

Examining the role of the U7 snRNA in histone pre-mRNA processing and the U7 snRNP  
dependent and independent roles of Lsm10 and Lsm11, two novel Lsm proteins in  
*Drosophila*

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## ABSTRACT

Ashley C. Godfrey: Examining the role of the U7 snRNA in histone pre-mRNA processing and the U7 snRNP dependent and independent roles of Lsm10 and Lsm11, two novel Lsm proteins in *Drosophila*.  
(Under the direction of Robert J. Duronio)

Cell cycle regulated histone gene expression ensures that the correct amounts of histones are synthesized each S phase, and is controlled in large part by the unique 3' end of histone mRNA, which terminates in a conserved stem-loop structure generated by an endonucleolytic cleavage, rather than a polyA tail. Histone 3' end formation involves a pre-mRNA processing reaction requiring a protein that binds the stem loop (SLBP), and the U7 snRNP, which interacts with a purine rich sequence, called the HDE (Histone Downstream Element), downstream of the cleavage site in histone pre-mRNA. The U7 snRNP is related to the spliceosomal snRNPs, small noncoding RNAs bound by a seven member Sm protein ring, but its protein ring contains two unique proteins, Lsm10 and Lsm11, and it's only known function is in histone pre-mRNA processing. Much of this molecular model has been obtained from *in vitro* studies. In this thesis we characterize the U7 snRNP and its two unique proteins, Lsm10 and Lsm11, genetically and molecularly in order to determine their role in cell cycle regulated histone expression *in vivo*, and during development in *Drosophila melanogaster*.

We have created null alleles of the U7 snRNA and found that they result in the production of polyadenylated histone mRNA from the use of cryptic polyadenylation signals downstream of the normal processing site. A similar molecular phenotype also results from

mutation of *Slbp*, but *U7* null mutants survive to adulthood and are male and female sterile while *Slbp* null mutants are lethal. This difference in terminal phenotype may reflect a later onset of the histone pre-mRNA processing defect in *U7* null mutants compared to *Slbp* null mutants. In *Slbp* null mutants, misprocessed histone mRNA is seen as early as the embryo stage of development while in *U7* null mutants the misprocessed histone mRNA does not appear until the second instar stage of development, due to the maternal stores of U7 snRNA.

We have also analyzed mutations of the *Lsm10* and *Lsm11* genes and found that those mutations result in the same misprocessed histone mRNA phenotype as *U7* null mutants, but both mutations are lethal. We have shown that the difference in terminal phenotype is not due to an earlier onset of misprocessed histone mRNA, but instead could be due to a role(s) for Lsm10 and Lsm11 outside of histone pre-mRNA processing that is U7 independent. We have also shown that there is U7 snRNA still present in an *Lsm11* null mutant. The RNA can be pulled down using TMG coupled beads suggesting that the U7 snRNA is bound by snRNP proteins even though Lsm10 and Lsm11 are not present. However this snRNA does not localize to the Histone Locus Body (HLB) suggesting that both Lsm10 and Lsm11 are required for U7 snRNP localization to the HLB.

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## LIST OF ABBREVIATIONS

asn	Asparagine
asp	Aspartic acid
CB	Cajal Body
Cdk 2	Cyclin-Dependent Kinase 2
CDK	Cyclin-Dependent Kinase
CNS	Central Nervous System
CPSF 73	Cleavage and Polyadenylation Factor number 73
CPSF 100	Cleavage and Polyadenylation Factor number 100
cDNA	complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
ds	Down stream
Df	Deficiency
EDTA	Ethylenediamine Tetraacetic Acid, Disodium Salt Dihydrate
Eip63E	Ecdysone induced puff on chromosome area 63 E
EMS	Ethyl Methyl Sulfate
FISH	Fluorescent In Situ Hybridization
Gly	Glycine
Glu	Glutamic acid
G	Gap
HBP	Hairpin Binding Protein
HDE	Histone Downstream Element

HLB	Histone Locus Body
Lsm	Like-sm
Lsm10	Like-sm protein number 10
Lsm11	Like-sm protein number 11
M	Mitosis
Mep 50	WD repeat domain 45 protein
mis-proc	misprocessed
MOPS	3-(N-Morpholino) Propane-Sulfonic Acid
MPM-2	Mitotic Protein Monoclonal #2
mRNA	messenger Ribonucleic Acid
MF	Morphogenetic Furrow
NP40	Nonidet P 40
PCR	Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
PMSF	PhenylMethaneSulphonylFluoride
PRMT5	Protein Argine Methyl-Transferase number 5
PSEA	Proximal Sequence Element A
PSEB	Proximal Sequence Element B
RG	Arginine Glycine
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
S	Synthesis
S2	Schneider 2

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLBP	Stem Loop Binding Protein
Sm	Stephanie Smith
SMN	Spinal Muscular
SMA	Spinal Muscular Atrophy
snRNA	small nuclear Ribonuclear Acid
snRNP	small nuclear Ribonuclear Protein
TILLING	Targeted Induced Local Lesions In Genomes
TMG	TriMethlyGuanosine cap
U7	small nuclear ribonuclear acid number 7
UTR	UnTranslated Region
V5	Simian Virus 5
ZFP 100	Zinc Finger Protein 100
zn	Zinc finger
WT	Wild Type

## **CHAPTER I**

### **INTRODUCTION**

One of the most important processes in the cell cycle is the replication of DNA. Every S-phase the amount of DNA is doubled so that each daughter cell that results from the cell division has the same amount of DNA as its parent cell. This is important for maintaining genomic integrity. The DNA itself is extremely long and must be compacted in order to fit into the small space of the nucleus. This higher order structure of DNA, also known as chromatin, is made by wrapping the DNA around histone octamers which form a nucleosome. Each nucleosome is then further compacted and looped until a chromosome is made. Since histones are the core proteins that make up the nucleosome, histone biosynthesis is an important process in all cells. The histones that make up the core nucleosome octomer, H2A, H2B, H3, H4, as well as the H1 linker, are synthesized only in S-phase when they are needed for chromatin synthesis. Histone expression outside of S-phase has been shown in budding yeast to lead to genomic instability (Gunjan & Verreault, 2003). This tight S-phase regulation in animal cells is accomplished mainly through posttranscriptional regulation of the histone mRNA.

#### **Mechanism of Cell Cycle Regulated Histone Biosynthesis**

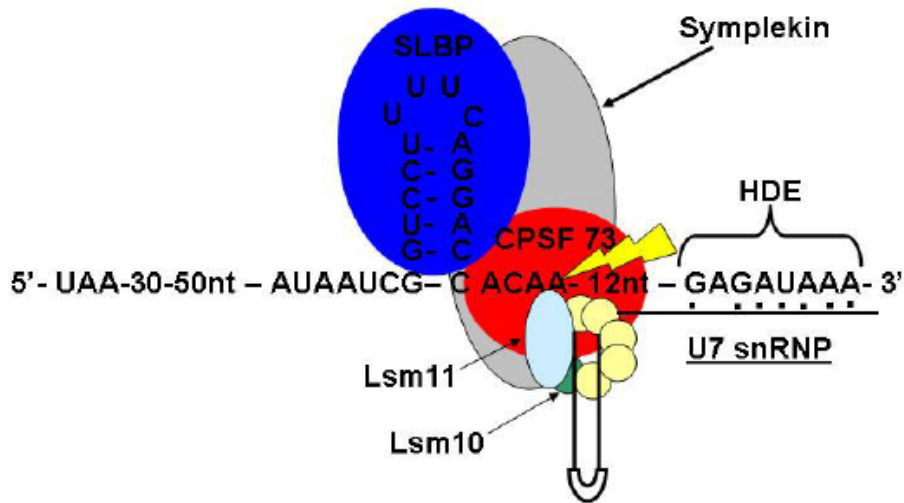
Cell cycle regulated histone biosynthesis is an essential aspect of genome duplication during cell proliferation, and is controlled primarily by the cell cycle regulated biosynthesis of histone mRNAs (Luscher & Schumperli, 1987; Stauber & Schumperli, 1988; Harris et al., 1991). The 3' end of histone mRNAs is required for this cell cycle regulation (Birchmeier et

al., 1983). However, in animal cells the histone mRNAs are unique: unlike other mRNAs, the histone mRNA ends in a conserved 26-nt sequence that forms a stem-loop rather than a poly A<sup>+</sup> tail (Marzluff, 2005). Since histone genes lack introns, the only processing step required for mature histone mRNA production is endonucleolytic cleavage of the pre-mRNA to form the 3' end of the mRNA (Dominski & Marzluff, 1999). A specialized set of molecular machinery is needed to generate histone mRNAs, and understanding how this unique and necessary machinery functions is the goal of my thesis.

Cell cycle regulated histone pre-mRNAs undergo a one step processing reaction which utilizes their unique 3' end (Fig. 1.1). The 3' end contains two cis acting elements which are required for this processing reaction: a conserved stem loop followed by a purine rich region termed the Histone Downstream Element (HDE) (Mowry & Steitz, 1987a). The pre-mRNA is cleaved between the stem loop and the HDE forming a mature, processed mRNA. The stem loop is recognized by a protein called Stem Loop Binding Protein (SLBP) (Wang et al., 1996), or Hairpin Binding Protein (HBP) (Martin et al., 1997) which binds to the stem loop and then helps to recruit the U7 Small Nuclear RNA (snRNA). The U7 snRNP recognizes and binds to the HDE via base pairing between U7's 5' end and the HDE in the histone pre-mRNA (Galli et al., 1983; Schaufele et al., 1986; Mowry & Steitz, 1987a; Bond et al., 1991). In mammals the position of the HDE, and thus the binding site for U7 snRNP, determines the cleavage site. Insertion of additional nucleotides between the normal cleavage site and the HDE results in the cleavage site moving farther 3' of the stem-loop, while decreasing the efficiency of processing (Scharl & Steitz, 1994). It has been shown that compensatory insertions of nucleotides in the U7 snRNA can compensate for both defects seen by insertions of nucleotides between the normal cleavage site and the HDE. It has also been



shown that compensatory mutations in the U7 snRNA can restore processing both in *vitro* and in *vivo* when mutations in the HDE abolish processing (Bond et al., 1991) (Scharl & Steitz, 1996). In *Drosophila* however, the U7 snRNP does not function as a molecular ruler, as increasing the distance between the stem-loop and HDE does not result in a corresponding shift of the cleavage site. SLBP instead plays a critical role in specifying the cleavage site as it is essential for processing all five *Drosophila* histone pre-mRNAs (Dominski et al., 2005b). In mammals a 100 kDA zinc finger protein, ZFP100 binds to U7 snRNP and helps to stabilize the whole complex on the pre-mRNA (Dominski et al., 2002). The cleavage endonuclease in mammals has recently been identified as CPSF73 (Dominski et al., 2005a), which is part of the cleavage and polyadenylation complex which cleaves and polyadenylates all other mRNAs. This result was somewhat surprising since it was believed that the histone endonuclease would be unique to histone pre-mRNA processing. There are other novel components of the processing reaction that have recently been discovered, but how they assemble into the complex and what function they play is unknown. For example, a heat labile factor, Symplekin, has been shown to be part of the histone pre-mRNA processing reaction in mammals (Kolev & Steitz, 2005), and has also been shown to be necessary in flies as part of an elaborate RNAi screen in S2 cells to identify factors necessary for histone pre-mRNA processing in *Drosophila* (Wagner et al., 2007). Once processed, the mature histone mRNAs exit the nucleus with SLBP still bound and are translated in the cytoplasm (Sanchez & Marzluff, 2002). Figure 1.1 shows a diagram of the histone pre-mRNA 3' end and the known processing factors in *Drosophila*.



**Figure 1.1 Structure of the histone pre-mRNA.** The *Drosophila* histone 3' end is shown. The dots below the HDE represent nucleotides that can base pair between U7 snRNP's 5' end and the HDE. The histone pre-mRNA is cleaved after the last A of the terminal ACAA (yellow arrow).

### Structure and synthesis of snRNP particles

The U7 snRNP is necessary for histone pre-mRNA processing and is related to the spliceosomal snRNPs (Galli et al., 1983; Gick et al., 1986; Mowry & Steitz, 1987b). A snRNP, small nuclear RiboNuclear Protein, is composed of a short, noncoding RNA sequence which is bound by proteins and functions in the nucleus. All of the spliceosomal snRNPs, except U6, are composed of a short, noncoding, nonpolyadenylated RNA bound by a heptameric ring of conserved proteins: SmD1, SmD2, SmE, SmF, SmG, SmB/B', and SmD3 (Luhrmann et al., 1990). (See Figure 1.2 for a pictorial representation of this spliceosomal ring complex.). These proteins are named after Stephanie Smith, the first patient from whom the systemic lupus erythematosus-associated anti-Sm autoimmune antibodies were discovered (Lerner & Steitz, 1979). These proteins bind to a conserved uridyl-rich sequence on the RNA termed the Sm binding site (Branlant et al., 1982)(Fig 1.2).

The structure of the Sm proteins consists of two conserved motifs, SM1 and SM2 with a linker of variable length in between (Hermann et al., 1995) (Seraphin, 1995). The crystal structure analysis of two Sm protein complexes, D3/B and D1/D2, reveals that the proteins have a common fold, termed the Sm fold, containing an N-terminal alpha-helix followed by a strongly bent five-stranded antiparallel beta sheet (Kambach et al., 1999). This data, along with the data from 3 other papers containing two related bacterial crystal structures: Lsm $\alpha$  from the thermophilic archaeon *Methanobacterium thermoautotrophicum*, and Sm1 and Sm2 from the hyperthermophilic euryarchaeon *Archaeoglobus fulgidus* (AF-Sm1 and AF-Sm2), suggest that the 7 proteins form a donut shaped ring structure where the RNA threads through the center hole (Toro et al., 2001) (Toro et al., 2002) (Collins et al., 2001). Kambach et al., proposed that the RNA is bound on the inside of this structure through an interaction with three conserved residues in each Sm protein (except D3) and the Uradines in the RNA sequence (Kambach et al., 1999). Crosslinking data and other studies have also shown that Sm D3, B, and G each contact one single residue in the Sm binding sequence (Urlaub et al., 2001) (Fury et al., 1997) (Stark et al., 2001).

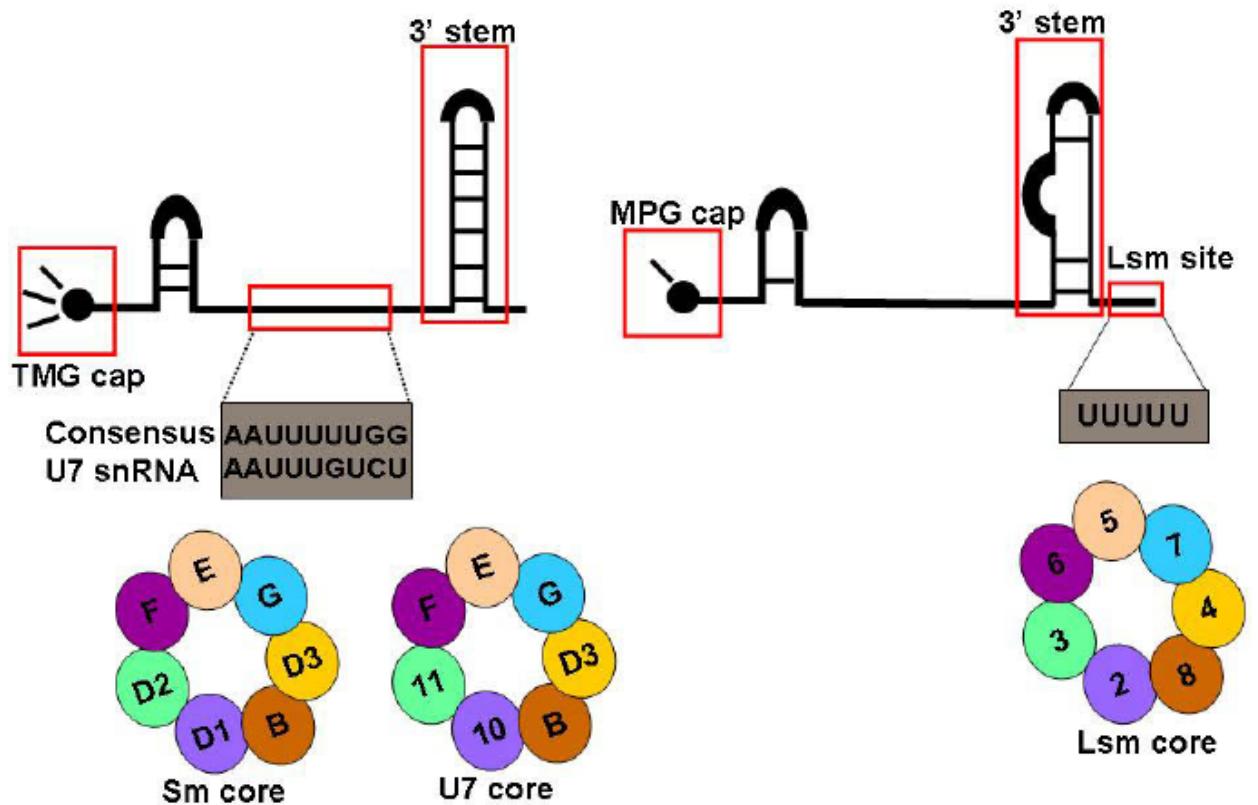
The U6 snRNA does not contain the Sm binding site and recruits a ring made of Lsm proteins 2-8 which are required for the U6 snRNP to accumulate to normal levels (Mayes et al., 1999; Salgado-Garrido et al., 1999)(see figure 1.2). The Lsm proteins are named for being Like-Sm proteins; they contain the 2 SM domains and are therefore similar in structure to Sm proteins. Although there is no Sm site in the U6 snRNA, there is a uridine tract at the 3' end, called the Lsm site, that was demonstrated to be essential for Lsm protein binding in both yeast and humans (Achsel et al., 1999; Vidal et al., 1999)(Fig. 1.2). Also unlike the other spliceosomal snRNAs, the U6 snRNA is transcribed by RNA polymerase III, acquires a

$\gamma$ -monomethyl cap and appears to be restricted to the nucleus (Kunkel et al., 1986; Reddy et al., 1987). The nuclear localization of the U6 snRNP has recently been shown to be determined by the complete Lsm2-8 complex in yeast (Spiller et al., 2007).

