loss of NS1 caused growth arrest in salivary glands. (A) Wildtype larval salivary glands showed a normal distribution of endoreplicated chromatin (B). RNAi expressing salivary glands contained what appeared to be under-replicated chromatin. This phenotype suggests an endomitotic arrest. Bars, 100 µm.
Figure 4.5 (A) Eye disc specific expression of NS1 is required for development of the *Drosophila* eye. The *ey-GAL4* driver line had normal eyes. (B) In some instances eye disc specific knockdown of NS1 caused a near complete loss of eye development (black circle).
Figure 4.6 NS1 is required for normal growth of *Drosophila* ommatidia. (A) Wildtype ommatidia were approximately 30% larger than those in which NS1 was depleted by RNAi (quantified in Adobe Photoshop). (B) Ommatidia size was reduced in ey-GAL4/+; RNAi-NS1-D1/+ adults. This phenotype appears to be a growth defect. These images were taken at the same magnification.
Figure 4.7 A. Depletion of NS1 affects larval midgut growth and differentiation. Wildtype larvae fed with BrdU showed new DNA synthesis in the Midgut Imaginal Island Cells (MICs) after a 2 hour pulse (red ovals). B. RNAi expressing larvae were completely devoid of the MICs suggesting that these cells had a higher requirement for NS1, and that their cell cycle was dependent on NS1. RNAi expressing larvae also showed new DNA synthesis in the polyploid midgut cells (red arrows). Delayed development may be the reason for the reduced endoreplication seen in these cells. Bars, 200 µm.
Figure 4.8 NS1 is required for normal development of midgut imaginal island cells (MICs) in third instar larvae. (A) Wildtype larval midgut showed an abundance of MICs (white arrows). These MICs proliferate rapidly during pupation to form the adult midgut. (B) RNAi expressing larvae showed an almost complete abolishment of these cells in the larval midgut, and only the polyploid cells were present (red arrows). Bars, 200 µm.
cell populations exhibiting different rates of protein synthesis. In the *Drosophila* wing imaginal disc, where it was discovered, cells with reduced ribosomal activity (loser cells) are eliminated by apoptosis, but only if situated amongst cells with wild-type activity (winner cells) (Moreno, 2008). In a few flies with underdeveloped heads, the phenotype appeared similar to the pinhead phenotype (Junger *et al*., 2003). The pinhead phenotype has been described for genes that normally function in growth regulation (Werz *et al*., 2009). Finally, ommatidia which expressed RNAi-NS1 in an ey-GAL4 pattern were about 30% smaller in size than normal (Figure 4.6B). The reduced ommatidia size was observed in all of the fly eyes that were viewed. These various phenotypes supported the hypothesis that a loss of NS1 disrupts normal growth.