The synthesis of snRNPs is a rather complex process that takes place primarily in the cytoplasm of cells. The snRNAs are transcribed by RNA polymerase II and co-transcriptionally given a m<sup>7</sup>G-cap which is specifically recognized by the cap-binding complex (CBC), which itself associates with other proteins to form the nuclear export complex (Askjaer et al., 1999). The Sm proteins themselves form heterooligomeric complexes composed of D1-D2, B-D3, and E-F-G (Raker et al., 1996) which are then bound by the methylosome, a large active complex containing Protein Arginine Methyl-Transferase 5 (PRMT5), WD repeat domain 45 (WD45), also called Mep50, and chloride conductance regulatory protein (pICln). Through the action of PRMT5, this complex catalyzes the formation of symmetrical dimethylation on arginine residues in RG repeats in the C-terminal tail of Sm proteins B, D1, and D3. The methylosome is then thought to bind the SMN complex. The Sm proteins are transferred to the SMN-complex and then put onto the snRNA around the Sm binding site (Friesen et al., 2001; Meister et al., 2001b; Friesen et al., 2002; Meister et al., 2002; Meister & Fischer, 2002; Miranda et al., 2004). The SMN-complex is named for its founding protein SMN (Survival Motor Neuron) whose reduced expression causes the neuromuscular disease spinal muscular atrophy (SMA) and it was originally found to transiently interact with U snRNAs in the cytoplasm but was not part of mature U snRNPs (Fischer et al., 1997). Other than the SMN protein, the SMN-complex is made up of at least eight key subunits, Gemins 2-8 and unrip (Meister et al., 2001a) (Pellizzoni et al., 2002) (Eggert et al., 2006) (Gubitz et al., 2004) (Otter et al., 2007). Once the Sm ring is in place

the m<sup>7</sup>G-cap gets hypermethylated by the TGS1 protein to become an m<sub>3</sub>G cap, also called the TMG cap (TriMethlyGuanosine) and this is essential for import of the UsnRNP back into the nucleus (Hamm et al., 1990; Mouaikel et al., 2002).

After being imported, the U snRNPs, presumably still attached to the SMN-complex, transiently accumulate in subnuclear domains termed Cajal bodies, where pseudouridylation and 2'0-methylation at specific sites in the snRNA completes the maturation process of the U snRNPs (Jady et al., 2003). Cajal bodies (CBs), are nuclear structures involved in the assembly and modification of the machinery needed for pre-mRNA splicing, pre-ribosomal RNA processing, and histone pre-mRNA processing (for reviews see Gall, 2000; Carmo-Fonseca, 2002; Gall, 2003; Matera, 2003; Cioce & Lamond, 2005). Some of these Cajal bodies are associated with histone genes, and may represent sites of histone mRNA biosynthesis. *Drosophila* cells also contain a Cajal body, but the Cajal body lacks U7 snRNP (Liu et al., 2006). *Drosophila* cells have a distinct body termed the histone locus body (HLB) which is invariably associated with the histone gene locus and where the U7 snRNP localizes (Liu et al., 2006; White et al., 2007). HLBs likely contain all of the factors necessary for histone mRNA transcription and pre-mRNA processing (Marzluff et al., 2008). It has been shown previously in our lab that the U7 snRNP specific protein Lsm11 can be seen in the HLB in *Drosophila* cells(White et al., 2007).



**Figure 1.2. Structure and features of Sm and Lsm class small nuclear RNAs.** The left part shows the structure of the Sm class of snRNPs. The three important structural elements are boxed in red. The seven member protein ring which binds to the Sm site is shown below. The Sm core is invariant in all spliceosomal snRNPs except for U6, while the U7 core is found only bound to the U7 snRNP. The right part shows the structure of the Lsm class of snRNPs. Again the three important structural elements are boxed in red and the seven member protein ring which binds to the Lsm site is shown below.

### **The U7 snRNP particle in *Drosophila***

The U7 snRNP has been shown to contain two unique proteins, Lsm10 and Lsm11. Lsm10 is closely related to SmD1, while Lsm11 is somewhat related to SmD2 (Pillai et al., 2001) (Pillai et al., 2003). U7 snRNA contains a unique Sm site which is believed to recruit

Lsm10 and Lsm11 specifically to the U7 particle (Fig. 1.2). When this unique site is mutated to the spliceosomal consensus Sm binding site, then SmD1 and SmD2 are inserted in place of Lsm10 and Lsm11 in the U7 snRNP (Pillai et al., 2003). Lsm11 is unique among all other Sm and Lsm proteins, containing an N-terminal region longer than any other known protein of these two families. It also contains a rather long linker sequence between Sm motif 1 and 2 (Schumperli & Pillai, 2004). Previous work has suggested a role for Lsm11 in the 3' processing reaction, by binding directly to ZFP 100 (Pillai et al., 2003) (Azzouz et al., 2005). Indeed, in mammalian cells the binding site for ZFP100 has been mapped to amino acids 30-60 in Lsm11's N-terminal region (Wagner et al., 2007). Other work has shown mutation of certain conserved amino acids in Lsm11's N-terminal region can impair processing (Azzouz et al., 2005).

Recently, the *Drosophila* U7 snRNA has been cloned and characterized (Dominski et al., 2003). The Lsm10 and Lsm11 homologues have also been recently discovered in *Drosophila* (Azzouz & Schumperli, 2003). Most of the knowledge we have about the U7 snRNP has been gained from nuclear extract systems that support the processing of synthetic histone pre-mRNAs and by examining the processing of histone pre-mRNAs injected into *Xenopus* oocytes (for review, see Dominski and Marzluff 1999). An *in vivo* study of the U7 snRNP and its two unique proteins, Lsm10 and Lsm11, has not been carried out to date. An *in vivo* knockout of the U7 snRNA has not been studied thus far and it is unclear what might happen to the whole animal when just the U7 snRNA is removed. An *in vivo* knockout of Lsm10 and Lsm11 protein(s) has also not been done and there is no evidence for how the structure of Lsm10 and Lsm11 affect each proteins function in a whole animal, and there is

also no evidence for how each protein contributes to histone pre-mRNA processing in a whole animal.



## Dissertation goals

In this thesis I will discuss work I performed with my collaborators studying the role of the U7 snRNP and its two unique proteins, Lsm10 and Lsm11, in histone pre-mRNA processing in *Drosophila*. In chapter II we generated and analyzed null mutations in the U7 snRNA. We found that the U7 snRNA is necessary for normal histone mRNA biosynthesis during *Drosophila* development and loss of U7 snRNA results in the production of polyadenylated histone mRNAs. We also found that loss of both the U7 snRNA and SLBP disrupt oogenesis as they are both required for normal histone mRNA biosynthesis in the female germ line. In chapter III we searched for and analyzed mutations in the Lsm10 and Lsm11 genes. We found that like the U7 snRNA, Lsm10 and Lsm11 are both necessary for normal histone mRNA biosynthesis during *Drosophila* development and loss of either protein results in the production of polyadenylated histone mRNAs. *Lsm10* and *Lsm11* mutants however, cause a more severe terminal phenotype than *U7* null mutants and we therefore hypothesize that Lsm10 and Lsm11 have a function outside of the U7 snRNP which is required for viability. We also found that in the absence of both Lsm10 and Lsm11 proteins, the U7 snRNA can bind to Sm proteins, but the snRNP formed is non-functional in histone pre-mRNA processing and does not localize to the HLB, demonstrating that Lsm10 and Lsm11 are required for proper localization of the U7 snRNP. In chapter IV, we analyzed the function of Lsm11's unique N-terminus in histone pre-mRNA processing and during development. We found that a full length, tagged copy of Lsm11 could fully rescue all phenotypes seen in an *Lsm11* null mutant, but surprisingly any deletion of any part of the N-terminus could not be stably expressed even when put in an *Lsm11* null mutant background. In chapter V these results are summarized and discussed in a broad context.

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## CHAPTER II

### **U7 snRNA MUTATIONS IN *DROSOPHILA* BLOCK HISTONE PRE-mRNA PROCESSING AND DISRUPT OOGENESIS**

#### **Preface**

This work was previously published and represents my first author paper with work contributed to it by members of my lab as well as member of the Marzluff lab. This work was carried out in collaboration with William F. Marzluff, a professor here at UNC.

My advisor, Robert J. Duronio, conceived the project with input on the project's direction from me and the other contributors. I completed the majority of the experimental work shown. A former graduate student in the lab, Jeremy M. Kupsco, discovered the original P-element allele of *U7* (Fig. 2.1C), did some of the *in situ* staining on imaginal eye discs (Fig. 2.5E and F), and did the H2a S1 assay (Fig. 2.8B). Jeremy also mentored a former undergraduate student in our lab, Ryan M. Zimmerman, who made *slbp*<sup>15</sup> germ line clones and did *in situ* staining on their egg chambers (Fig. 2.7I) and an H3 northern blot from female ovaries (Fig. 2.8A). A research assistant professor in the Marzluff lab, Zbigniew Dominski, did the H3/H4 double Northern blot on *U7*<sup>EY11305</sup> males and females (Fig. 2.3B) as well as the early embryo U7 Northern blot from wild-type embryos (Fig. 2.4A). A graduate student in the Marzluff lab, Brandon D. Burch, performed the H2a S1 nuclease protection assay on the *U7* alleles (Fig. 2.3E) and on *slbp* and *U7* double and single mutant alleles (Fig. 2.6B). Robert J. Duronio wrote most of the manuscript with input from me and the other authors.



**Godfrey, A.C., Kupsco, J.M., Burch, B.B., Zimmerman, R.M., Dominski, Z., Marzluff, W.F., and Duronio, R.J.** (2006). U7 snRNA mutations in *Drosophila* block histone pre-mRNA processing and disrupt oogenesis. *RNA* **12**, 396-409.

## **Abstract**

Metazoan replication dependent histone mRNAs are not polyadenylated, and instead terminate in a conserved 26 nt sequence that forms a stem loop structure. Generation of this unique 3' end requires an endonucleolytic cleavage involving the U7 snRNP, which interacts with histone pre-mRNAs through base pairing between U7 snRNA and a purine rich sequence in the pre-mRNA located downstream of the cleavage site. However, the contributions of U7 snRNA to histone mRNA biosynthesis in an intact animal, and the consequences to development of inactivating U7 snRNA, have not been examined. Here we generate null mutations of the single *Drosophila* U7 gene and demonstrate that U7 snRNA is required in vivo for processing all 5 replication associated histone pre-mRNAs. Mutation of U7 results in the production of poly A<sup>+</sup> histone mRNA in both proliferating and endocycling cells because of read-through to cryptic polyadenylation sites found downstream of each *Drosophila* histone gene. We previously reported that a similar molecular phenotype also results from mutation of *Slbp*, which encodes the protein that binds the 26 nt stem loop at the 3' end of histone mRNAs. U7 mutants are viable, but both males and females are sterile, and the females display defects during oogenesis similar to germ line clones of *Slbp* null cells. In contrast, *Slbp* null mutations cause lethality, and this difference in terminal phenotype likely results from a later onset of the histone pre-mRNA processing defect in the U7 mutants

compared to *Slbp* mutants, due to maternal stores of U7 snRNA. A double mutant combination of a viable, hypomorphic *Slbp* allele and a viable *U7* null allele is lethal, and these double mutants express polyadenylated histone mRNAs earlier in development than either single mutant. Together these data suggest that SLBP and U7 snRNP cooperate in the production of histone mRNA in vivo, and that disruption of histone pre-mRNA processing is detrimental to development.

## **Introduction**

Chromosome duplication during the cell cycle requires the production of histones during S phase to package newly replicated DNA into chromatin. Bulk histone production during S phase is achieved through the biosynthesis of replication-dependent histone mRNAs, which are cell-cycle regulated and accumulate only in S-phase. In animal cells these histone mRNAs are unique: the 3' end terminates in a conserved 26 nt sequence that forms a stem-loop rather than in a poly A<sup>+</sup> tail (Marzluff, 2005). As histone genes lack introns, the only processing step required for mature histone mRNA production is endonucleolytic cleavage of the pre-mRNA to form the 3' end of the mRNA (Dominski & Marzluff, 1999). Much of the cell cycle regulation of histone mRNAs is posttranscriptional and is mediated by the 3' end of the mRNA (Luscher et al., 1985; Harris et al., 1991; Zheng et al., 2003). Thus, a complete understanding of cell cycle regulated histone mRNA production requires a full understanding of the factors required for histone pre-mRNA processing.

The processing of histone pre-mRNAs requires two cis elements and a number of trans-acting factors. The cis elements are the stem-loop at the 3' end of histone mRNA, and a purine rich region downstream of the cleavage site, termed the histone downstream element

(HDE). A protein called stem loop binding protein (SLBP) (Wang et al., 1996) or hairpin binding protein (HBP) (Martin et al., 1997) specifically binds the 3' end of histone mRNA. SLBP is required for histone pre-mRNA processing in vivo (Sullivan et al., 2001; Kodama et al., 2002; Pettitt et al., 2002) and accompanies the mRNA to the cytoplasm (Erkmann et al., 2005) where it promotes the translation of the histone mRNA (Sanchez & Marzluff, 2002; Whitfield et al., 2004). The HDE binds U7 snRNP by base pairing with the 5' end of U7 snRNA (Schaufele et al., 1986; Mowry & Steitz, 1987; Cotten et al., 1988; Soldati & Schumperli, 1988). SLBP, the U7 snRNP, and a U7 snRNP-associated zinc finger protein called ZFP100 (Dominski et al., 2002) cooperate to recruit an endonuclease complex that cleaves the pre-mRNA. Recent evidence indicates that CPSF73, a component of the complex that mediates AAUAAA-directed cleavage prior to polyadenylation, is the likely endonuclease (Dominski et al., 2005; Kolev & Steitz, 2005). This revealed some unexpected overlap in the machinery carrying histone pre-mRNA processing and canonical polyadenylation.

The U7 snRNA is a small RNA (55-70 nts) that, like the spliceosomal snRNAs, contains both a trimethyl guanosine cap and an Sm binding site, which is essential for its function (Grimm et al., 1993; Schumperli & Pillai, 2004). The Sm site in these snRNAs stably binds a complex of seven related proteins of the LSm/Sm family to form the core snRNP particle. Proteins of the LSm/Sm family share a common tertiary structure called the Sm fold that assembles into hexameric or heptameric rings capable of binding single stranded RNA. The U snRNPs contain a heptameric Sm ring with each of the seven individual subunits making a specific contact with a residue in the Sm binding site of the snRNA (Khusial et al., 2005). The heptameric Sm ring of spliceosomal snRNPs contain the proteins

SmB/B', SmD1, SmD2, SmD3, SmE, SmF, and SmG. In contrast, the U7 snRNP contains five of these Sm proteins (B/B1, D3, E, F, G) and two novel Sm proteins called LSm10 and LSm11 that replace SmD1 and SmD2 of the spliceosomal snRNPs (Pillai et al., 2001; Pillai et al., 2003). The Sm site found in U7 snRNAs is distinct from the Sm site in spliceosomal snRNAs and is responsible for incorporation of LSm10 and LSm11 into the U7 snRNP (Schumperli & Pillai, 2004; Azzouz et al., 2005). In addition to the Sm fold that participates in ring formation, LSm11 contains an NH<sub>2</sub> terminal extension that makes contacts with ZFP100 and possibly other components of the histone pre-mRNA processing machinery (E. Wagner and W.F.M, unpublished)(Azzouz et al., 2005).

The role of U7 snRNP in histone pre-mRNA processing has been examined primarily in nuclear extract systems that support the processing of synthetic histone pre-mRNAs, and by monitoring the processing of histone pre-mRNAs injected into *Xenopus* oocytes (reviewed in (Dominski & Marzluff, 1999)). Complementary mutations in U7 snRNA and the HDE provided early evidence that base pairing between the 5' end of U7 and the HDE was an important part of U7 snRNP function (Schaufele et al., 1986; Bond et al., 1991). Furthermore, blocking the 5' end of the U7 snRNA with a complementary oligonucleotide specifically inhibits processing of synthetic histone pre-mRNAs in nuclear extracts (Cotten et al., 1991; Dominski et al., 2003). However, the contribution of U7 snRNA to endogenous histone mRNA biosynthesis, and whether this contribution is important for animal development, has not been examined. To explore these issues, we have generated and characterized U7 snRNA mutations in *Drosophila*.

*Drosophila* provides an excellent model to examine the complexities of histone mRNA biosynthesis in a multi-cellular organism (Sullivan et al., 2001; Lanzotti et al., 2002;

Marzluff & Duronio, 2002; Lanzotti et al., 2004; Lanzotti et al., 2004). *Drosophila* has a single set of replication-dependent histone genes which are present in ~100 tandem repeats of a 4.5kB unit containing one copy of each of the five histone mRNAs (Lifton et al., 1978). This gene cluster is subject to multiple mechanisms of regulation that provide the histone proteins for the different types of cell cycles that occur during *Drosophila* development, including the early syncytial cell cycles that lack gap phases, the endocycles of polyploid tissues, and the canonical cycles of proliferating diploid cells in the CNS and imaginal discs.

*Drosophila* SLBP (Sullivan et al., 2001), U7 snRNA (Dominski et al., 2003) and U7 snRNP specific proteins, Lsm10 and Lsm11 (Azzouz & Schumperli, 2003), have all been identified, and we have begun to characterize them genetically. Mutations in the *Drosophila* *Slbp* gene block normal histone pre-mRNA processing during embryonic development, and result in production of polyadenylated histone mRNAs as a consequence of read-through past the normal processing site (Sullivan et al., 2001; Lanzotti et al., 2002). This occurs because each of the five *Drosophila* histone genes contains cryptic polyadenylation sites downstream of the HDE that are utilized in the absence of SLBP (Lanzotti et al., 2002). Null mutations of *Slbp* cause lethality during larval and pupal stages, presumably because of the histone processing defects, although the precise cause of lethality is not known. *Slbp* mutant cells are capable of replicating, likely because the inappropriate polyadenylated mRNAs are translated. A hypomorphic *Slbp* mutant allele that produces reduced amounts of SLBP protein results in the production of both normal and poly A<sup>+</sup> histone mRNAs during embryogenesis, but does not cause lethality. However, these viable mutant females lay eggs that contain reduced amounts of histone mRNA and protein and do not develop (Sullivan &

Karpen, 2001; Lanzotti et al., 2002). Thus, SLBP is required during both zygotic development and oogenesis.

Here we report the study of mutations in the *U7* snRNA gene, and compare the resulting phenotypes with those caused by mutation of *Slbp*. Our results indicate that *U7* snRNA is required for normal histone mRNA biosynthesis during *Drosophila* development, and that, like *Slbp* mutations, loss of *U7* snRNA results in the production of polyadenylated histone mRNAs. However, unlike *Slbp* null mutants, *U7* null mutants are viable, but both male and female sterile. This difference in terminal phenotype is most likely because the maternal supply of *U7* snRNA delays the onset of the histone processing defect in *U7* mutants relative to *Slbp* mutants, which do not have a significant maternal supply of SLBP protein. Both *U7* and SLBP are required for normal histone mRNA biosynthesis in the female germ line, and mutation of either gene disrupts oogenesis. These data indicate that loss of SLBP and *U7* cause similar molecular phenotypes in *Drosophila*, and suggest that expression of this molecular phenotype prevents normal development.

## Materials and Methods

### P-element Excision

The EY11305 insertion site was determined by sequencing flanking genomic DNA obtained via PCR using a primer that hybridizes to the 5' terminal repeat of the P-element (SP1: 5'ACACAACCTTTCCTCTCAACA3') and a primer 5' of the *U7* coding region (U7F1: see Fig. 1B). EY11305 third chromosome excisions were recovered over TM6b as white-eyed male progeny from *yw; P[EY11305]/ry<sup>506</sup> Sb P[ry<sup>+</sup> Δ2-3]* fathers. These single males were crossed to *w; Df(3L)E1/TM3 Ser P[act-GFP]* females. *Df(3L)E1* removes

*Eip63E* but not *U7*, and 17 balanced stocks were made from those excision events that complemented the lethality of *Df(3L)E1*. Twelve of the excision chromosomes were viable and five were lethal when homozygous. Seven of the twelve homozygous viable lines were fertile, and five were sterile. Sequencing of PCR products obtained using primers that flank the EY11305 insertion site (see Fig. 1B) was used to precisely characterize the *U7*<sup>14</sup>, *U7*<sup>20</sup>, *U7*<sup>9</sup> excision alleles, and to confirm that the seven viable and fertile flies contained precise excision events. In the two cases where a PCR product using the flanking primers was not recovered (*U7*<sup>1</sup> and *U7*<sup>23</sup>), primers SP1 and 5'CAAGCATACGTTAAGTGGATGTC3', which hybridize to the 5' and 3' end of the P element, respectively, were paired with the flanking primers to assess if the P element ends remained intact. By this assay, the *U7*<sup>1</sup> allele retained both the 5' and 3' ends of the P element, and likely sustained an internal deletion of EY11305 that inactivated the mini *w*<sup>+</sup> gene. *U7*<sup>23</sup> similarly retained the 3' end of EY11305, but sustained a deletion of the 5' end of the P. However, very little if any *U7* coding sequence was removed, since a primer just upstream of the EY11305 insertion site was able to amplify flanking DNA from *U7*<sup>23</sup> animals (Fig. 1B). Each of the five homozygous lethal chromosomes contained a precise excision of EY11305, suggesting that in each case a lethal was induced elsewhere on the chromosome during transposition. The *Slbp*<sup>15</sup> null and *Slbp*<sup>10</sup> hypomorphic alleles are described in (Sullivan et al., 2001). *w*<sup>1118</sup> was used throughout as a *U7* and *Slbp* wild type control.

### **U7 Transgenic Rescue**

DNA containing the *U7* gene (Fig. 1B) was amplified by PCR from adult female *w*<sup>1118</sup> genomic DNA and subcloned into the pCaSpeR 4 transformation vector and confirmed

by sequencing. A  $w^{1118}$ ; P[U7]/P[U7]; +/- transgenic line was crossed to  $w$ ; +/-;  $U7^{14}/TM3$ , and males of the genotype P[U7]/+;  $U7^{14}/+$  were crossed to  $w$ ;  $Df(3L)LY/TM6B$  females. Single P[U7]/+; ( $U7^{14}$  or +)/TM6B male progeny were crossed to U7EY11305/TM3 females, and the non-balanced progeny (i.e. those containing  $U7^{EY11305}$  and either + or  $U7^{14}$ ) were analyzed for the presence or absence of  $U7^{14}$  by PCR. The  $w^+$  (i.e. containing P[U7]) and  $w^-$  (i.e. not containing P[U7])  $U7^{EY11305}/U7^{14}$  classes were then tested for fertility.

### Northern analysis

For northern blots, total cellular RNA was isolated using TRIzol Reagent (Gibco). For the analysis of adult U7 snRNA, 15  $\mu$ g/lane of RNA denatured with 8M urea was subject to electrophoresis in 1X TBE through an 8% polyacrylamide gel containing 7M urea. For the analysis of adult histone mRNA, 10ug/lane of RNA was subject to electrophoresis in 1X TBE through a 1% agarose gel containing 0.01M MOPS pH 7.0 and 6.75% formaldehyde. Separated RNAs were transferred in 0.5X TBE to an N+ nitrocellulose membrane (Amersham) using a Genie Blotter (Idea Scientific).  $\alpha[^{32}P]$ -UTP was used for in vitro transcription reactions to label U7 or U1 antisense RNA probes. DNA containing histone or *rp49* coding regions were labeled with  $\alpha[^{32}P]$ -dCTP using a random primer labeling kit (Stratagene). Hybridizations were performed at 58°C for snRNA probes and at 60°C for histone and *rp49* probes.

Homozygous single or double mutant larvae were identified as the GFP negative class from heterozygous parents containing either *TM3 Ser P[act-GFP]* or *TM3 Sb P[kr-GFP]* balancer chromosomes. Agarose gel northern analysis was performed as above, except that 2  $\mu$ g of RNA per lane was used and the RNA was transferred via the wick method in 20X SSC.



For the embryo analysis in Fig. 4, a collection of eggs from *U7<sup>14</sup>/TM3 Ser P[act-GFP]* parents was allowed to age 3 hours and then subjected to RNA preparation. *U7* mutant embryos were not selected because we previously demonstrated that we could detect polyadenylated H3 mRNA in a total embryo population where only ¼ of the embryos are *Slbp<sup>15</sup>* homozygous mutant (Lanzotti et al., 2002).

### **S1 Protection Analysis**

A BspE I restriction fragment containing the histone H2a gene was end-labeled at 25 °C for 20 minutes with Klenow (New England Biolabs) in a reaction containing  $\alpha$ -P<sup>32</sup>-dCTP. After removing unincorporated deoxynucleotides using a ProbeQuant G-50 Micro Column (Amersham), the labeled DNA was digested with Hind III. The resulting 650nt, 3' end labeled fragment was recovered by gel extraction (Qiagen) after electrophoresis through 2 % agarose. For the S1 nuclease protection assay, 5 µg of total RNA was hybridized at 52 °C overnight with 1 µl of the labeled histone H2A probe in 40 mM Pipes, pH 6.4, 500 mM NaCl, 1 mM EDTA, 80 % deionized formamide. PolyA RNA was purified from total RNA using oligo(dT) cellulose (Ambion). The hybridized samples were diluted 10 fold and treated with S1 nuclease (Promega) for 1.5 hours at 25°C. The hybrids were recovered by ethanol precipitation, denatured in formamide, and subjected to electrophoresis through a 6 % acrylamide gel in 1X TBE. The gel was dried using a SGD200 Slab Gel Drier (Savant) for 1.5 hours at 80 °C and subject to autoradiography.

## **In situ hybridization**

Eye discs were dissected from wandering 3<sup>rd</sup> instar larvae and fixed with either 4% formaldehyde for 15 min for *Slbp* mutants or 10% formaldehyde for 20 min for *U7* mutants. Ovaries were dissected from 1-2 day old females and fixed with 10% formaldehyde for 20 minutes. In situ hybridization was performed with digoxigenin-labeled riboprobes complimentary to the coding region of H3 or to the region downstream of the normal H3 pre-mRNA processing site (H3-ds) as previously described (Lanzotti et al., 2002).

## **Generation of germ line clones**

Mosaic *Slbp*<sup>15</sup> ovaries were generated using the dominant female sterile technique (Chou et al., 1993). Clones were induced by heat shocking P[hsFLP]/w; P[neoFRT]82B P[ovo<sup>D1-18</sup>]/P[neoFRT]82B *Slbp*<sup>15</sup> larvae for 1 hour at 37°C on the third and fourth days of development. Ovaries were dissected from the resulting adults and either fixed with 10% formaldehyde for 20 minutes for in situ hybridization analysis or subjected to northern analysis as described above. *Slbp*<sup>15</sup> mutant clones were identified because they developed past the ovo<sup>D1-18</sup> developmental arrest point.

## **Results**

### ***U7* snRNA null mutants are viable but sterile**

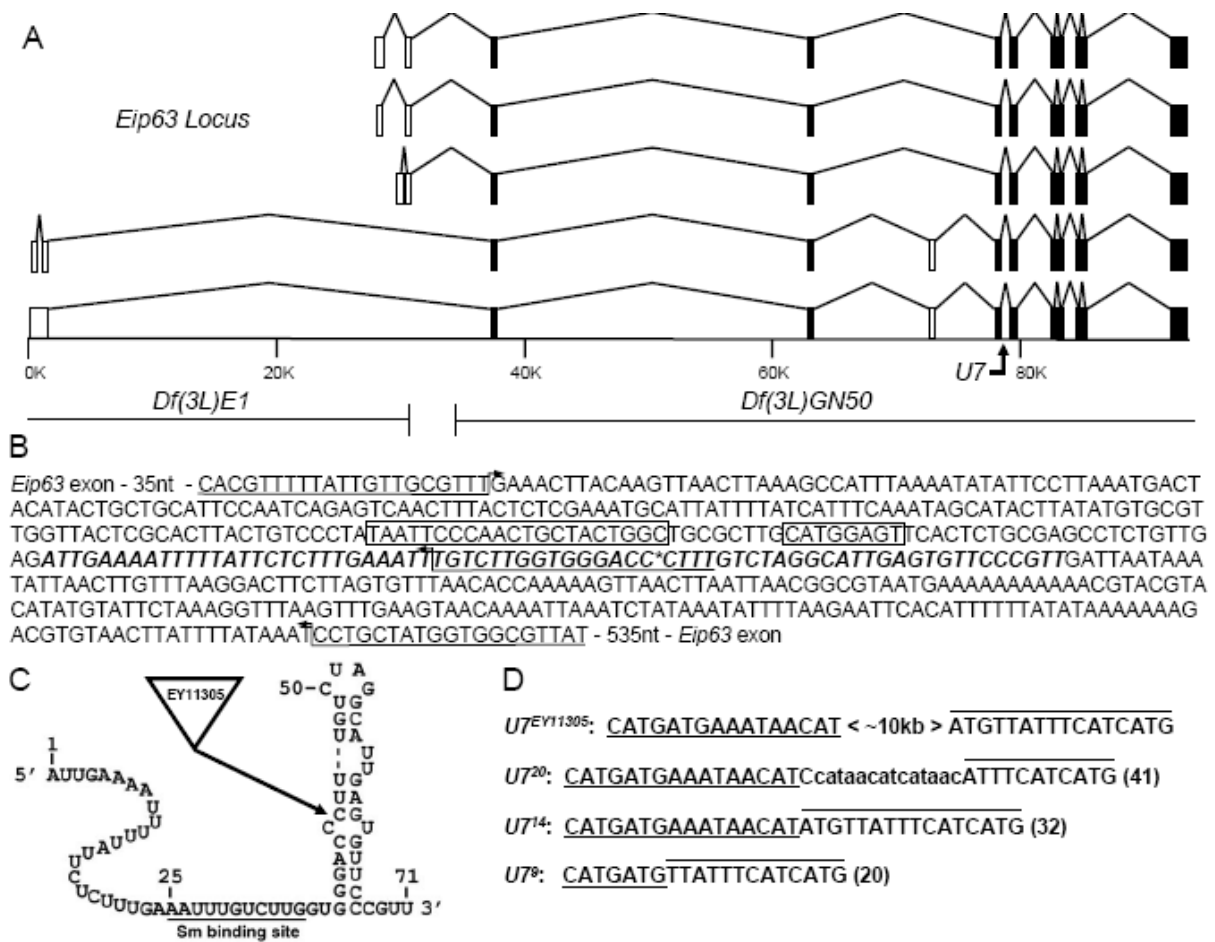
To examine the contribution of *U7* snRNA to histone mRNA production during animal development, we generated null alleles in the *Drosophila U7* gene. The *U7*

locus is located within an intron of the differentially spliced *Eip63E* gene (Dominski et al., 2003), which encodes a CDK-like Ser/Thr kinase that is induced in response to the fly hormone ecdysone (Fig. 2.1A) (Stowers et al., 2000; Rasclé et al., 2003). We obtained from the *Drosophila* Gene Disruption Project (Bellen et al., 2004) a stock carrying a P element transposon (EY11305) that inserted 8 nucleotides downstream of the Sm protein binding site between nucleotides 43 and 44 of the 71 nucleotide U7 snRNA (Fig. 2.1B, C). *U7<sup>EY11305</sup>* homozygous animals develop into adult flies with no overt morphological defects. However, both males and females of this genotype are completely sterile. Precise excision of the EY11305 P element restored male and female fertility, indicating that the *U7<sup>EY11305</sup>* homozygous adult sterility was caused by the P element insertion.

We generated five additional *U7* alleles by genetically mobilizing the EY11305 P element (see Materials and Methods). Three of these (*U7<sup>9</sup>*, *U7<sup>14</sup>*, and *U7<sup>20</sup>*) contain an insertion ranging from 20 to 41 nucleotides, including sequence from both the 5' and 3' end of the P element (Fig. 2.1D). The insertions are located downstream of the Sm site and after the first 5 nts of the terminal stem. The other two alleles (*U7<sup>1</sup>* and *U7<sup>23</sup>*) retain a larger portion of the P element that we did not precisely determine. These events result from imprecise repair of the double strand break induced by transposase during P-element excision (Adams et al., 2003). Like the original *U7<sup>EY11305</sup>* mutation, each of the five imprecise excision mutations is viable when homozygous, but causes complete male and female sterility.

To test whether these mutations specifically disrupt *U7* and not *Eip63* function, we performed genetic complementation analyses with various deletions of the *Eip63* locus. *Df(3L)E1* deletes the 5' half of *Eip63*, but not *U7*, and *Df(3L)GN50* deletes the entire *U7*

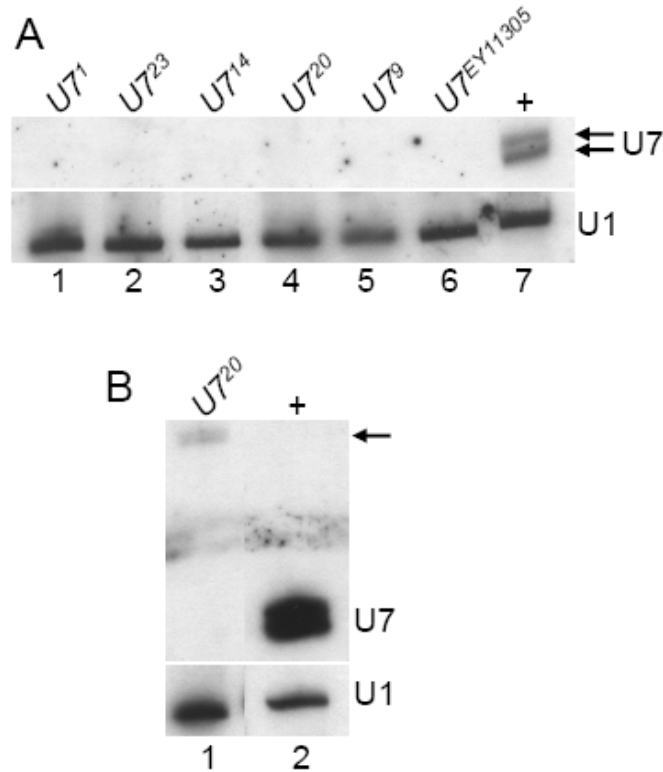
gene and the 3' half of *Eip63* (Fig. 2.1A). *Df(3L)E1/Df(3L)GN50* heterozygotes are lethal because of the loss of *Eip63* function (Stowers et al., 2000). As expected if our mutations do not affect *Eip63*, each of the six *U7* mutant alleles complements the *Df(3L)E1* lethality, and the resulting adult male and female flies are fertile. In contrast, all six mutations result in viable, sterile flies when present in trans to *Df(3L)GN50* because it removes *U7*. In addition, a P element transgene containing only the *U7* gene (Fig. 2.1B) rescues the sterility of *U7<sup>14</sup>/U7<sup>EY11305</sup>* mutant females and males. We conclude from these data that all of our mutant alleles affect *U7* function without affecting *Eip63*, consistent with the *U7* snRNA being derived from an independent transcript distinct from that producing *Eip63E* mRNA.



**Figure 2.1. Mutation of the *Drosophila* U7 gene.** A) Schematic of the *Eip63* locus. The five identified *Eip63* mRNAs species are shown, with open boxes indicating variably spliced exons and closed boxes indicating invariant exons. The scale bar indicates genomic DNA in kilobase pairs. The *U7* gene is located at position ~79K. The right break point of *Df(3L)E1* and the left breakpoint of *Df(3L)GN50*, located at position ~31K and ~34K, respectively, are shown below the scale bar. B) Sequence of the genomic fragment used to rescue the *U7* mutant phenotype. The *U7* snRNA transcript is indicated in bold italic. The asterisk indicates the location of the ~10kb EY11305 P element. The large and small boxes respectively indicate the PSEA and PSEB essential snRNA promoter elements (Zamrod et al., 1993). The bent arrows indicate primers used to analyze the excision mutations. Note that the middle and top primers were able to detect a product in *U7<sup>23</sup>* homozygous mutant animals. C) Putative U7 snRNA secondary structure indicating the location of the EY11305 insertion. The underlined sequence indicates the Sm protein binding site. D) Molecular characterization of *U7* imprecise excision mutations. Each sequence shown

To determine whether the *U7* mutants represent null alleles, total RNA was extracted from 1-2 day old adult female flies and subjected to northern blot analysis. In wild type female flies, the U7 probe detects a characteristic doublet representing RNAs of 71 and 74 nucleotides that result from differential processing of the 3' end of U7 snRNA (Fig. 2.2A, lane 7) (Dominski et al., 2003). None of the mutant samples contained detectable levels of either wild type U7 snRNA (Fig. 2.2A, lanes 1-6). Interestingly, the *U7<sup>20</sup>* allele produced very low levels of an RNA that hybridizes with the U7 probe, but that is larger in size, consistent with the 41 nucleotide insertion (Fig. 2.1D and arrow in Fig. 2.2B, lanes 1). Based on the lack of production of wild type U7 snRNA, the sterility phenotype, and the histone mRNA molecular phenotypes described below, we conclude that all of these alleles are null.

The short insertions in  $U7^9$ ,  $U7^{14}$ , and  $U7^{20}$  are in a region of the U7 snRNA that does not have a defined function, and they likely affect the assembly and/or stability of the U7 snRNP.



**Figure 2.2. Northern analysis of  $U7$  mutants.** A) Top: Total RNA extracted from 1-2 day old adult females was fractionated through an 8% polyacrylamide-7M Urea gel, transferred to nitrocellulose, and probed with  $^{32}\text{P}$ -labeled U7 RNA. Lanes 1-6: RNA from the indicated homozygous mutant genotypes. Lane 7:  $w^{1118}$  control RNA. Note the lack of detectable U7 snRNA in the mutant samples. Bottom: The same blot hybridized with a  $^{32}\text{P}$ -labeled U1 probe as a loading control. B) Long exposure of mutant samples. The arrows indicate the low abundance, aberrantly large mutant U7 snRNAs produced by the excision mutants 14 and 20. The U7 snRNAs produced by  $U7^{20}$  are more abundant than those produced by  $U7^{14}$ , since we could easily see the band for  $U7^{20}$  (top arrow), but had to overexpose the blots to faintly see the band for  $U7^{14}$  (bottom arrow).