**NS1 Is Required for Maintenance of Larval Midgut**

BrdU labeling revealed that the polyploid cells within the midguts of RNAi expressing larvae had slightly more DNA synthesis than the wildtype controls (Figure 4.7B). Chromatin within polyploid tissues often appeared under-replicated. In fact, third instar larval polyploid intestinal cells contained DNA with an under-replicated phenotype. These polyploid tissues often showed more on-going DNA synthesis than the same cells from wildtype larvae. Increased incidence of new DNA synthesis suggested that these tissues were “behind” compared to endoreplication within the wildtype cells, and therefore were still undergoing endoreplication to “catch up”. These phenotypes suggested a perturbation in the endoreplication cycle of these polyploid cells. In both the P-element disruption line and the RNAi transgenic line, the most striking phenotype seen was loss of the MICs (see Figures 4.7B and 4.8B). These cells arise in embryonic stage 11. They undergo limited cell division during the larval stages, but then upon ecdysone stimulation proliferate extensively during pupation to form the adult midgut (Hartenstein, 1992; Hayashi *et al*., 2005; Li and White, 2003; Yee and Hynes, 1993).
Figure 4.9 NS1 is required for export of Rpl11. (A) Phase contrast image of wildtype larval anterior midgut cells that over-expressed GFP-Rpl11. (B) Fluorescence microscopy showed predominantly cytoplasmic localization of GFP-Rpl11. (C) Phase contrast image of RNAi-NS-D1/GFP-Rpl11 showed nucleolar morphology was effectively wildtype. (D) Fluorescence microscopy of GFP-Rpl11/+; RNAi-NS-D1/da-GAL4 larval midgut cells showed increased nucleolar labeling suggesting that the large ribosomal subunits were not released from the nucleoli. Bars, 100 µm.
Figure 4.10 NS1 is required for export of RpL26. (A) Phase contrast of wildtype larval anterior midgut that over-expressed mRFP-RpL26. (B) Fluorescence microscopy showed ubiquitous localization of mRFP-RpL26. (C) Phase contrast image of mRFP-RpL26/+;RNAi-NS-D1/+ showed nucleolar hypertrophy, which was a phenotype often seen in the RNAi expressing lines. (D) Fluorescence microscopy of mRFP-RpL26/+;RNAi-NS-D1/+ larval midgut showed increased nucleolar labeling suggesting that large ribosomal subunits failed to be released from the nucleoli. There was also a slight accumulation on mRFP-RpL26 on the interior side of the nuclear envelope (white arrows). Bars, 100 µm.
In concert with the proliferation of MICs at the pupal stage, the larval midgut degenerates and undergoes apoptosis (Dorstyn et al., 1999). Therefore, MICs are preparing to undergo rapid divisions to replace the larval midgut, but they are also actively proliferating at a slow rate even in the third instar larval stage (Jiang and Edgar, 2009). This was confirmed with BrdU pulse labeling followed with immunohistochemistry with an anti-BrdU antibody (Figure 4.7A). These clusters of small cells, which were usually abundant in the midgut of third instar larvae, were reduced in number and often completely missing in the RNAi expressing transgenic larvae. This result is an interesting one in that these cells also appeared to have a higher relative abundance of NS1 than other cells within the larval midgut. Thus, MICs are actively dividing precursor cells that have an apparent requirement for high levels of NS1. This result bears similarity to the mammalian NS model in that the MICs are precursor cells.

**NS1 Is Required for LSU Export**

In yeast, Nug1 is required for export of the large ribosomal subunit (Du et al., 2006). To investigate whether or not NS1 is similarly required for export of the large subunit, GFP-RpL11 and mRFP-RpL26 transgenic lines were prepared to monitor LSU export. A separate fly line was prepared to express mRFP-RpS6. When GFP-RpL11 was ubiquitously expressed using da-GAL4 in an otherwise wildtype background, GFP-RpL11 was found predominantly in the cytoplasm, with only slight nucleolar labeling. Similarly, RpL26 was fairly ubiquitous within the cell, with a slightly more intense nucleolar labeling (Rosby et al., 2009). However, when either one of these two fluorescent ribosomal proteins were expressed in a NS1-depleted background, nucleolar fluorescence increased, which suggested nucleolar accumulation of the LSU. GFP-RpL11 labeled nucleoli much more intensely in the NS1-depleted background (Figure 4.9D) than in the wildtype background (Figure 4.9B). Likewise, mRFP-RpL26 was more prominent within nucleoli.
in the NS1-depleted background (Figure 4.10D) when compared to the labeling seen in a wildtype background (Figure 4.10B). Interestingly, RpL26 also accumulated just on the underside of the nuclear envelope (white arrows in Fig. 4.10D). Localization of mRFP-RpS6 was unaffected by RNAi targeting NS1. These data indicate that like yeast Nug1, NS1 is necessary for export of the large ribosomal subunit, and therefore has a similar conserved role in the large ribosomal subunit biosynthetic pathway.

**Loss of NS1 leads to Autophagy**

Fluorescence microscopy showed that GFP-RpL11 and mRFP-RpL26 accumulated within nucleoli in cells depleted of NS1. This suggested that export of the LSU failed. If this is true, there should be concomitant loss of cytoplasmic ribosomes. Transmission electron microscopy revealed that the depletion of NS1 resulted in the loss of cytoplasmic ribosomes (Figures 4.12 and 4.13). In addition, the development of stacked membranes was observed in the NS1 depleted Malpghian tubules (Figure 4.12). These membranes appeared to be autophagic isolation membranes (Yamamoto et al., 1990). Autophagy is a mechanism that cells use to recycle organelles and nutrients. Autophagy can be programmed or induced by physiological conditions such as nutrient starvation. During regular *Drosophila* development, programmed autophagy often occurs in larval stages, and results in cell death within structures that will be lost in the adult form (Kundu and Thompson, 2005). Autophagy usually progresses in distinct steps: pre-autophagosome formation, mature autophagosome formation, and finally delivery of the autophagosomes to lysosomes for final digestion (Yamamoto et al., 1990). Lysosomes were evident in some of the cells that had autophagosome formation (Figure 4.13). Therefore, this observation encompassed the full autophagic pathway in that autophagosomes will deliver their contents to the lysosomes for degradation (Kundu and Thompson, 2005).
Figure 4.11 Ultrastructural analysis of wildtype Malpighian tubules showed an abundance of cytoplasmic ribosomes with clearly discernable rough ER (circled regions).
Figure 4.12 Loss of NS1 in the Malpighian tubules resulted in reduced cytoplasmic ribosomes and formation of complex membrane stacks reminiscent of isolation membranes that indicate autophagy. These stacks had partially encircled a mitochondrion (arrow).
Figure 4.13 Depletion of NS1 leads to a depletion of cytoplasmic ribosomes. It appeared that autophagy is the cellular stress response to this loss of ribosomes in the Malpighian tubules of third instar larvae. Pre-autophagosome (P) formation, mature autophagosomes (AS) and lysosomes (LS) could be seen in the cytoplasm.
Conclusions

NS has been described as a stem cell maintenance protein (Tsai and McKay, 2002). This is a reasonable conclusion based on its loss in stem cells that were induced to differentiate with Ciliary Neurotrophic Factor. Upon induction, NS levels drop just prior to terminal differentiation. Its association with several tumor suppressor proteins (Ma and Pederson, 2007; Tsai and McKay, 2002) such as p53 and MDM2 also suggests a role in the regulation of cell replication.

*Drosophila* NS1 turned out to be an essential protein for organismal development. It was found in virtually all tissue types and is likely required for their growth, differentiation and viability. For example, MICs which are precursor cells for the adult midgut appeared to have a high relative abundance and perhaps requirement for NS1. This observation coincides with the role of mammalian NS in progenitor cell maintenance. MICs were virtually abolished with the loss of NS1. In addition, the location of NS1 in MICs was slightly different than that in cells which have exited the cell cycle: there was a greater nucleoplasmic concentration of NS1 when viewed by immuno-fluorescence. This observation suggests that NS1 has more shuttling activity between the nucleolus and nucleoplasm in actively dividing cells. This conclusion makes perfect sense when coupled with the observation that NS1 is required for nucleolar release of the large subunit. In the nucleoplasm, NS1 would have a higher incidence of chromatin contact, and therefore NS1 would also be in potential contact with nascent mRNA transcripts as well. This also reinforces the hypothesis that aberrant RNA binding might be the cause of midgut melanization seen when GFP-NS1 was severely over-expressed.

This work has shown that NS1 is required for either maturation or export of the large ribosomal subunit. Large ribosomal subunit fusion proteins, GFP-RpL11 and mRFP-RpL26
accumulated within nucleoli when NS1 was depleted by RNAi expression. This result mimicked those observed in yeast when Nug1 was absent. Specifically, fluorescently labeled RpL11 accumulated in the nucleolus of S. cerevisiae nug1 mutants (Du et al., 2006). These comparable results observed in yeast and now in Drosophila suggest that Nug1 and NS1 associate relatively late with pre-ribosomes within the nucleolus, and that they function in LSU maturation or export. The location of NS1 in the granular regions also supports a role in late maturation/export.

Grn1 (the S. pombe NS homologue) mutants showed growth and cell cycle arrest phenotypes. These mutant cells elongated, but failed to divide (Du et al., 2006). In C. elegans loss of NST-1 caused growth arrest, specifically at the L1 or L2 larval stage (Kudron and Reinke, 2008). Cell cycle arrest was also observed in C. elegans germline stem cells (Kudron and Reinke, 2008). When morpholino oligonucleotides directed against NS transcripts were injected into the neural plate of X. laevis embryos, there was a net loss of mitotic cells that accompanied the knockdown of NS. In mouse models, blastocysts that were ns-/ns- were embryonic lethal and also displayed cell cycle arrest phenotypes (Romanova et al., 2009). In Drosophila, depletion of NS1 by RNAi expression caused growth arrest phenotypes, and phenotypes that included loss of ommatidia, reduced ommatidia size, and loss of MICs (Rosby et al., 2009). The salivary glands appeared under-developed in terms of growth and endoreplication of the chromatin. Thus, the major common phenotypes of the NS depletion included growth arrest in quiescent cells and cell cycle arrest in dividing cells.