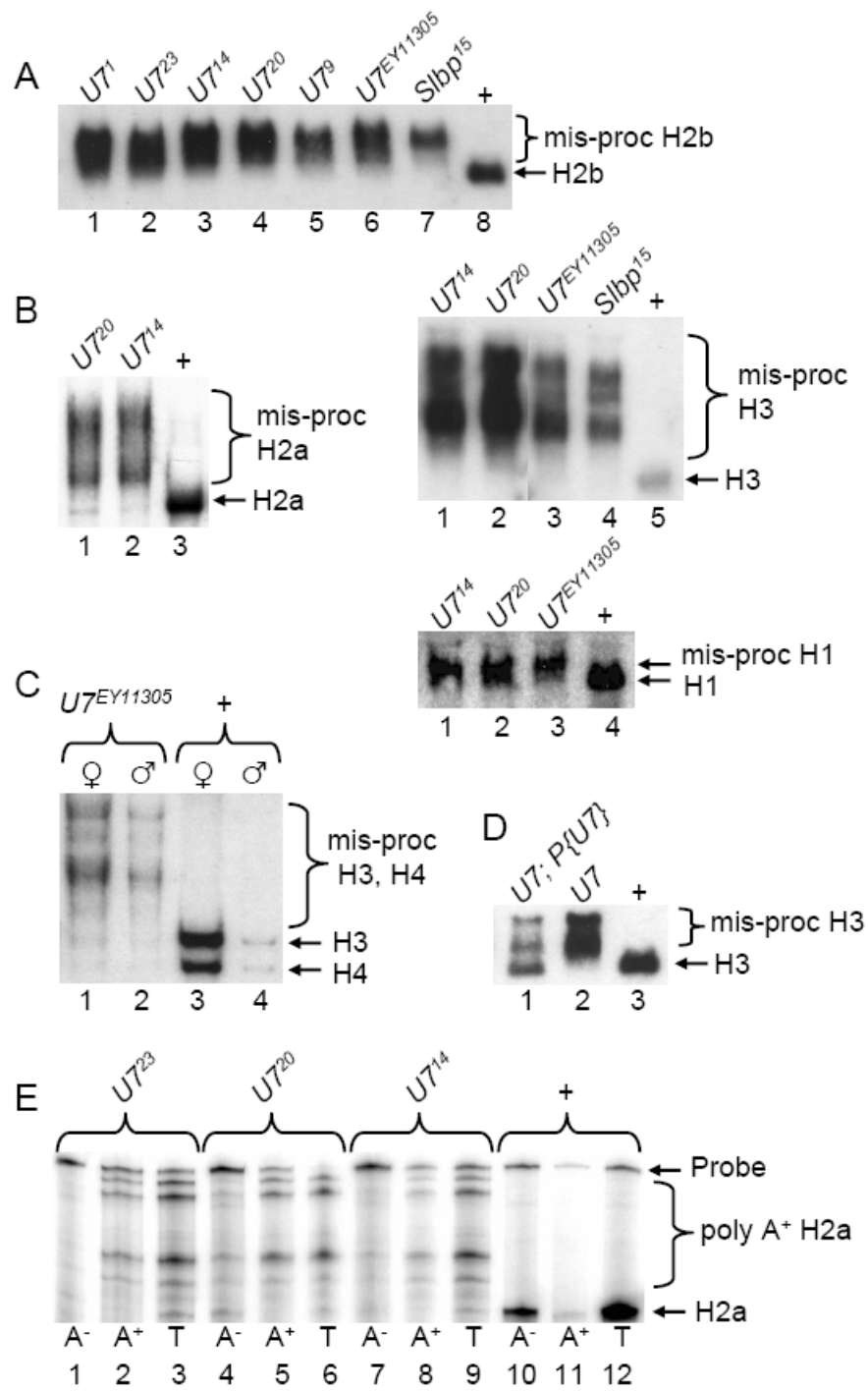
To determine how the loss of U7 snRNA affects the biosynthesis of histone mRNA, total RNA isolated from wild type or mutant adult female flies was subjected to Northern

blot analysis. A probe derived from the histone H2b gene hybridizes with a single mRNA in wild type control RNA samples (Fig. 2.3A, lane 8). In contrast, H2b mRNAs that migrate more slowly are detected in all of the *U7* mutants (Fig. 2.3A, lanes 1-6). An identical phenotype is observed in *Slbp*<sup>15</sup> null mutant larvae (Fig. 2.3A, lane 7) (Lanzotti et al., 2002), indicating that the longer mRNAs in the *U7* mutants likely represent inappropriately polyadenylated histone mRNAs. Each of the *U7* mutants produces a similar pattern of aberrant H2b mRNAs (Fig 2.3A), suggesting that they all disrupt histone pre-mRNA processing to the same extent. Longer, aberrantly processed mRNAs are also detected in *U7* mutants with probes derived from each of the other four replication associated histone genes (Fig 2.3B-C). Aberrantly processed histone mRNA is detected in *U7* mutant males as well as females, consistent with the sterility of both genders (Fig. 2.3C). Proper histone H3 processing is partially restored in *U7* mutant flies containing a P element transgene harboring the wild type *U7* gene (Fig. 2.3D, lane 1), and these flies are no longer sterile. The incomplete restoration of H3 processing is likely because the transgene does not restore wild type levels of *U7* snRNA (see Fig. 2.4C, lanes 8-10). These data indicate that the *U7* locus we have identified is required for processing all five of the replication dependent histone mRNAs, and thus likely represents the only *U7* snRNA encoded in the *Drosophila* genome.

To more precisely assess the contribution of *U7* snRNA to histone pre-mRNA processing, and to confirm that the aberrantly processed histone mRNAs in *U7* mutants were polyadenylated, S1 nuclease protection analysis of H2a mRNA was performed with total RNA isolated from wild type or *U7* mutant females and separated into poly A<sup>+</sup> and poly A<sup>-</sup> fractions. In wild-type flies a single protected fragment was observed that corresponds to the expected 3' end of histone H2a mRNA (Fig. 2.3E, lane 12), and that partitions primarily into

the poly A<sup>-</sup> fraction (Fig. 2.3E, lanes 10, 11). In the *U7* mutant RNA samples, multiple H2a mRNA protected fragments were detected, almost all of which are longer than the single species detected in wild type samples (Fig. 2.3E, lanes 3, 6, 9). All of the aberrant mRNAs were enriched in the polyA<sup>+</sup> fraction (Fig. 2.3E, lanes 1, 4, 7 versus lanes 2, 5, 8). These data are consistent with the use of multiple, inefficient polyadenylation signals downstream of the normal site of H2a pre-mRNA processing, as we observed previously in *Slbp* mutants (Lanzotti et al., 2002). Importantly, very little, if any, normally processed H2a was detected in *U7* mutant adult female RNA samples. We conclude from all of the data presented in Figure 3 that U7 snRNP is an essential histone pre-mRNA processing factor in *Drosophila*.





**Figure 2.3. Aberrant histone mRNA production in *U7* mutants.** Total RNA extracted from 1-2 day old adult females was fractionated through agarose gels, transferred to nitrocellulose, and probed with a <sup>32</sup>P-labeled histone probes. A) H2b probe. Lanes 1-7: equal amounts of RNA from the indicated homozygous mutant genotypes. Lane 8: WT control RNA. Since *Slbp*<sup>15</sup> homozygotes are lethal at the pupal stage, *Slbp*<sup>15</sup> mutant RNA samples were isolated from homozygous wandering 3<sup>rd</sup> instar larvae identified as Tb<sup>+</sup> progeny from *Slbp*<sup>15</sup>/TM6b Tb parents. B) Left: H2a probe. Lanes 1-2: RNA from the indicated homozygous mutant genotypes. Lane 3: WT control RNA. Right top: H3 probe. Lanes 1-4: RNA from the indicated homozygous mutant genotypes. Lane 5: WT control RNA. Right bottom: H1 probe. Lanes 1-3: RNA from the indicated homozygous mutant genotypes. Lane 4: WT control RNA. Note that because H1 mRNA is longer than the other histone mRNAs, and because the cryptic polyadenylation site is near the normal processing site (Lanzotti et al., 2002), there is a less pronounced difference in migration between the wild type and polyadenylated mRNA. C) A mixture of histone H3 and H4 probes hybridized to RNA isolated from adult males or females of the indicated genotypes. Note that the females contain more histone mRNA than males because of the large amounts of production during oogenesis. D) Transgenic rescue of H3 processing. Lane 1: RNA from *U7*<sup>14</sup>/*U7*<sup>EY11305</sup> adult females carrying a U7 P element transgene. Lane 2: RNA from *U7*<sup>14</sup>/*U7*<sup>EY11305</sup> female siblings of those in Lane 1. Lane 3: RNA from *w*<sup>1118</sup> control adult females. E) H2a S1 nuclease protection of mRNA isolated from 1-2 day old adult females. The RNA was separated into poly A<sup>+</sup> and poly A<sup>-</sup> fractions prior to analysis. Note the multiple protected fragments in the mutant poly A<sup>+</sup> RNA samples, representing the use of multiple cryptic polyadenylation signals located downstream of the normal pre-mRNA processing site (Lanzotti et al., 2002).

### **Loss of U7 and SLBP affects *Drosophila* development differently**

Because zygotic mutation of *Slbp* and *U7* results in a similar molecular phenotype with respect to histone mRNA biosynthesis, we expected that development of the animal would be similarly affected as well. However, *Slbp* null mutations cause lethality while *U7* null mutations cause adult sterility. This difference in terminal phenotype may reflect differences in the maternal contribution of SLBP and U7. SLBP protein is not contributed maternally (as assessed by western blotting (Lanzotti et al., 2002)), and zygotic expression of

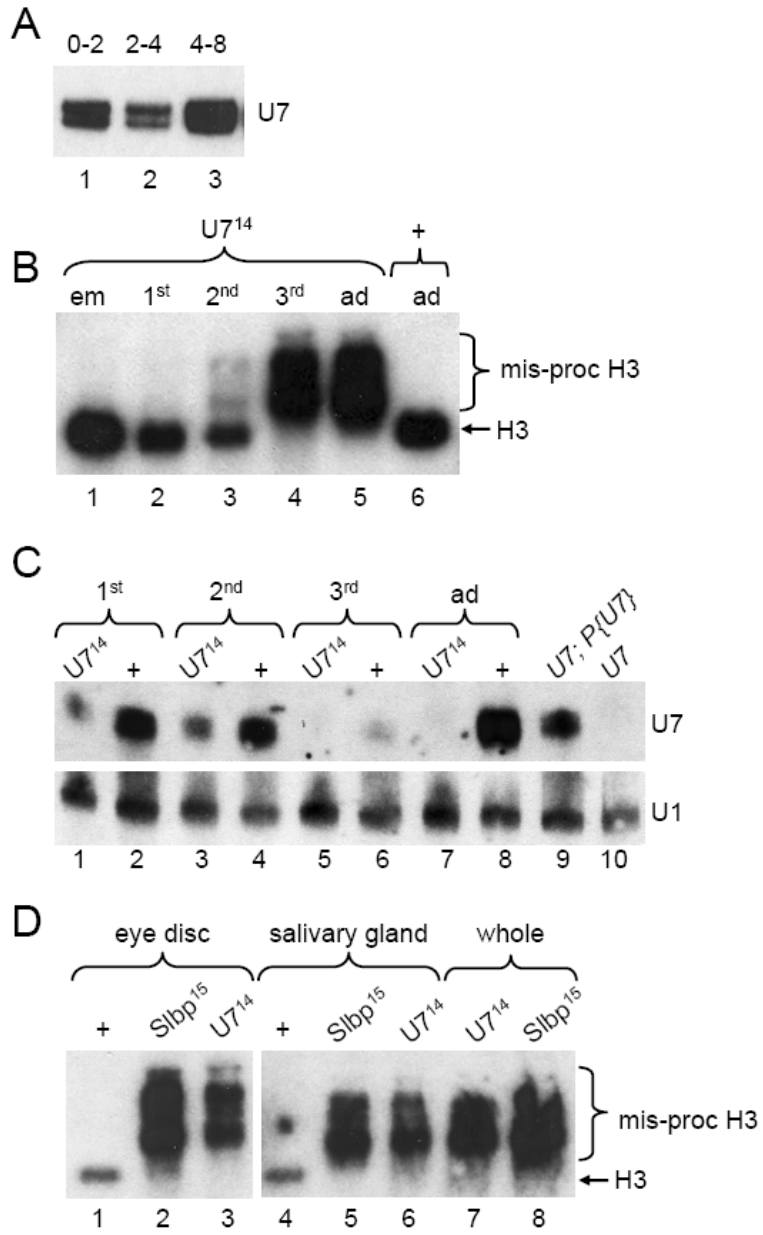
SLBP is required for processing of histone mRNAs transcribed in the early embryo. In contrast, there is a substantial amount of U7 snRNA stored in the egg, and the total amount of U7 snRNA changes relatively little during the first 8 hrs of development as assayed by northern blotting (Fig. 2.4A). This maternal U7 snRNA contribution may be sufficient to support normal histone mRNA synthesis during early development of *U7* zygotic mutants, resulting in a delay in onset of the histone pre-mRNA processing defect compared to *Slbp* mutants. This later onset of the mutant phenotype likely explains the difference in developmental outcome.

To test this possibility we analyzed histone H3 mRNA production at different stages of development in *U7* mutant animals (Fig. 2.4B). Mis-processed (i.e. poly A<sup>+</sup>) histone H3 mRNA was not detected in *U7* mutant embryos by in situ hybridization using a probe derived from sequences downstream of the normal H3 processing site (called H3-ds, for “H3-downstream”) that specifically detects poly A<sup>+</sup> histone H3 mRNA in *Slbp* mutant embryos (not shown) (Lanzotti et al., 2002). Poly A<sup>+</sup> H3 mRNA was also not detected in *U7* mutant embryos or first instar larvae by northern blotting (Fig. 2.4B, lanes 1 and 2). We first detected aberrantly processed, poly A<sup>+</sup> H3 mRNA in second instar *U7* mutant larvae, although these larvae contain primarily normal and only small amounts of poly A<sup>+</sup> histone mRNAs (Fig. 2.4B, lanes 3). By the wandering third larval instar stage only aberrant, poly A<sup>+</sup> H3 mRNA was detected, and thus the molecular phenotype was identical to that observed in *U7* mutant adult female flies (Fig. 2.4B, lanes 4 and 5).

The gradual onset of mis-processed, poly A<sup>+</sup> H3 mRNA during development is consistent with a gradual depletion of maternal U7 snRNA. To test this we analyzed U7 snRNA levels in wild type and *U7* mutant larvae by northern analysis. U7 snRNA is clearly

detectable but reduced relative to wild type in both 1<sup>st</sup> and 2<sup>nd</sup> instar *U7* mutant larvae, and is undetectable in 3<sup>rd</sup> instar mutant larvae (Fig. 2.4C). Unexpectedly, wild type 3<sup>rd</sup> instar larvae had greatly reduced amounts of U7 compared to younger larvae or adult females (Fig. 2.4C, lane 6), but we believe this result is due to the age of the third instar larvae picked in this experiment and we found that this result did not repeat when younger 3<sup>rd</sup> instar larvae were picked (Fig. 3.5A). These data indicate that both maternal stores of U7 as well as zygotic expression contribute to the total level of U7 snRNA in early larvae. The reduced amount of U7 snRNA in 1<sup>st</sup> and 2<sup>nd</sup> instar *U7* mutant larvae is sufficient to process all of the H3 pre-mRNA, indicating that U7 snRNA is normally present in excess. Taken together, our results suggest that the *U7* mutant animals have a later onset (i.e. not until 3<sup>rd</sup> instar) of the histone pre-mRNA processing defect than *Slbp* mutants because of maternal stores of U7 snRNA.

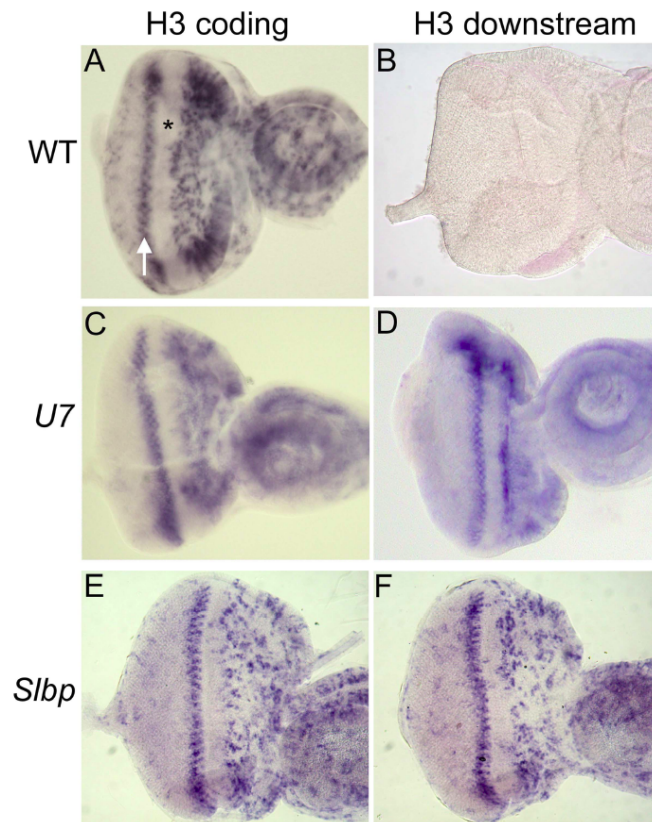
We used northern analysis of RNA prepared from specific tissues as well as in situ hybridization to characterize the *U7* larval mutant phenotypes in more detail, and to compare them to *Slbp* mutant phenotypes. Mis-processed, poly A<sup>+</sup> H3 mRNA was present in RNA isolated from third instar eye imaginal discs and salivary glands dissected from both *U7* and *Slbp* mutants (Fig. 2.4D). This indicates that U7 snRNP and SLBP are required for histone pre-mRNA processing in both proliferating (imaginal discs) and endocycling (salivary glands) cells.



**Figure 2.4. Developmental onset of the *U7* mutant phenotype.** A) RNA isolated from *w<sup>1118</sup>* control embryos and subjected to northern analysis with a <sup>32</sup>P-labeled *U7* RNA probe. Lane 1, 2 and 3: equal numbers of 0-2, 2-4, and 4-8 hour old embryos, respectively. B) Equal amounts of total RNA extracted from different stages of development and subjected to northern analysis with a <sup>32</sup>P labeled *H3* probe. Lane 1: Embryos collected overnight from *U7<sup>14</sup>/TM3* heterozygous parents and allowed to age for 3 hours. Lane 2, 3, and 4: Homozygous 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> instar *U7<sup>14</sup>* mutant larvae, respectively. Lane 5 and 6: 1-2 day old homozygous *U7<sup>14</sup>* and *w<sup>1118</sup>* control adult females, respectively. Note that the poly A<sup>+</sup> *H3* mRNA is first detectable in small amounts at the 2<sup>nd</sup> larval instar stage, and that wild type *H3* mRNA is absent by the 3<sup>rd</sup> larval instar stage. C) *U7* northern analysis of RNA isolated from homozygous *U7<sup>14</sup>* or *w<sup>1118</sup>* control larvae and adults. Odd lanes: *U7<sup>14</sup>* homozygous mutants collected from *U7<sup>14</sup>/TM3* heterozygous parents. Note that the detectable *U7* snRNA is maternally derived. Even lanes: *w<sup>1118</sup>* control animals. Hybridization of the blot with a *U1* probe was used as a loading control. D) *H3* northern analysis of RNA isolated from eye imaginal discs (lanes 1-3), salivary glands (lanes 4-6), or whole larvae (lanes 7 and 8) of the indicated genotypes.