Formation of stacked membranes and their subsequent engulfing of cytoplasmic organelles suggested an autophagic response in Drosophila Malpighian tubule cells that were depleted for NS1. The loss of cytoplasmic ribosomes suggested that this stress response may be
due to loss of protein synthesis. The autphagic pathway is predominantly governed by the master regulator, metazoan Target of Rapamycin (mTOR). mTOR is a large kinase that regulates several cell functions such as ribosome biosynthesis and Pol II transcription. mTOR also suppresses autophagy. mTOR serves as a major stress response sensor in the cell. Thus, the loss of ribosome biosynthesis may induce a feedback signal to the TOR regulatory pathway, inducing a suppression of TOR signaling, which in turn would promote the progression of autophagy. Interestingly, a link has been established for TOR signaling and Nog1, another small nucleolar GTPase in yeast that functions in ribosome biosynthesis. Specifically, Nog1 shuttles between the nucleolus and the nucleoplasm during ribosome biogenesis, but it was tethered to the nucleolus by both nutrient depletion and TOR inactivation, causing cessation of the late stages of ribosome biogenesis (Honma et al., 2006).

The common theme among most nucleolar proteins is that they have a role in ribosome biosynthesis and this could certainly be true for NS1. In yeast, loss of Nog1 caused aberrant processing of the 35S rRNA. In C. elegans, loss of NST-1 caused decreased levels of 18S and 26S rRNA, which suggested that the 35S rRNA was not properly processed. In mouse models, loss of NS correlated with inefficient processing of the 35S rRNA intermediate. And finally, in Drosophila, NS1 is required for maturation or export of the large ribosomal subunit, and its loss leads to a net deficiency of cytoplasmic ribosomes. These two lines of evidence (processing and export) suggest that NS homologues have a conserved function in the large ribosomal subunit biosynthetic pathway. The precise roles that NS has in LSU processing or export, however, need to be further clarified. With the large amount of data implicating NS in ribosome biosynthesis, it is hard to fathom that vertebrate NS is any different from these other homologues.
Chapter 5

Conclusions and Future Direction

The Nucleolus and Small Nucleolar GTPases

The nucleolus is a very dynamic organelle in the nucleus. Although, the nucleolus has been ascribed many unique and novel functions, its major role is ribosome biosynthesis. There are relatively few nucleolar proteins that actually lack a function in ribosome biosynthesis.

Chapter 1 described basics of nucleolar function and ribosome biosynthesis, with an emphasis on possible roles for mammalian NS in cell cycle progression. Chapter 1 also described roles for other nucleolar GTPases in ribosome maturation and export. The YawG GTPases are represented throughout all species, and have an amazingly conserved structure of permuted GTP-binding domains. The conserved domains suggest conserved functions in NS-like proteins (Reynaud et al., 2005). The wealth of data unanimously suggests that the small nucleolar GTPases have a function in ribosome biosynthesis. Chapter 1 described the yeast nucleolar GTPases, Nug1, Nog1, Nog2 and Lsg1; all have been ascribed roles in ribosome biosynthesis.

*Drosophila NS1 Is Structurally Related to Human NS*

Chapter 2 described *Drosophila* NS1. NS1 is 581 amino acids in length, and it is 50% similar to human NS, with the highest conservation in the N-terminal basic domain and the permuted GTP binding domains. Like human NS, NS1 localized in the granular component of nucleoli, where ribosomal subunits undergo final maturation/packaging events.