We next examined the accumulation of mis-processed *H3* mRNA in the highly regulated cell cycles of the developing eye. During larval stages asynchronous cell proliferation supports eye imaginal discs growth. In late third instar larvae, a wave of differentiation called the morphogenetic furrow (MF) sweeps across the eye imaginal epithelium from posterior to anterior. All cells within the MF arrest in G1 phase (Fig. 2.5A, asterisk). Certain of these cells remain arrested and begin to differentiate. The remaining cells synchronously enter a final, single cell division cycle called the second mitotic wave, which can be visualized with the *H3* coding probe (Fig. 2.5A, arrow), and then subsequently differentiate. Whereas control eye discs do not stain with the *H3*-ds probe (Fig. 2.5B), in situ hybridization of *U7* or *Slbp* mutant third instar eye imaginal discs with the *H3*-ds probe produced a staining pattern similar to that obtained using a histone *H3* coding probe in wild type discs (Fig. 2.5C-F). This includes expression in the asynchronously proliferating cells

anterior to the MF, and the synchronously replicating cells of the second mitotic wave just posterior to the MF. Mis-processed, poly A<sup>+</sup> H3 mRNAs were not detected in G1 arrested cells within the MF, or in the G1 arrested terminally differentiating cells posterior to the MF. This essentially normal staining pattern suggests that poly A<sup>+</sup> H3 mRNAs are regulated properly with respect to accumulation during the mitotic cell cycle, including efficient down-regulation in cells exiting the cell cycle in G1. However, we cannot exclude the possibility that in these cells the poly A<sup>+</sup> mRNAs are not as rapidly down-regulated at the end of S-phase as wild type histone mRNAs (Lanzotti et al., 2002).



**Figure 2.5. *U7* mutant eye imaginal discs display a wild type pattern of polyadenylated H3 mRNA.** Eye imaginal discs were dissected from *w<sup>1118</sup>* control (A, B), *U7<sup>14</sup>* (C,D), or *Slbp<sup>15</sup>* (E,F) mutant larvae and subjected to in situ hybridization with an H3 coding probe (A, C, E) or the H3-ds probe (B, D, F), which detects only misprocessed, polyA<sup>+</sup> H3 mRNA. The asterisk indicates the morphogenetic furrow, which contains cells arrested in G1 phase. The arrow indicates S phase of the second mitotic wave. Asynchronously dividing, undifferentiated cells are located anterior of the MF (right of the asterisk), and differentiating cells that have exited the cell cycle and that will make up the adult eye are located posterior to the MF (left of the arrow).

### **SLBP and U7 snRNA cooperate in histone pre-mRNA processing**

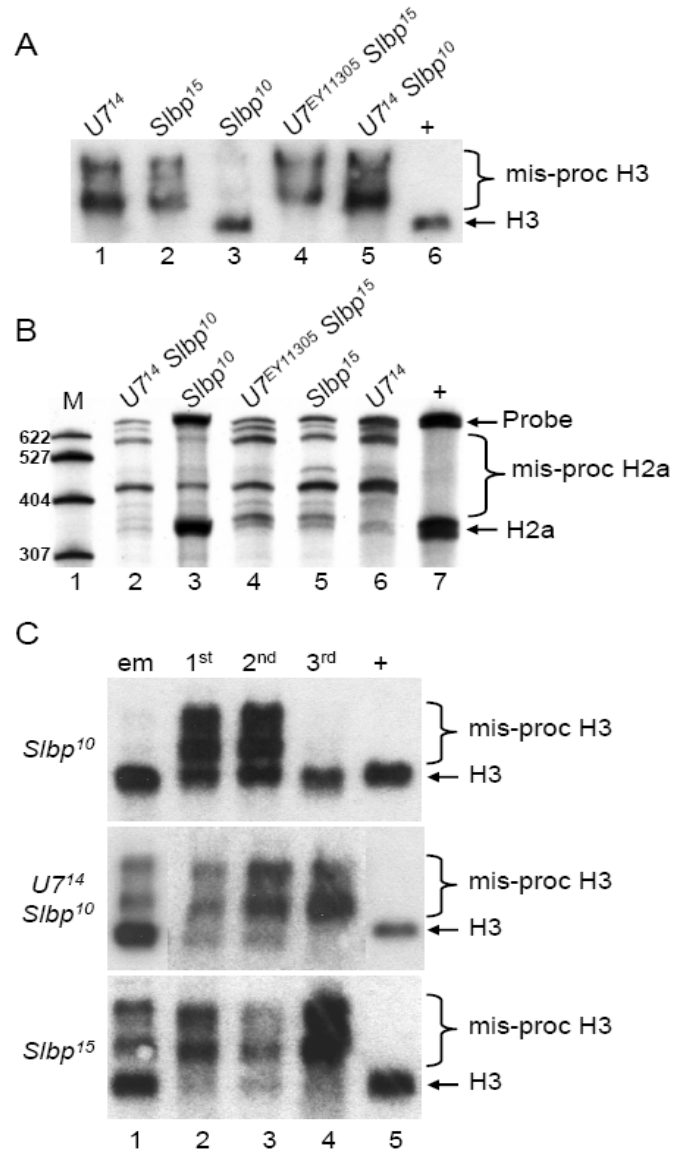
Synthetic histone pre-mRNA substrates are efficiently processed in mammalian nuclear extracts. When there is high complementarity between U7 snRNA and the HDE in the synthetic histone pre-mRNA, these extracts will support processing after SLBP depletion as well as the processing of mutant RNAs that cannot bind SLBP (Spycher et al., 1994; Dominski et al., 1999). This suggests that in vivo the U7 snRNP may be capable of supporting processing in the presences of very low amounts of SLBP. To examine this issue, we used the partially functional *Slbp<sup>10</sup>* allele, which produces SLBP protein at ~10% the amount of wild type. As a result, *Slbp<sup>10</sup>* mutant embryos produce a small amount of normally processed histone mRNA in addition to misprocessed mRNA (Lanzotti et al., 2002). In contrast, at the third larval instar stage *Slbp<sup>10</sup>* mutants contain almost entirely normally processed histone H3 and H2a mRNA, as detected by northern and S1 nuclease protection, respectively (Fig. 2.6A, B, lane 3). To test whether production of normally processed histone mRNA in *Slbp<sup>10</sup>* mutants was U7 dependent, we engineered *U7<sup>14</sup> Slbp<sup>10</sup>* double mutants and analyzed histone H3 and H2a mRNA isolated from whole third instar larva, the earliest time



during development at which the *U7* mutant phenotype is fully expressed (see Fig. 2.4B). As assayed by northern blotting, *U7<sup>14</sup> Slbp<sup>10</sup>* double mutant third instar larvae produce very little if any processed H3 mRNA, similar to *U7* single mutants (Fig. 2.6A). This demonstrates that the H3 pre-mRNA processing occurring in *Slbp<sup>10</sup>* mutants is *U7* dependent. In contrast to the *Slbp<sup>10</sup>* hypomorph, *Slbp<sup>15</sup>* null mutant third instar larvae contain very little if any processed histone H3 mRNA (Fig. 2.6A, lane 2), and mutating *U7* in this background does not alter the phenotype as would be expected (Fig. 2.6A, lane 4). Very similar results were obtained by analyzing H2a mRNAs in these different genotypes by S1 nuclease protection (Fig. 2.6B). These data suggest that the *U7* snRNP can stimulate histone processing with a reduced amount of SLBP, but that in the absence of SLBP *U7* snRNP cannot support processing.

Interestingly, the *U7<sup>14</sup>* null and *Slbp<sup>10</sup>* hypomorphic mutations display synthetic lethality: whereas both *U7* and *Slbp<sup>10</sup>* single mutant animals develop into adults, the *U7 Slbp<sup>10</sup>* double mutants do not eclose as adults. This result suggests that the severity of the processing defects may be enhanced as a result of limiting both SLBP and *U7* snRNA, causing increased mis-processing of histone mRNA earlier in development. Indeed, *U7<sup>14</sup> Slbp<sup>10</sup>* double mutants produce a greater proportion of mis-processed, poly A<sup>+</sup> H3 mRNA at 1<sup>st</sup> and 2<sup>nd</sup> larval instar stages than either of the *Slbp<sup>10</sup>* or *U7<sup>14</sup>* single mutants (Fig. 2.6C and Fig. 2.4B, respectively). In addition, the H3 mRNA expression profile of *U7<sup>14</sup> Slbp<sup>10</sup>* double mutants is similar to that of *Slbp<sup>15</sup>* null mutants, which are lethal (Fig. 2.6C). This is most striking at embryonic stages, where there is a marked increase in poly A<sup>+</sup> H3 mRNA in the *U7<sup>14</sup> Slbp<sup>10</sup>* double mutants relative to either single mutant (lane 1 in Figs. 2.6C and Fig. 2.4B). Thus, the severity of the histone pre-mRNA processing defect is enhanced as a result

of limiting both SLBP and U7 snRNA, and this increase in severity of the histone pre-mRNA processing defect may contribute to the lethality of *Slbp*<sup>10</sup> *U7*<sup>14</sup> double mutants.



**Figure 2.6. *U7-Slbp* double mutant analysis.** RNA isolated from the indicated genotypes was subjected to Northern analysis with a [<sup>32</sup>P]-labeled H3 probe (A, C) or S1 nuclease protection analysis with a [<sup>32</sup>P]-labeled H2a probe (B). A) H3 Northern of 3<sup>rd</sup> instar larvae. Lanes 1-5: RNA from the indicated homozygous single or double mutant genotypes. Lane 6: *w<sup>1118</sup>* control RNA. B.) H2a S1 nuclease protection analysis of 3<sup>rd</sup> instar larvae. Lane 1: [<sup>32</sup>P]-labeled marker (M) RNAs of the indicated lengths. Lanes 2-6: RNA from the indicated homozygous single or double mutant genotypes. Lane 7: *w<sup>1118</sup>* control RNA. C) Lane 1: RNA from embryos collected overnight from heterozygous parents. Note that ¼ of the embryos will be homozygous mutant for the indicated genotypes. Lane 2, 3, and 4: RNA from 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> instar larvae, respectively, homozygous mutant for the indicated genotypes. Lane 5: Control RNA from *w<sup>1118</sup>* 3<sup>rd</sup> instar larvae.

### **Histone pre-mRNA processing is necessary for oogenesis**

*U7* mutant female flies are sterile and lay very few eggs, none of which hatch. These eggs are often smaller than wild type, some have a defective chorion (e.g. fused dorsal appendages), and most are desiccated (not shown). These data suggest that proper histone pre-mRNA processing is required during oogenesis, and we therefore analyzed histone mRNA expression in *U7* and *Slbp* mutant ovaries.

The basic unit of *Drosophila* oogenesis is the egg chamber, which consists of a cyst of 16 interconnected germ cells surrounded by a single layer of somatic follicle cells (Spradling, 1993). Fifteen of the germ cells differentiate into nurse cells, which become highly polyploid, and which synthesize and transport RNA and protein (including histones and histone mRNA) through cytoplasmic bridges into the single developing oocyte. The follicle cells contribute to oogenesis by synthesizing yolk and the egg shell, and by participating in dorsal/ventral and anterior/posterior axis formation. Both the follicle cells

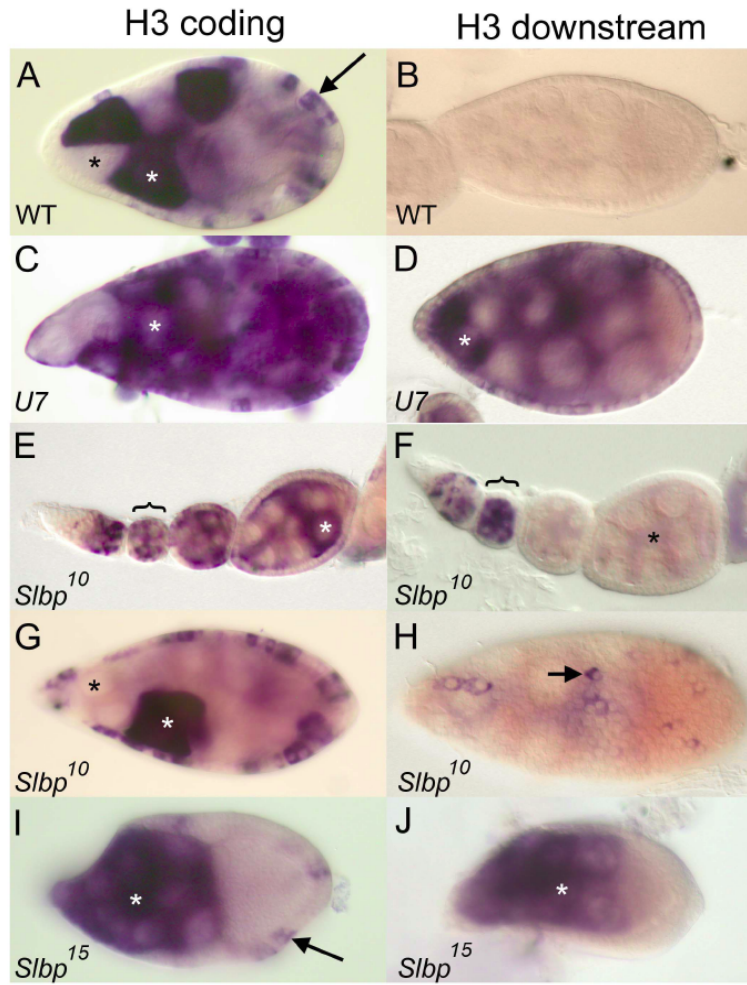
and the nurse cells become polyploid via endocycles, which consist of repeated rounds of S phase interrupted by a gap phase with no intervening mitosis (Lilly & Duronio, 2005). Histone mRNA accumulation during endocycles correlates with S phase (Sullivan et al., 2001). Because the cells do not replicate in synchrony, this appears as a mosaic in situ hybridization staining pattern in both nurse cells and follicle cells using a histone H3 coding probe (Fig. 2.7A) (Ambrosio & Schedl, 1985; Ruddell & Jacobs-Lorena, 1985; Walker & Bownes, 1998). Late in oogenesis, there is a burst of histone mRNA synthesis in the nurse cells that is not associated with DNA replication (Ambrosio & Schedl, 1985; Ruddell & Jacobs-Lorena, 1985). These mRNAs are transported into the oocyte and may be used to support early embryonic development (Sullivan et al., 2001; Lanzotti et al., 2002).

To examine the cause of *U7* mutant sterility, we dissected ovaries from *U7* mutant females and examined egg chamber development. *U7* mutant egg chambers appear morphologically similar to wild type until stage 10, as assessed by phalloidin and DAPI staining, which detect f-actin and DNA, respectively (not shown). However, few mature oocytes are produced and many egg chambers eventually degenerate, consistent with the lack of egg production. Hybridization of *U7* mutant egg chambers with the histone H3 coding probe detects mRNA in a subset of follicle cells and nurse cells, both of which are endocycling at the stages shown (Fig. 2.7C). Hybridization with the H3-ds probe, which detects only misprocessed, poly A<sup>+</sup> H3 mRNA (Fig. 2.7B), produces a similar staining pattern (Fig. 2.7D). Thus, *U7* snRNA is required for histone pre-mRNA processing in both germ line and somatic tissue. This is consistent with the Northern analysis described above, which showed that essentially all of the H3 histone mRNA produced in *U7* mutant females is misprocessed (Fig. 2.3).

The *U7* mutant sterility is reminiscent of that of *Slbp*<sup>10</sup> hypomorphic mutants, which are also viable but female sterile (although *Slbp*<sup>10</sup> males are fertile). In contrast to *U7* mutants, *Slbp*<sup>10</sup> mutant females lay normal numbers of eggs that are wild type in appearance. These eggs can be fertilized, but do not hatch because of severe mitotic defects during the syncytial cycles (Sullivan et al., 2001). This is likely the result of reduced deposition of maternal histone mRNA (Sullivan et al., 2001; Lanzotti et al., 2002) and protein (P. Fort, W.F.M, and R.J.D, unpublished) into these eggs. While reduced ~10 fold in amount relative to wild type, the histone mRNA deposited into eggs by *Slbp*<sup>10</sup> mutant females is processed normally, and we did not detect any poly A<sup>+</sup> histone mRNAs (Sullivan et al., 2001; Lanzotti et al., 2002). To test whether this reflected a defect in histone mRNA synthesis during oogenesis, we analyzed RNA isolated from ovaries dissected from *Slbp*<sup>10</sup> mutant females by northern blotting and S1 nuclease protection. These assays revealed reduced amounts of normally processed H3 and H2a mRNA, but no misprocessed H3 or H2a mRNA (Fig. 2.8A, B, respectively).

To examine this in more detail, *Slbp*<sup>10</sup> mutant egg chambers were dissected and hybridized with the H3 coding and H3-ds probes. *Slbp*<sup>10</sup> mutant egg chambers are morphologically normal, and produce H3 mRNA in both the nurse cells and the follicle cells in patterns similar to wild type (Fig. 2.7E, G). In contrast, the H3-ds probe stained *Slbp*<sup>10</sup> egg chambers more sporadically and much more weakly (Fig. 2.7F, H). Strong staining was only usually detected in young (i.e. stage 2-3) egg chambers in nurse cells undergoing endoreduplication cycles (Fig. 2.7F, bracket). In general, egg chambers older than stage 2-3 did not stain with the H3-ds probe (compare Figs. 2.7E and F, asterisks), although we could detect sporadic follicle cell staining and sometimes nurse cell staining in stage 9 egg

chambers (shown for follicle cells in Fig. 2.7H). These in situ data indicate that misprocessed H3 mRNA represents a small fraction of the total H3 mRNA that accumulates in *Slbp*<sup>10</sup> mutant ovaries, and are consistent with our inability to detect misprocessed H3 mRNA by northern blot or S1 nuclease analysis. Taken together, our molecular and cytological analyses of *Slbp*<sup>10</sup> mutant ovaries indicate that reduction of SLBP function during oogenesis causes a reduction of histone mRNA biosynthesis, likely because of defects in histone pre-mRNA processing.