All four members *Drosophila* nucleostemin family have permuted GTP binding domains. This suggests that these proteins may have evolved from a common ancestral gene by
duplication and then divergence (Tsai and Meng, 2009). Immuno-fluorescence showed labeling of every cell type tested, which in turn showed that NS is a ubiquitous protein. Furthermore, NS1 was often up-regulated in cells that have a high requirement for ribosomes. This included cells that are undergoing rapid cell divisions, such as the S2 cultured cells. NS1 was also up-regulated in cells that are preparing to undergo rapid divisions as seen in the MICs which undergo a rapid proliferation during pupation. In addition, NS1 was abundant in adult nurse cells which are terminally differentiated cells that provide enormous ribosome stores to the developing oocyte (Dapples and King, 1970). Up-regulation of nucleolar proteins in rapidly dividing cells or in metabolically active cells has been well described in the past, however, and it is not a novel observation (Sommerville, 1986).

Like mammalian NS, NS1 was most abundant within the granular regions of the nucleolus. This localization suggests that NS1 associates late with maturing ribosomal subunits. In mammals, yeast and C. elegans NS, there was aberrant pre-rRNA processing associated with loss of the respective NS homologues. Interestingly, this result has been described for the yeast GTPase, Lsg1, which associates with ribosomes in the nucleoplasm (Kallstrom et al., 2003). Therefore, although NS may play its most important role in LSU maturation and nucleolar export, a loss of NS may also cause a feedback block in pre-rRNA processing. Thus the aberrant pre-rRNA processing seen in NS depleted organisms may not be directly related to NS function.

In addition to nucleolar localization, over-expressed NS1 associated with the polytene salivary chromosomes. This association may have caused the lethality seen in the larvae that over-expressed NS1. If NS1 associates with nascent mRNA transcripts, it may inhibit mRNA processing or translation which in turn could lead to aberrant cell cycle arrest, apoptosis or necrosis in those affected tissues. Furthermore, this might explain the apoptosis described
when NS was over-expressed in cultured mammalian cells, which already have a high abundance of endogenous NS. Cells that already express NS1 might be adversely affected by over-expression of NS, especially if they are at or near the physiological threshold for NS1 expression.

In Chapter 4, RNAi was used to deplete NS1 mRNA levels, and several phenotypes were observed. First and foremost was lethality when the da-GAL4 driver was used. Tissue specific expression of RNAi using tissue specific GAL4 drivers avoided this lethality. Upon close inspection, growth arrest and developmental defects were apparent in these tissues. For example, loss of NS in the wing disc caused a crumpled wing phenotype. Targeted RNAi expression in the eye discs resulted in reduced ommatidia size, and sometimes partial or complete loss of the ommatidia. In the salivary glands there was severe growth arrest, which was characterized by an approximately 60% size reduction. These larvae adhered weakly to the container walls which suggested that the salivary glands were inefficient at synthesizing glue protein. Thus, this observation supports the hypothesis of the loss of ribosomes and protein synthesis. In addition, the polytene chromosomes within the salivary glands had a phenotype reminiscent of under-replication that has been described for salivary gland specific p21 over-expression (Edgar and Orr-Weaver, 2001).

To investigate the role of NS1 in Drosophila ribosome biosynthesis, we used fluorescently tagged ribosomal proteins (GFP-RpL11 or mRFP-RpL26), and found that these proteins accumulated in the nucleolus when NS1 was depleted. Thus, like yeast Nug1, Drosophila NS1 is required for export of the large ribosomal subunit (Rosby et al., 2009).

Cell cycle arrest and apoptosis are associated with NS depletion and have been primarily ascribed to the action of p53 and MDM2 (Ma and Pederson, 2007; Tsai and McKay, 2002), but it
is important to remember that flies lack p14^{ARF}, MDM2, and that p53 function is mechanistically different from vertebrate p53 in that *Drosophila* p53 is required for DNA repair, but not for cell cycle arrest (Brodsky *et al*., 2004; Sogame *et al*., 2003). This challenges the various p53 links that have been described for mammalian NS. Furthermore, the model for apoptosis associated with NS1 knockdown assumes that nucleolar disruption occurs, yet there are no solid reports of a redistribution of nucleolar components from the nucleolus upon NS depletion (Beekman *et al*., 2006; Ma and Pederson, 2007).

In p53 null mouse blastocysts, loss of NS still lead to cell cycle arrest and lethality, which suggests that p53 was not the causative agent in the described growth and cell cycle defects (Romanova *et al*., 2009). A similar result was observed in *C. elegans* (Kudron and Reinke, 2008). Another discrepancy in the NS deficient blastocysts was the lack of caspase activation which should accompany a p53 mediated apoptotic cascade (Beekman *et al*., 2006). Activation of the caspase proteinases is the central event in the effector phase of apoptosis (Schuler and Green, 2001). These observations suggest that there is a separate underlying mechanism in which the cell is sensing a problem with ribosome biosynthesis, and in turn shutting down other metabolic events.