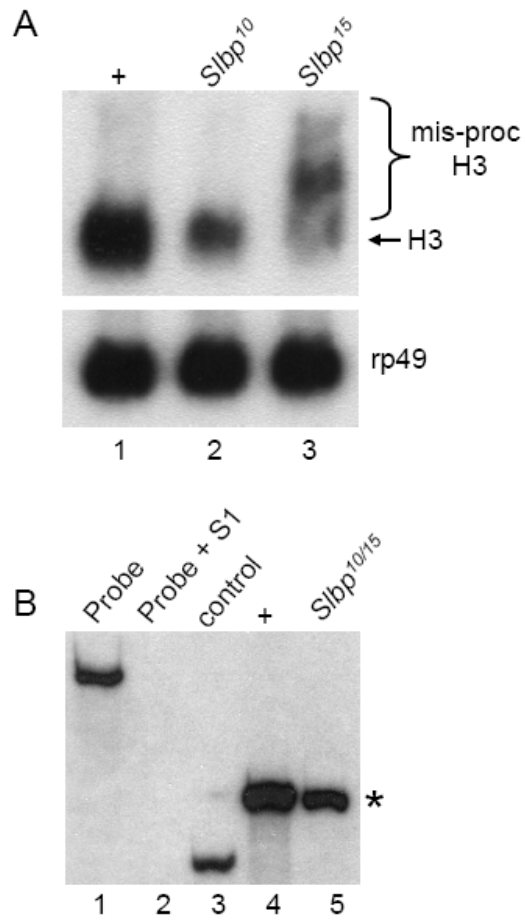


**Figure 2.7. U7 and SLBP are required for histone pre-mRNA processing during oogenesis.** A)  $w^{1118}$  control egg chamber hybridized with a histone H3 coding probe. In this and subsequent panels, white and black asterisks indicate individual nurse cells with and without H3 mRNA, respectively, and arrows indicate follicle cells. B)  $w^{1118}$  control egg chamber hybridized with an H3-ds probe. Note the lack of staining because H3-ds only detects misprocessed, poly A<sup>+</sup> histone H3 mRNA. C, D)  $U7^{14}$  mutant egg chambers hybridized with H3 coding and H3-ds probes, respectively. E, F) Early stage  $Slbp^{10}$  mutant egg chambers, each from an individual ovariole, hybridized with the H3 coding and H3-ds probes, respectively. The brackets indicate a stage 2 or 3 egg chamber with staining in the nurse cells undergoing endocycles. Note the absence of H3-ds probe in the older egg chambers (asterisk in F). G)  $Slbp^{10}$  mutant egg chamber stained with the H3 probe indicating production of H3 mRNA in both nurse and follicle cells. H)  $Slbp^{10}$  mutant egg chamber hybridized with an H3-ds probe, with focus on the follicle cells. The arrow indicates a follicle cell expressing misprocessed, poly A<sup>+</sup> histone mRNA. I, J) Mosaic egg chambers containing  $Slbp^{15}$  mutant germ cells hybridized with a histone H3 coding probe (I) or an H3-ds probe (J). Because this is a mosaic egg chamber, the follicle cells are phenotypically wild type and do not stain with the H3-ds probe because they express only wild type H3 mRNA (arrow in I). All egg chambers except panels E and F stage 9. Anterior at left, and posterior at right in all panels.

To directly test whether the variable H3-ds staining in  $Slbp^{10}$  mutant ovaries reflected reduced but not absent SLBP function, we generated egg chambers containing  $Slbp^{15}$  null mutant germ cells using FLP/FRT-mediated mitotic recombination (see Materials and Methods). In contrast to the  $Slbp^{10}$  mutants, the H3 coding and H3-ds probes stained  $Slbp^{15}$  null mutant germ cells similarly, suggesting that a greater fraction of the H3 mRNA produced was misprocessed (Fig 2.7I, J). This result was confirmed by northern analysis of RNA isolated from  $Slbp^{15}$  mosaic ovaries (Fig. 2.8, lane 3). These data indicate that SLBP function is required in the germ line to process histone mRNAs, and that the germ line is not intrinsically incapable of utilizing the cryptic polyadenylation signals downstream of the normal H3 pre-mRNA processing site. Females with  $Slbp^{15}$  mutant germ cells lay very few



eggs that do not hatch, and that have chorion defects including fusion of the dorsal appendages, similar to the small number of eggs produced by *U7* mutants (not shown). Taken together, our genetic analysis of *U7* and *SLBP* function in ovaries suggests that histone pre-mRNA processing is necessary to complete oogenesis.



**Figure 2.8. SLBP is required for histone pre-mRNA processing during oogenesis.** A) RNA was isolated from whole ovaries dissected from adult females and subjected to northern analysis with <sup>32</sup>P-labeled H3 (top) or rp49 (bottom) coding probes. Lane 1: *w<sup>1118</sup>* control. Lane 2: *Slbp<sup>10</sup>* homozygotes. Lane 3: RNA from ovaries containing mitotic germ line clones of *Slbp<sup>15</sup>* cells. Rp49 serves as a loading control. B) Detection of H2a mRNA by S1 nuclease protection. Lane 1: 3' end-labeled 650-nt H2a probe. Lane 2: H2a probe incubated with S1 nuclease and non-specific yeast tRNA. Lane 3: H2a probe + S1 and a synthetic, partial H2a mRNA that yields a protected 265-nt fragment that is shorter than the 340-nt protected fragment resulting from full length H2a mRNA (asterisk). Lane 4: H2a probe + S1 and total RNA isolated from ovaries dissected from wild type females. Wild type histone H2a mRNA protects a 340-nt fragment (asterisk). Lane 5: H2a probe + S1 and total RNA isolated from ovaries dissected from *Slbp<sup>10</sup>/Slbp<sup>15</sup>* mutant females.

## Discussion

Probing the function of snRNAs using genetic approaches in metazoans has been difficult since most of the snRNAs are encoded by multiple genes. Exceptions are the snRNAs in the minor spliceosome and U7 snRNA, each of which are present in a single copy in all organisms whose genome has been sequenced. The U12 snRNA has been disrupted in *Drosophila* and is essential for proper development (Otake et al., 2002). Here we report the first example of mutating a U7 snRNA gene as part of our studies to understand the in vivo function of factors involved in histone mRNA metabolism.

### The Role of U7 and SLBP in histone mRNA metabolism

We find that *Drosophila* U7 snRNA is essential for normal histone mRNA biosynthesis during development, and that all five replication associated histone mRNAs are improperly processed in *U7* mutant animals. The histone mRNAs produced in *U7* mutants

are longer than wild type mRNAs and are polyadenylated, consistent with the use of cryptic downstream polyadenylation signals located within each histone gene. This phenotype is identical to the phenotype we previously described for mutations in *Slbp* (Sullivan et al., 2001; Lanzotti et al., 2002). The similar poly A<sup>+</sup> histone mRNA phenotype in *Slbp* and *U7* mutants suggests that neither the presence of SLBP in *U7* mutants, nor the presence of *U7* snRNP in *Slbp* mutants, impedes the ability of the polyadenylation machinery to utilize histone pre-mRNAs as a substrate. Polyadenylation occurs even though SLBP likely binds to the nascent transcripts in the *U7* mutants. In addition, very recent evidence indicates that cleavage of histone pre-mRNA is carried out by some of the components that mediate pre-mRNA cleavage prior to polyadenylation of canonical mRNAs (Dominski et al., 2005; Kolev & Steitz, 2005). How then is polyadenylation of histone mRNAs normally prevented in *Drosophila*? In vitro studies of *Drosophila* histone gene transcription have suggested that RNA polymerase II pausing just 3' of the normal processing site may contribute to efficient histone 3' end formation (Adamson & Price, 2003). The polymerase pauses before the cryptic polyadenylation signals are encountered, and hence must resume transcription to allow the production of polyadenylated histone mRNAs. Since we do not see any polyadenylated histone mRNA in wild-type animals or cultured cells, it is possible that the presence of both SLBP and *U7* snRNP promote both processing and transcription termination such that the downstream cryptic poly A signals are never encountered.

*U7* snRNP likely acts in a catalytic fashion, and is recycled after release from the downstream cleavage product, which is degraded in a *U7*-dependent reaction (Walther et al., 1998). *U7* snRNA is present at very low levels in the cell and is largely confined to the Cajal bodies, nuclear organelles described in vertebrates that are responsible for snRNP maturation

and function. Cajal bodies are often found in association with histone loci, although in vertebrates U7 snRNA is associated with all the Cajal bodies irrespective of whether they are close to histone loci (Wu & Gall, 1993; Frey & Matera, 1995; Gall, 2003). U7 snRNP is thus normally in excess in the cell, and therefore cells may be able to tolerate a reduction in U7 snRNA levels. In *Drosophila* cells, the U7 snRNP also localizes to the histone locus (J. Gall, personal communication).

Consistent with the idea that U7 is present in excess, our data indicate that normal levels of H3 processing occur in 1<sup>st</sup> and 2<sup>nd</sup> instar larvae that contain reduced amounts of wild type U7 snRNA. There is then a re-accumulation of U7 snRNA in wild type adult females, most likely from production during oogenesis. Thus, *Drosophila* U7 snRNA is present in substantial excess over what is functionally required during much of zygotic development. SLBP may also be capable of acting efficiently in histone pre-mRNA processing in vivo even when it is present in reduced amounts. For instance, the majority of histone H3 and H2a mRNAs is processed in *Slbp*<sup>10</sup> hypomorphic mutant 3<sup>rd</sup> instar larvae even though the *Slbp*<sup>10</sup> allele produces only 10% the amount of wild type SLBP protein (Sullivan et al., 2001). Perhaps a small nuclear pool of SLBP, possibly associated with Cajal bodies (Abbott et al., 1999), accumulates preferentially and is sufficient to carry out histone pre-mRNA processing.

Interestingly, the proportion of mis-processed:wild type H2a in *Slbp*<sup>10</sup> embryos (Lanzotti et al., 2002) and 1<sup>st</sup> and 2<sup>nd</sup> instar larvae is substantially higher than we observed in the 3<sup>rd</sup> instar larvae (Fig. 2.6C). This suggests that the demand for SLBP is greater in early development. Because the demand for histone mRNA is determined by the rate of DNA replication, the *Slbp*<sup>10</sup> phenotype may reflect differences in replication rates, and hence

demand for histone proteins, between the early and later stages of development. A similar situation may be occurring early during oogenesis when misprocessed H3 mRNAs preferentially accumulate in *Slbp*<sup>10</sup> mutants. We envision that SLBP binds the nascent transcript and rapidly recruits the U7 snRNP to initiate processing very soon after transcription of the 3' end (possibly while the polymerase is stalled at the processing site). In *Slbp*<sup>10</sup> mutant cells with a high rate of histone gene transcription (e.g. early in zygotic development or oogenesis), there is likely not sufficient SLBP to recruit U7 snRNP to the transcripts, and read-through of many transcripts occurs. In the 3<sup>rd</sup> instar larval cells of *Slbp*<sup>10</sup> mutants the rate of histone transcription may be less, and the small amounts of SLBP and U7 snRNP are sufficient to cooperate to stimulate a substantial amount of histone pre-mRNA processing. Recent in vitro studies indicate that both U7 and SLBP are required for processing in *Drosophila* nuclear extracts, as they are in the animal (Dominski et al., 2005). Thus, in the complete absence of either SLBP or U7 snRNA, no accumulation of processed histone mRNA occurs.

### **U7 and SLBP are required during oogenesis**

During oogenesis there are two distinct modes of histone mRNA biosynthesis. The first is replication dependent and results in the accumulation of histone mRNA specifically during S phase. The second is a burst of replication-independent nurse cell expression late in oogenesis that generates the maternal mRNAs that are transported to the oocyte (Ambrosio & Schedl, 1985; Ruddell & Jacobs-Lorena, 1985; Walker & Bownes, 1998). Both modes of expression are affected by mutation of *Slbp*. *Slbp* mutant nurse cells and follicle cells contain misprocessed, poly A<sup>+</sup> H3 mRNA in replicating cells, and the total amount of histone mRNA

produced during oogenesis is reduced relative to wild type. *U7* mutants also contain misprocessed, poly A<sup>+</sup> H3 mRNA in replicating nurse and follicle cells, although the total amount of mRNA is not reduced relative to wild type.

Mutation of either *Slbp* or *U7* prevents the completion of oogenesis, and results in the production of very few eggs, none of which develop. Although the egg chambers in each mutant ovary appear to form properly and contain the appropriate cell types, most do not progress to late stages. The *U7* mutant egg chambers typically do not develop past the “dumping” stage where the nurse cell cytoplasmic components are rapidly transferred to the oocyte. The basis for this developmental defect is not known, and we have not pursued a detailed analysis of the phenotype.

### **Mutations in *U7* and *Slbp* affect *Drosophila* development differently**

*Slbp* null mutations result in lethality while *U7* null mutations result in viable adults that are sterile. Because SLBP and U7 snRNP both play essential roles in the same molecular process, histone pre-mRNA processing, we expected that abolishing the function of these factors would cause very similar terminal phenotypes in the whole animal. However, there is maternal pool of U7 snRNA that is sufficient to support histone pre-mRNA processing until the third larval instar. In contrast, there is very little if any maternal SLBP, and poly A<sup>+</sup> histone mRNAs appear in *Slbp* mutants as soon as zygotic histone transcription begins (Lanzotti et al., 2002). Thus, the simplest explanation for the difference in terminal phenotypes between *Slbp* and *U7* null mutants is the maternal pool of U7 snRNA, which results in a later onset of the mutant phenotype. The consequence of combining the viable

*Slbp*<sup>10</sup> hypomorphic allele with a viable *U7* null mutation is consistent with this: histone pre-mRNA processing is disrupted sooner in the double mutant than either single mutant, leading to lethality. The relative severity of the *Slbp* mutant phenotype compared to *U7* may also result in part from the participation of SLBP but not U7 snRNP in aspects of histone mRNA metabolism other than pre-mRNA processing. SLBP is part of the mature histone mRNP (Sanchez & Marzluff, 2002; Whitfield et al., 2004) and contributes to efficient histone mRNA translation (Sanchez & Marzluff, 2002) and the rapid destruction of histone mRNAs after replication inhibition (Kaygun & Marzluff, 2005). These processes do not involve U7 snRNP, and thus loss of SLBP might cause more severe defects.

The precise molecular and cellular basis for *Slbp* mutant lethality and *U7* mutant sterility is not known. The correlation between the extent and time of onset of the histone pre-mRNA processing defects and the severity of the developmental phenotype strongly suggests that the developmental defects result from the disruption of histone mRNA metabolism. One possibility is that the presence of poly A<sup>+</sup> histone mRNA is somehow detrimental, or that the poly A<sup>+</sup> mRNAs cannot produce the correct amount or correct stoichiometry of the canonical histones during S phase. However, we cannot exclude the possibility that the mutant phenotypes are due to the participation of SLBP and U7 snRNP in processes other than histone pre-mRNA processing. For instance, in the *Xenopus* oocyte germinal vesicle some SLBP1 is found in Cajal bodies, as expected for a role in histone pre-mRNA processing, and some SLBP1 is also found in association with the genes encoding 5S rRNA and U1 and U2 snRNAs (Abbott et al., 1999). However, there is no direct evidence that SLBP1 is required for the function or biosynthesis of these RNA molecules. A

continued examination of *Slbp* and *U7* mutant phenotypes should contribute to a more complete understanding of the roles played by these factors during animal development.

### **Acknowledgements**

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## CHAPTER III

# **THE *DROSOPHILA* U7 snRNP PROTEINS LSM10 AND LSM11 PLAY AN ESSENTIAL ROLE IN DEVELOPMENT INDEPENDENT OF HISTONE PRE-mRNA PROCESSING**

### **Preface**

This work is under revision for RNA and represents my second, first author paper with work contributed to it by members of my lab. This work was carried out in collaboration with William F. Marzluff, a professor here at UNC.

Robert J. Duronio, my thesis advisor, and I conceived of this project with input and advice from our collaborator and other authors. I completed the majority of the experimental work shown. Anne E. White, a graduate student in my lab, performed the U7 snRNA FISH on third instar larval brains as well as the confocal imaging of those preparations (Fig 3.6 C thru F). Deirdre C. Tatomer, a graduate student in my lab, stained Lsm11 V5 tagged embryos with the V5 antibody and took the confocal images of those preparations (Fig3.6 A and B). I wrote most of the manuscript with comments from Robert J. Duronio and other contributors.

**Ashley C. Godfrey, Anne E. White, Deirdre C. Tatomer, William F. Marzluff, and Robert J. Duronio.** (2009). The *Drosophila* U7 snRNP Proteins Lsm10 and Lsm11 Play an Essential Role in Development Independent of Histone pre-mRNA Processing. RNA.

## ABSTRACT

Metazoan replication-dependent histone mRNAs are not polyadenylated, and instead terminate in a conserved stem-loop structure generated by an endonucleolytic cleavage of the pre-mRNA involving U7 snRNP. Unlike spliceosomal snRNPs, U7 snRNP contains two like-Sm proteins, Lsm10 and Lsm11, which replace SmD1 and SmD2 in the canonical heptameric Sm protein ring that binds snRNAs. Here we show that mutations in either the *Drosophila Lsm10* or the *Lsm11* gene disrupt normal histone pre-mRNA processing, resulting in production of poly A<sup>+</sup> histone mRNA as a result of transcriptional read-through to cryptic polyadenylation sites present downstream of each histone gene. This molecular phenotype is indistinguishable from that which we previously described for mutations in other components of the histone pre-mRNA processing machinery, including U7. Lsm10 protein fails to accumulate in *Lsm11* mutants, consistent with prior observations suggesting that a stable pool of Lsm10-Lsm11 dimers provides precursors for U7 snRNP assembly. Unexpectedly, U7 snRNA accumulates in *Lsm11* mutants. This U7 can be precipitated with anti-trimethylguanosine antibodies, suggesting that it assembles into a snRNP particle in the absence of Lsm10 and Lsm11. However, it cannot be detected at the histone locus body, suggesting that Lsm10 and Lsm11 are necessary for U7 snRNP localization. In contrast to U7 snRNA null mutants, which are viable, *Lsm10* and *Lsm11* mutants do not survive to adulthood. When considered in the context of the histone mRNA phenotype, this difference in developmental phenotype suggests that Lsm10 and Lsm11 perform an essential function that is distinct from histone pre-mRNA processing and independent of U7 snRNA.

## INTRODUCTION

The histones that make up the core nucleosome octomer, H2A, H2B, H3, H4, as well as the H1 linker, are synthesized only during S-phase of the cell cycle when they are needed for packaging newly replicated DNA into chromatin. This replication-coupled histone biosynthesis is an essential aspect of genome replication during cell proliferation, and is controlled primarily by regulating histone mRNA abundance. The 3' end of histone mRNA is required for cell cycle regulation (Luscher & Schumperli, 1987; Stauber & Schumperli, 1988; Harris et al., 1991). However, unlike all other metazoan mRNAs, the histone mRNA 3' end contains a conserved terminal stem-loop structure rather than a poly A tail (Marzluff et al., 2008). Specialized machinery is needed to generate this unique mRNA 3' end, and determining how this machinery functions is necessary for fully understanding replication-coupled histone mRNA biosynthesis.

Histone pre-mRNA utilizes two cis-acting elements for proper 3' end processing: the stem-loop and a purine rich region downstream of the cleavage site termed the Histone Downstream Element (HDE) (Mowry & Steitz, 1987a). The stem loop is recognized by a protein called Stem Loop Binding Protein (SLBP) (Wang et al., 1996), or Hairpin Binding Protein (HBP) (Martin et al., 1997), and U7 snRNP interacts with histone pre-mRNA by base pairing between the 5' end of U7 snRNA and the HDE (Galli et al., 1983; Schaufele et al., 1986; Mowry & Steitz, 1987a; Bond et al., 1991). These factors recruit a complex that triggers endonucleolytic cleavage between the stem loop and the HDE, forming the mature mRNA. After processing, the mature histone mRNA bound to SLBP exits the nucleus and enters the cytoplasm, where SLBP stimulates translation (Sanchez & Marzluff, 2002).



Histone pre-mRNA cleavage is catalyzed by the CPSF73 endonuclease (Dominski et al., 2005). Interestingly, CPSF73 and three other factors necessary for histone pre-mRNA processing, including CPSF100, which directly binds CPSF73, and Symplekin, are members of a large multi-protein complex that cleaves and polyadenylates all other mRNAs (Kolev & Steitz, 2005; Wagner et al., 2007). These observations demonstrate remarkable similarity in the machinery that generates different mRNA 3' ends. How these factors assemble into functionally different complexes is unknown.

Because U7 snRNP functions to recognize histone pre-mRNA and to recruit the appropriate cleavage factors, it is essential for histone pre-mRNA processing *in vitro* and *in vivo* (Galli et al., 1983; Gick et al., 1986; Mowry & Steitz, 1987b; Godfrey et al., 2006). U7 snRNP is related to spliceosomal snRNPs, all of which are composed of a core particle containing a short, noncoding, nonpolyadenylated snRNA bound by seven structurally similar Sm proteins: SmD1, SmD2, SmE, SmF, SmG, SmB/B', and SmD3 (Luhrmann et al., 1990; Matera et al., 2007). These proteins bind to a conserved uridine-rich sequence in the snRNA termed the Sm binding site (Branlant et al., 1982). Sm proteins consist of two conserved motifs, SM1 and SM2, separated by a linker region of variable length (Hermann et al., 1995; Seraphin, 1995). Structural analysis of the SmD3/SmB and SmD1/SmD2 complexes reveals that the SM1 and SM2 domains together form a common fold, termed the Sm fold, containing an NH<sub>2</sub>-terminal alpha-helix followed by a strongly bent five-stranded antiparallel beta sheet (Kambach et al., 1999). These data, along with structural information from two related bacterial proteins, Lsm $\alpha$  from the thermophilic archaeon *Methanobacterium thermoautotrophicum* and AF-Sm1 and AF-Sm2 from the hyperthermophilic euryarchaeon *Archaeoglobus fulgidus*, suggest that the seven Sm proteins assemble into a donut shaped,

heptameric ring and that the snRNA may thread through the center hole (Collins et al., 2001; Toro et al., 2001; Toro et al., 2002).

U7 snRNP differs from spliceosomal snRNPs in an important way. U7 snRNP contains two unique Sm-like proteins, Lsm10 and Lsm11, which replace SmD1 and SmD2 in the canonical heptameric Sm ring. Lsm10 and Lsm11 contain the SM1 and SM2 motifs and are very similar in structure to Sm proteins. Lsm10 is closely related to SmD1, whereas Lsm11 is related to SmD2 (Pillai et al., 2001; Pillai et al., 2003). U7 snRNA contains a non-canonical Sm binding site that recruits Lsm10 and Lsm11 specifically to the U7 particle. Mutation of the U7 Sm binding site to a spliceosomal consensus Sm binding site disrupts U7 snRNA function (Stefanovic et al., 1995), and results in replacement of Lsm10 and Lsm11 with SmD1 and SmD2 in the U7 snRNP (Pillai et al., 2003). This finding demonstrates that Lsm10 and Lsm11 confer functional properties to U7 snRNP that are essential for histone pre-mRNA processing. Lsm11 contains an NH<sub>2</sub>-terminal domain larger than that in any other Sm or Lsm protein, as well as a rather long linker sequence between Sm motif 1 and 2 (Schumperli & Pillai, 2004). Previous work suggests that one role for Lsm11 in histone 3' end processing is to bind directly to ZFP100 (Pillai et al., 2003; Azzouz et al., 2005), a 100 kDa zinc finger protein that is part of U7 snRNP and helps to stabilize the cleavage complex on histone pre-mRNA (Dominski et al., 2002). A specific binding site for ZFP100 was mapped to amino acids 63-82 in the NH<sub>2</sub>-terminal domain of human Lsm11 (Wagner et al., 2006). Lsm11 likely plays additional roles in the processing reaction, since mutation of other conserved amino acids in the NH<sub>2</sub>-terminal domain of Lsm11 impairs processing without affecting binding to ZFP100 (Azzouz et al., 2005).

How Lsm10 and Lsm11 contribute to histone pre-mRNA processing *in vivo*, or which aspects of their structure are necessary *in vivo*, has not been determined. For instance, ZFP100 has not been identified in invertebrate species like *Drosophila*, which contain a single U7 snRNA (Dominski et al., 2003) and single genes encoding Lsm10 and Lsm11 (Azzouz & Schumperli, 2003; Dominski et al., 2003). It is unclear whether *Drosophila* Lsm11 binds a highly diverged but functionally analogous protein, or whether it functions in the processing reaction independently of a ZFP100-like protein. In addition, it is not known whether Lsm10 or Lsm11 participate in other aspects of RNA metabolism, as do other Lsm proteins. For instance, a complex of Lsm2-8 binds to U6 snRNA and is required for the U4/U6 formation during splicing (Achsel et al., 1999), while a complex of Lsm1-7 functions in cytoplasmic mRNA decay (Bouveret et al., 2000). We hypothesized that if Lsm10 and Lsm11 only function in histone pre-mRNA processing as part of U7 snRNP, then mutations in *Drosophila Lsm10*, *Lsm11*, and *U7* would cause identical phenotypes. We previously demonstrated that *U7* null mutants fail to properly process histone mRNA beginning at the third larval instar stage, but nonetheless develop into fully formed but sterile adults. Here we identify mutations of *Lsm10* and *Lsm11* and demonstrate that, like U7 snRNA mutations, disruption of Lsm10 and Lsm11 function results in the production of mis-processed histone mRNAs beginning at the third larval instar stage. However, unlike *U7* null mutants, *Lsm10* and *Lsm11* mutants do not survive to adulthood, dying as non-pharate pupae. This strongly suggests that Lsm10 and Lsm11 are required for an essential process distinct from histone pre-mRNA processing.

## MATERIAL AND METHODS

### Western Blots

Tissue extracts from 3<sup>rd</sup> instar larval brains plus salivary glands or adult flies were prepared in NET buffer (0.05 M Tris pH 7.5, 0.4 M NaCl, 0.005 M EDTA, and 1% NP40) with 100 mM PMSF, 1 mg/ml Leupeptin, and 0.5 mg/ml Pepstatin and cleared by centrifugation at 10,000g for 10 min at 4°C. GFP-negative, homozygous *U7* mutant larvae were collected from *U7<sup>20</sup>/TM3 P[act-GFP]* parents. *Lsm11* mutant larvae were collected from *Lsm11<sup>c02047</sup>/CyO P[twist-GFP]* x *Df(2R)M073/CyO P[twist-GFP]* parents. *Lsm10* mutant larvae were collected from *Lsm10<sup>f06616</sup>/CyO P[twist-GFP]* or *Lsm10<sup>G40E</sup>/CyO P[twist-GFP]* x *Df(2R)17/CyO P[twist-GFP]* parents. Proteins were separated through either a 12% (*Lsm11*) or a 15% (*Lsm10*) acrylamide gel (BIO-RAD) and transferred to a 0.45 µm Pure Nitrocellulose Membrane (BIO-RAD). Membranes were probed with anti-*Lsm11*, anti-*Lsm10* (1:1,000; graciously donated by Dr. Joe Gall), or anti- $\alpha$ -tubulin (1:1,000; Sigma). Horseradish Peroxidase linked secondary (Amersham Biosciences) was used at 1:5,000 for *Lsm10* and 1:1,000 for *Lsm11* and  $\alpha$ -Tubulin and visualized with ECL (Amersham Biosciences).

### Northern Analysis

For the detection of U1 or U7 snRNA with an  $\alpha$ [<sup>32</sup>P]-UTP anti-sense RNA probe, 15 µg/lane of total cellular RNA isolated using TRIzol Reagent (Gibco) was denatured with 8M urea and subjected to electrophoresis through an 8% polyacrylamide gel containing 7M urea. Separated RNA were transferred to an N+ nitrocellulose membrane (Amersham) using a Genie Blotter (Idea Scientific). For the analysis of histone mRNA, 2 µg/lane of RNA were

subjected to electrophoresis through a 1% agarose gel containing 0.01 M MOPS (pH 7.0) and 6.75% formaldehyde and transferred via the wick method to N+ nitrocellulose membrane. DNA containing histone coding regions were random primer labeled with  $\alpha$ [<sup>32</sup>P]-dCTP (Stratagene). Hybridizations were performed at 58° C for snRNA probes and at 60° C for histone probes.

### ***Drosophila* Genetics**

The *U7*<sup>20</sup> and *U7*<sup>EY11305</sup> null alleles are described in Godfrey et al. (2006). *w*<sup>1118</sup> was used throughout as a wild-type control. To assess relative fertility, eggs were collected overnight from broods of *Lsm10* mutant males and virgin females. 100 eggs from each genotype were transferred to a fresh grape juice plate and the total number that hatched within three days was determined. The data is reported as the average and standard deviation of six independent measurements. P values for mutant-wild type comparisons were determined using a paired student T-test. A chi-squared test was used to determine significance of deviations from expected Mendelian ratios.

P element transgenes were constructed for rescue of *Lsm10* and *Lsm11* mutant phenotypes. DNA containing either the *Lsm10* or *Lsm11* locus (Fig. 1 A) was amplified by PCR from adult female *w*<sup>1118</sup> genomic DNA and subcloned into the pCaSpeR 4 transformation vector and confirmed by sequencing. The V5 epitope was added to the NH<sub>2</sub>-terminus of Lsm11 by using a 5' primer with the V5 sequence immediately downstream of the ATG start codon. All phenotypic rescue experiments employed strains containing a second chromosome recombinant of genotype *Lsm11*<sup>c02047</sup>, P[V5-Lsm11<sup>+</sup>] or *Lsm10*<sup>G40E</sup>,

P[*Lsm10*<sup>+</sup>]. Each recombinant chromosome was confirmed molecularly by PCR (*Lsm11*<sup>c02047</sup> allele) or sequencing (*Lsm10*<sup>G40E</sup> allele).

### **Immunoprecipitations and RT-PCR**

20 µg of total cellular RNA was added to anti-TMG antibody coated beads (EMD Biosciences, INC.) in 1ml of buffer A (20mM HEPES-KOH, pH 7.6, 0.2 mM EDTA, 0.5 mM DTT, 0.2 M KCl, 5% glycerol) plus 80 units of RiboLock RNase Inhibitor (Fermentas). Samples were incubated at 4°C for 2 hours and the beads were recovered by centrifugation at 2300 RPM for 1 min., and then washed 3X with buffer A at 0.3 M KCl. Bound RNA was eluted by incubation in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS for 10 minutes at 60°C and subsequently precipitated with PCI( Phenol Chlorophorm Isoamly ) (Invitrogen). Recovered RNA was treated with DNase (Promega) prior to generating cDNA by using an N6 random Hexamer (IDT) and SuperScriptII Reverse Transcriptase (Invitrogen). U7, U1, and rp49 cDNA was detected in separate PCR reactions.

### **Immunostaining, in situ Hybridization, and Microscopy**

To detect transgenic *Lsm11*, *Lsm11*<sup>c02047</sup>, *P[V5-Lsm11<sup>+</sup>]* homozygous embryos were dechorionated, fixed in a 1:1 mixture of 7% formaldehyde / heptane for 25 min, and incubated with primary and secondary antibodies overnight at 4°C and for 1hr at 25°C respectively. Primary antibodies used were monoclonal mouse anti-V5 (1:1000; Invitrogen, Carlsbad, CA), monoclonal mouse anti-Discs Large (1:1000, Developmental Studies Hybridoma Bank, U. of Iowa). Secondary antibodies used were goat anti-mouse IgG-Cy3 (Jackson Immuno Research Laboratories, West Grove, PA). DNA was detected by staining

embryos with 4,6-diamidino-2-phenylindole (DAPI) (1:1000 of 1mg/mL stock, Dako North America, Carpinteria, CA) for 1 min.

Brains were dissected from 3rd instar larvae in PBS, fixed in 4% formaldehyde for 20 min, permeabilized in 1% Triton X-100 for 1 hr, and incubated with primary antibodies at 37°C for 1 hour or overnight and secondary antibodies at 25°C for 1 hr or overnight at 4°C. Brains were fixed again in 4% formaldehyde for 20 min prior to *in situ* hybridization. The following primary and secondary antibodies were used: monoclonal mouse anti-Ser/Thr-Pro MPM-2 (1:1000; Millipore, Temecula, CA), affinity-purified polyclonal rabbit anti-Lsm10 (1:1000; Liu *et al.* 2006), goat anti-mouse Cy5 and goat anti-rabbit Cy2 (both from Jackson Immuno Research Laboratories, West Grove, PA). Brains were hybridized with anti-sense *U7* digoxigenin-labeled RNA probes as described previously (Lanzotti *et al.* 2002; White *et al.* 2007). Probes to *U7* were derived from a clone containing a cDNA for *U7* (Dominski *et al.* 2003). Hybrids were detected using the Cyanine 3 tyramide signal amplification system (PerkinElmer Life and Analytical Sciences, Boston, MA).

Confocal images were taken at a zoom of 1.0-5.0 with a 40X (numerical aperture 1.30) Plan Neofluor objective on a Zeiss 510 laser scanning confocal microscope using the LSM data acquisition software (Carl Zeiss, Jena, Germany). Image false coloring and contrast was adjusted using Photoshop (Adobe Systems, Mountain View, CA).

## RESULTS

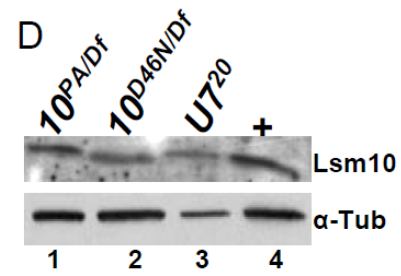
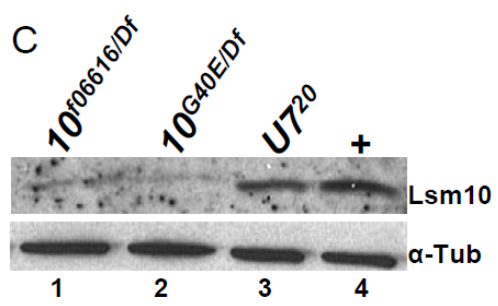
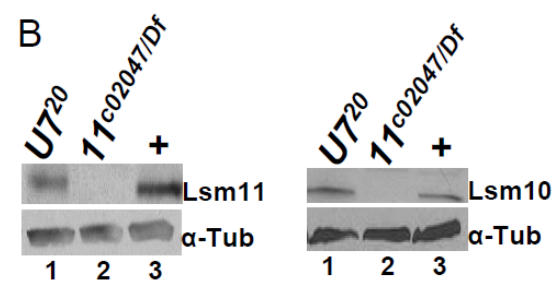
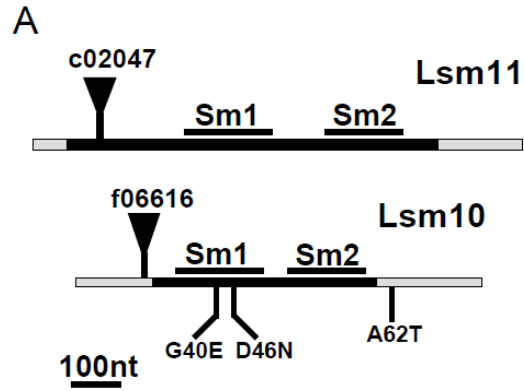
### Identification of *Lsm10* and *Lsm11* mutations

To examine the contribution of Lsm10 and Lsm11 proteins to histone pre-mRNA processing during development, we isolated mutations in each gene. We first identified from the Exelixis collection (Thibault et al., 2004) a single PiggyBac (pBac) insertion allele of both *Lsm10* and *Lsm11*. pBac transposons have a higher incidence of inserting into the coding sequence of genes relative to P-element transposons, which more often insert into the 5' UTR (Thibault et al., 2004). Accordingly, *Lsm11*<sup>c02047</sup> contains a pBac insertion near the 5' end of the coding sequence (Fig. 3.1A), and mutant animals containing this mutation in trans with a deficiency do not contain detectable amounts of Lsm11 protein by Western analysis (Fig. 3.1B, left panel). This suggests that *Lsm11*<sup>c02047</sup> is a null allele. *Lsm10*<sup>f06616</sup> contains a pBac insertion in the 5' UTR (Fig. 3.1A), and this mutant is a hypomorph that expresses strongly reduced amounts of Lsm10 protein (Fig. 3.1C, lane 1). In an effort to identify additional *Lsm10* alleles, including a null, we employed the TILLING (Targeting Induced Local Lesions in Genomes) strategy. TILLING is a high throughput method to molecularly identify EMS-induced mutations in specific regions of the genome (Till et al., 2003). The strategy was adapted for use with a collection of *Drosophila* strains that carry an EMS-mutagenized chromosome 2 (Koundakjian et al., 2004). We used this method to screen a ~800 nucleotide region containing the entire *Lsm10* gene from ~3000 of these mutagenized lines. Three mutations were recovered that we suspected might affect Lsm10 function: a Glu substitution for an evolutionarily invariant Gly (*Lsm10*<sup>G40E</sup>) needed for a sharp bend in  $\beta$ -strand 3 of the SM1 domain (Luhrmann et al., 1990), an Asn substitution for an evolutionarily invariant Asp that, based on structural analysis of related Sm proteins (Toro et



al., 2001; Toro et al., 2002), is predicted to contact the snRNA (*Lsm10<sup>D46N</sup>*), and a single nucleotide change in the 3' UTR that alters a canonical poly A signal sequence (AATAAA→TATAAA; *Lsm10<sup>PA</sup>*)(Fig. 3.1A). The *Lsm10<sup>G40E</sup>* mutant expressed a small amount of Lsm10 protein (Fig. 3.1C), suggesting that the G40E substitution disrupts folding of the SM1 domain resulting in reduced protein accumulation. The *Lsm10<sup>PA/Df</sup>* and *Lsm10<sup>D46N/Df</sup>* mutants did not show any reproducible reduction in Lsm10 protein compared to wild type (Fig. 3.1D, lanes 1,2).