An alternative explanation for cell cycle arrest upon NS1 depletion is that the loss of NS1 and subsequent loss of protein synthesis can lead to a loss of cyclin protein translation. Cyclins are proteins that have an oscillating expression pattern that regulate the progression through the cell cycle. Loss of Cyclin-D function for instance can induce arrest in the G1 phase (Weng *et al*., 2001). Since cyclins are regulated by their expression levels, they are good candidate proteins for NS-depletion mediated cell cycle arrest.
The induction of an autophagic response may also be the missing link to understanding how loss of NS seemingly escapes the p53 pro-apoptotic machinery. mTOR serves as a response element to many types of cellular stresses. mTOR has been shown to initiate responses to many stresses including hypoxia, nutrient starvation and some genotoxic stresses (Wullschleger et al., 2006). In a normal healthy cell, mTOR function prevents autophagy, while promoting ribosome biosynthesis and transcription of several Pol II genes that function in cell growth (Wullschleger et al., 2006). Under stress conditions such as inhibition of ribosome biosynthesis, however, the same stress sensing mechanism of mTOR may serve to promote autophagy in an attempt to rescue the organism from the loss of protein synthesis. mTOR signaling has been implicated in cell cycle arrest and apoptosis (Qi et al., 2008; Wanner et al., 2006), but again due to the lack of caspase activation, the question of apoptosis remains. Currently, the precise mechanism which would relate mTOR to NS is an unexplored avenue, but NS may be interacting with mTOR through a mechanism similar to that of Nog1 (Honma et al., 2006).

**Future Work**

This thesis has described *Drosophila* NS1, which is the homologue of human NS. Using the *Drosophila* system has offered several novel and key observations. First, NS1 shares many similarities with all NS homologues both structurally and functionally. Potential roles for human NS interacting with p53 has been produced by a relatively few labs. The *Drosophila* data described here has focused on different aspects of NS activity and has established a correlation with ribosome biosynthesis. Immunoprecipitation experiments should provide information regarding the association of NS1 with preribosomes, as well as more data about its role in cell cycle arrest and/or apoptosis. Determination of interacting genes will provide a wealth of information.
Establishing a strong link between mTOR signaling and NS would be a very novel and important finding. There are many TOR signaling mutants available in *Drosophila* that could be utilized for this study. For example, since TOR is an essential gene, a logical experiment would be to over-express TOR in flies depleted for NS1, and see if the autophagy phenotype persists. Alternatively knocking down NS1 in a mutant TOR signaling background should compound the autophagy and lethal phenotype.

There is a NS1 mutant stock available that has a P-element inserted in the NS1 promoter region. Rescue experiments were attempted, but the P-element that disrupts NS1 also disrupts the promoter of a divergent gene that is a mitochondrial ribosomal protein. GFP-NS1 over-expression did not rescue the P-element line, but further experiments should include making a rescue transgene for the second disrupted gene.

If NS1 functions in pre-rRNA processing, then Northern blot analysis of RNAi and wildtype flies would provide information with regards to a relative point of association for NS1 in the processing pathway. These experiments would also establish the aberrant processing event that might be associated with depletion of *Drosophila* NS1.

Finally, establishing a link between *Drosophila* NS1 and p53 would help understand some of the conflicting reports concerning human NS and p53. This is a very important facet of NS function that needs to be thoroughly characterized. There are p53 antibodies available, but p53 is expressed in very low levels in *Drosophila*, so an alternative approach such as over-expressing p53 or using p53 target genes as references would need to be used. If a p53-NS1 interaction could be confirmed, it would either suggest that *Drosophila* has a gene that functions as MDM2, or conversely, it would show that there in an alternative mechanism by
which loss of NS1 could affect p53. *NS1* is a gene that deserves rigorous study to relate its roles in ribosome biogenesis, cell cycle progression, and cell stress response.
Literature Cited


Kallstrom, G., Hedges, J., and Johnson, A. (2003). The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. Mol Cell Biol 23, 4344-4355.


### Appendix A: Chromosome Maps of Transgenic Stocks and Description

<table>
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<th>Insert</th>
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<th>Chromosome</th>
<th>Comments</th>
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</thead>
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<td>Normal</td>
<td>?</td>
<td>Mugsy insert is not on second chromosome</td>
</tr>
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<td>A2</td>
<td>Normal</td>
<td>3</td>
<td>Mugsy insert is on third chromosome</td>
</tr>
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<td>Normal</td>
<td>2</td>
<td>Insert is on second chromosome</td>
</tr>
<tr>
<td>GFP-NS</td>
<td>B2</td>
<td>Larval Lethal</td>
<td>?</td>
<td>1st instar lethal+slow development</td>
</tr>
<tr>
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<td>Normal</td>
<td>3</td>
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<td>Normal</td>
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<td>GFP-NS</td>
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<td>Normal</td>
<td>Multiple</td>
<td>lethal from larvae to pupae, mostly larvae a few adults survive, abdomen is asymmetric in pattern</td>
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<td>Semi-Lethal</td>
<td>X maybe multiple</td>
<td>several make it to adults but some die as larvae and as pupae</td>
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<td>Lethal</td>
<td>Multiple</td>
<td>2nd instar, 3rd instar and pupae lethal</td>
</tr>
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</table>

<p>| RNAiNS | A1   | Lethal    | 3          | On third |
| RNAiNS | A2   | Semi-Lethal | 3          | Ectopic Bristle and eye defects |
| RNAiNS | A3   | Lethal    | 3          | on third chromosome |
| RNAiNS | A4   | Lethal    | 3          | Probably multiple insertions on 3rd or a semi lethal insertion as I only see a few that are homozygous |
| RNAiNS | A5   | Lethal    | 3          | on third chromosome |
| RNAiNS | B1   | Lethal    | 2          | On second |
| RNAiNS | B2.1 | Lethal    | 2          | On second |
| RNAiNS | B2.2 | Lethal    | 2          | On second |
| RNAiNS | B2.3 | Lethal    | 2          | On second |
| RNAiNS | B3   | Lethal    | 2          | On second |
| RNAiNS | B4   | Lethal    | x          | insert is on X chromosome stock is balanced over FM4.B |
| RNAiNS | C1   | Lethal    | 3          | on third chromosome |
| RNAiNS | C2   | Lethal    | 2          | Balanced over cyo |
| RNAiNS | C2.2 | Lethal    | ?          | |
| RNAiNS | C4.2 | Lethal    | 2          | 90% Lethal |
| RNAiNS | C5   | Lethal    | 3          | on third chromosome 100% Lethal |
| RNAiNS | D1   | Lethal    | 3          | on third chromosome 90% Lethal |
| RNAiNS | D1.2 | Lethal    | 3          | on third chromosome |
| RNAiNS | E1   | Lethal    | ?          | |
| RNAiNS | E2   | Lethal    | ?          | |
| RNAiNS | E3   | Lethal    | ?          | |
| RNAiNS | E4   | Lethal    | ?          | |
| RNAiNS | E5   | Lethal    | ?          | |
| RNAiNS | F1   | Lethal    | ?          | |
| RNAiNS | G1   | Lethal    | 2          | 90% Lethal |
| RNAiNS | H1   | Lethal    | x          | on X Chromosome Lethal |
| RNAiNS | H2   | Lethal    | x          | on X Chromosome Lethal |
| RNAiNS | H3   | Lethal    | x          | on X Chromosome Lethal |</p>
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<tr>
<td>RFP-Rpl26</td>
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<td>Normal</td>
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Vita

Raphyel Rosby was born in Landstuhl, Germany. He is the son of Carl and Allene Rosby. After completing high school at Baton Rouge Magnet High School, he started at Louisiana State University in 1994. He took some time away from school, but returned to finish his Bachelor of Science degree in 2005. After his bachelor’s degree, Raphyel immediately started graduate school at Louisiana State University to work on his doctorate in Biological Sciences. Raphyel will receive the degree of Doctor of Philosophy in May of 2010, from Louisiana State University. Raphyel will start his post-doctoral training at Brown University in February of 2010 with Dr. Susan Gerbi.