To test whether the accumulation of Lsm10 and Lsm11 are interdependent, we examined Lsm10 and Lsm11 accumulation in *U7* mutants, and Lsm10 accumulation in *Lsm11* mutants. Both Lsm10 and Lsm11 protein were present at wild type levels in the *U7<sup>20</sup>* null mutant (Fig. 3.1B-D), indicating that a pool of these proteins can exist independent of U7 snRNA. In contrast, Lsm10 protein was undetectable in the *Lsm11<sup>c02047</sup>* null mutant (Fig. 3.1B, right panel). We previously obtained a similar result when Lsm11 was depleted by RNAi in cultured *Drosophila* S2 cells (Wagner et al., 2007). These data indicate that Lsm10 accumulation depends on Lsm11, and suggest that a free pool of Lsm10-Lsm11 dimers exist in cells. This is consistent with current models of snRNP assembly, which suggest that SmD1/SmD2 dimers are precursors for U7 snRNP assembly in the cytoplasm and that both SmD1/SmD2 and Lsm10/Lsm11 are located adjacent to one another in the Sm ring of their respective snRNP particles (Schumperli & Pillai, 2004).



**FIGURE 3.1. Identification of *Lsm10* and *Lsm11* mutations.** (A) Schematic diagram of the *Lsm11* and *Lsm10* genes. The black bars represent the coding sequence and the gray bars represent the 5' and 3' UTRs. Note there are no introns in these two genes. The position of the Sm1 and Sm2 domains are marked with black bars above the coding sequence. The positions of the pBac insertions are indicated by black triangles. The positions of the three *Lsm10* EMS alleles are indicated below the gene. (B-D) Protein extracts of brain and salivary gland tissue from third instar larvae or adult flies (D) of the indicated genotypes were probed with anti-Lsm11 or anti-Lsm10 antibodies by Western blotting. *w<sup>118</sup>* was used as a normal control here and in subsequent figures (+).  $\alpha$ -Tubulin is used as a loading control.

### ***Lsm10* and *Lsm11* mutants fail to properly process histone pre-mRNA**

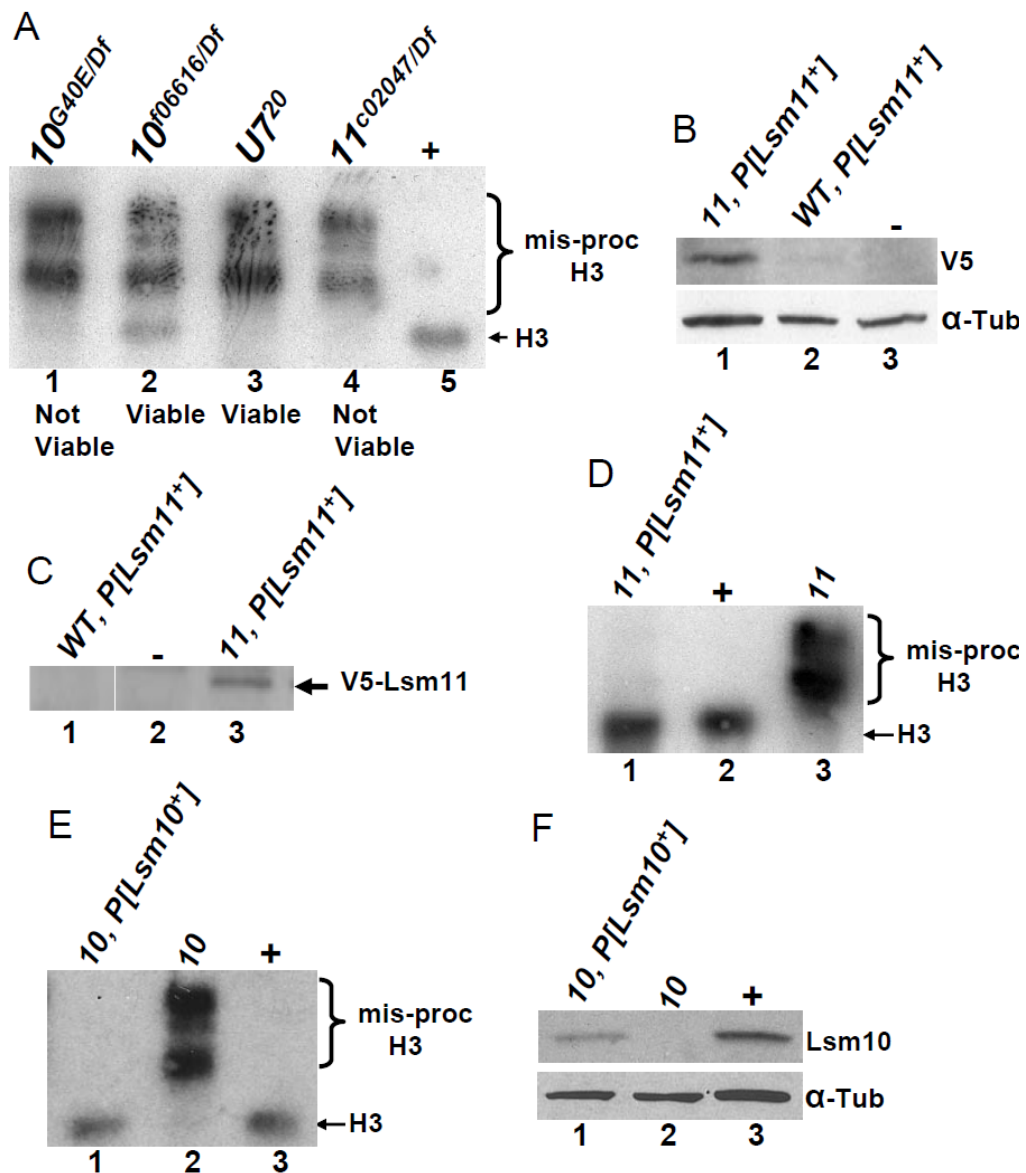
We have previously shown that mutations in U7 snRNA prevent normal histone pre-mRNA processing, resulting in aberrantly long histone mRNAs that are polyadenylated due to the use of cryptic polyadenylation signals downstream of the cleavage site in each histone gene (Godfrey et al., 2006). To test whether a similar phenotype occurs after loss of Lsm10 and Lsm11, we used Northern blotting to hybridize total RNA samples prepared from *Lsm10* or *Lsm11* mutant third instar larvae with a histone H3 probe, and compared the results to a *U7<sup>20</sup>* null mutant (Fig. 3.2A). As with *U7<sup>20</sup>*, we found that the strong *Lsm10<sup>G40E</sup>* and *Lsm11<sup>c02047</sup>* alleles caused nearly complete misprocessing of H3 mRNA, with very little wild-type H3 mRNA present in these mutants (Fig. 3.2A, compare lanes 1 and 4 with lanes 3 and 5). In the *Lsm10<sup>f06616</sup>* mutant, which expresses a small amount of Lsm10 protein, we observed misprocessed H3 mRNA, but unlike the other mutants, we could also detect a small amount of correctly processed wild-type H3 mRNA (Fig. 3.2A, lane 2). These data indicate that Lsm10 and Lsm11 are required for normal histone pre-mRNA processing *in vivo*.

## **Lsm10 and Lsm11 are necessary for development**

The strong *Lsm10*<sup>G40E/Df</sup> and *Lsm11*<sup>c02047/Df</sup> alleles cause lethality: the mutants progress through larval stages and die as non-pharate pupae. This result was surprising, since *U7* null mutants are fully viable yet display the same H3 mRNA misprocessing phenotype (Godfrey et al., 2006). We therefore tested if the *Lsm10* and *Lsm11* mutant phenotypes could be complemented with P element transgenes containing a functional copy of the respective genes. We first engineered a transgene expressing an NH<sub>2</sub>-terminal V5 epitope-tagged Lsm11 with the endogenous *Lsm11* promoter. Curiously, we could detect little if any transgenic V5-Lsm11 in a wild type background using anti-V5 on a Western blot (Fig. 3.2B, lane 2), immunoprecipitation (Fig. 3.2C, lane 1), or immunofluorescent staining of fixed tissue (data not shown). In contrast, we readily detected V5-Lsm11 using all of these methods when the V5-Lsm11 transgene was present in the *Lsm11*<sup>c02047</sup> mutant background (Fig. 3.2B, lane 1; Fig. 3.2C, lane 3; Fig. 3.7A,B). One explanation for this result is that V5-Lsm11 is a poor substrate for U7 snRNP assembly relative to normal Lsm11, and the resulting free V5-Lsm11 is degraded. In spite of this, V5-Lsm11 completely rescued the H3 misprocessing defect and the lethality of *Lsm11*<sup>c02047</sup> mutants, indicating that V5-Lsm11 is fully functional (Fig. 3.2D) and that Lsm11 function is necessary for *Drosophila* development.

Because of the V5-Lsm11 results, we elected not to epitope tag Lsm10, and instead generated a transgene carrying a genomic DNA fragment containing the wild type *Lsm10* gene. This transgene completely rescued both the H3 misprocessing defect and lethality of *Lsm10*<sup>G40E</sup> mutants (Fig. 3.2E). Note that we did not achieve wild type expression of Lsm10

protein in this experiment because the genotype we constructed only contained a single copy of the wild-type *Lsm10* transgene (Fig. 3.2F). This suggests that *Lsm10* is normally present in functional excess. These data show that reintroducing a functional copy of *Lsm10* and *Lsm11* can rescue both the lethality and histone mRNA misprocessing phenotypes of *Lsm10* and *Lsm11* mutants, confirming that these phenotypes are a direct consequence of the loss of each gene.



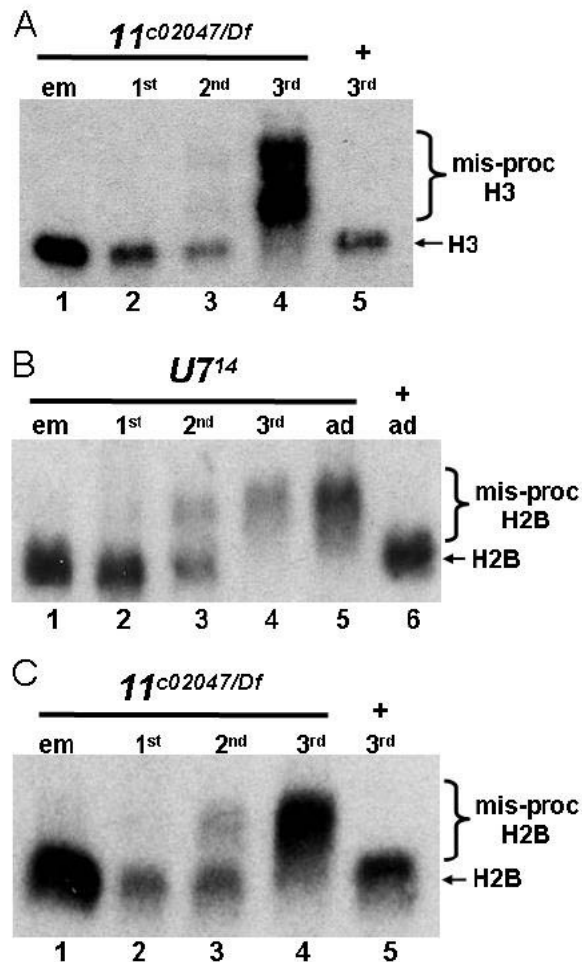
**FIGURE 3.2. *Lsm10* and *Lsm11* mutants fail to properly process histone pre-mRNA and are necessary for development.** (A) Total RNA isolated from whole third instar larvae of the indicated genotypes was subjected to Northern analysis with a <sup>32</sup>P-labeled H3 probe. Note that the severity of the misprocessed H3 phenotype is similar in *U7<sup>20</sup>*, *Lsm10<sup>G40E</sup>*, and *Lsm11<sup>c02047</sup>* mutants while the terminal developmental phenotype of *U7<sup>20</sup>* is different. (B) Protein extracts prepared from embryos of the indicated genotypes were probed with anti-V5 antibodies by Western blotting. P[Lsm11<sup>+</sup>] is a transgene expressing a V5-Lsm11 with the endogenous *Lsm11* promoter. “11” refers to the homozygous *Lsm11<sup>c02047</sup>* genotype. Lane 3 contains protein from a non-transgenic control. Note that in a wild-type *Lsm11* background there is very little accumulation of V5-Lsm11 protein.  $\alpha$ -Tubulin is used as a loading control. (C) Protein extracts isolated from whole third instar larvae of the indicated genotypes were subjected to immunoprecipitation then Western blot analysis with anti-V5 antibody. (D and E) RNA isolated from whole third instar larvae of the indicated genotypes was subjected to Northern analysis with <sup>32</sup>P-labeled H3 probe. “10” refers to the *Lsm10<sup>G40E/Df</sup>* mutant genotype. Note that there is very little misprocessed H3 in both *Lsm11<sup>c02047</sup>*, P[Lsm11<sup>+</sup>] and *Lsm10<sup>G40E/Df</sup>*, P[Lsm10<sup>+</sup>] genotypes. (F) Protein extracts prepared from whole third instar larvae of the indicated genotypes were probed with anti-Lsm10 antibodies. Note that the lane 1 genotype contains a single copy of P[Lsm10<sup>+</sup>], accounting for the reduction in Lsm10 accumulation relative to wild-type (+).

### ***Lsm11* mutant lethality is independent of histone mRNA misprocessing**

Because *U7* null mutants are viable, we were surprised to find that our strongest *Lsm10* and *Lsm11* alleles caused lethality. We previously showed that a maternal supply of *U7* snRNA is sufficient for normal histone pre-mRNA processing through the first larval instar stage of development, such that in *U7* mutants misprocessed histone mRNAs first appear in second instar larvae and wild type histone mRNA is undetectable by the third larval instar stage (Fig. 3.3A) (Godfrey et al., 2006). In contrast to *U7*, *Slbp* null mutations cause lethality in late larval or pupal stages, and this is likely due to an earlier onset of the histone mRNA misprocessing mutant phenotype: there is no functional maternal supply of SLBP and misprocessed histone mRNA can be detected as soon as zygotic transcription begins in *Slbp*

mutant embryos (Lanzotti et al., 2002). Based on these observations, we hypothesized that an earlier onset of the histone mRNA misprocessing phenotype in *Lsm10* and *Lsm11* mutants relative to *U7* mutants might account for the *Lsm10* and *Lsm11* mutant lethality. To test this we examined histone mRNA processing at different stages of development in *U7* and *Lsm11*<sup>c02047</sup> mutants (which also lack Lsm10 protein; Fig. 3.1B). Total RNA was extracted from mutant embryos as well as 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> instar larvae and hybridized with a histone H3 mRNA probe. As we previously reported for histone H3 (Godfrey et al., 2006), in *U7* mutants a small amount of misprocessed histone H2B mRNA was expressed in second instar larva and only misprocessed histone mRNA was present in 3<sup>rd</sup> instar larvae (Fig. 3.3B). In *Lsm11* mutants, the longer, misprocessed H3 mRNA was not detected until the 2<sup>nd</sup> larval instar stage of development, and the correctly processed, wild-type H3 mRNA was barely detectable by the third instar stage (Fig. 3.3A). Similar results were obtained with histone H2B mRNA (Fig. 3.3C) as well as histone H2A, H4, and H1 mRNAs (not shown). For all of these genes we could never detect an onset of misprocessing in *Lsm11*<sup>c02047</sup> mutants any earlier than the 2<sup>nd</sup> larval instar stage of development. Since this is a qualitative comparison of the timing of the onset misprocessed histone mRNA between *U7* and *Lsm11* mutants, we did a titration Northern blot on 1<sup>st</sup> instar larvae from wild-type, *U7* and *Lsm11* null mutants for the H3 (Fig. 3.4 B) and H2B (Fig. 3.4 A) histone genes. Comparing the amount of histone mRNA, which is all processed at this stage in both mutants, in *U7* mutants versus *Lsm11* mutants, relative to our U1 loading control, we did not detect any difference between the two mutants (Fig. 3.4). Thus, *Lsm11* mutants do not begin to accumulate misprocessed histone mRNA earlier in development than *U7* mutants and there is no detectable difference in the amount of normally processed histone mRNA present. We conclude from these data

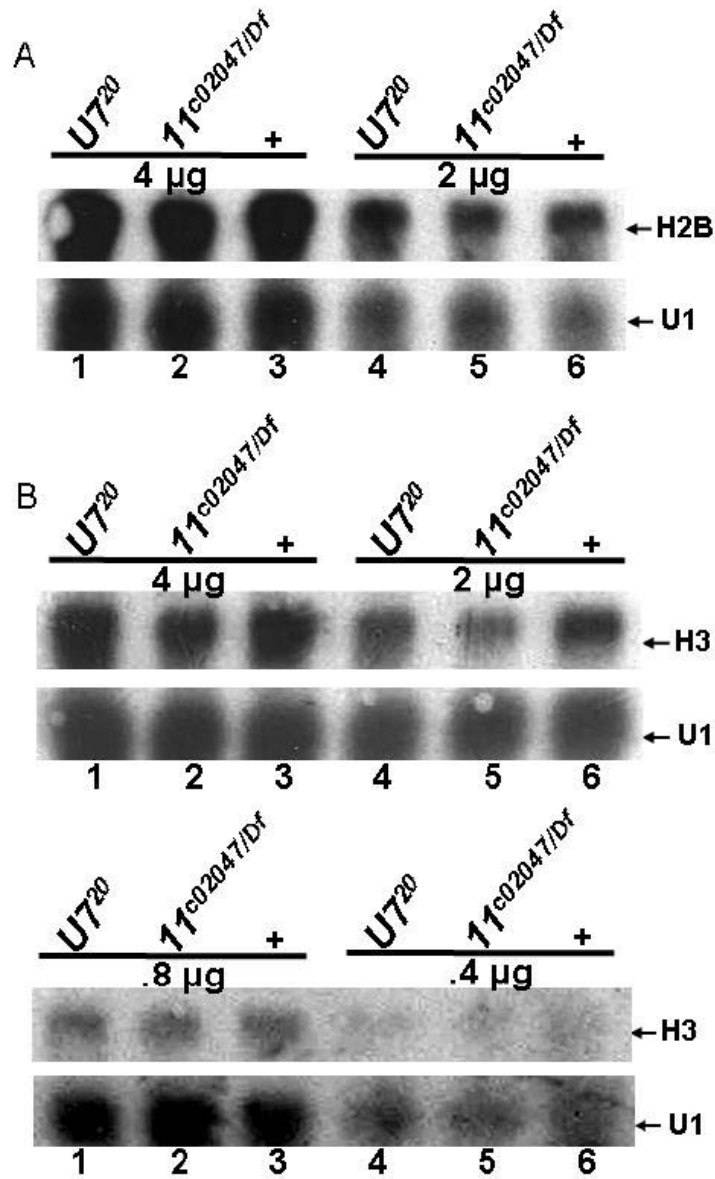
that the lethality caused by mutation of *Lsm11* probably does not result from defects in histone pre-mRNA processing. Because strong *Lsm10* mutants are also not viable, these data suggest that Lsm10 and Lsm11 perform an essential function during development independent of histone mRNA metabolism.



**FIGURE 3.3. *Lsm11* mutant lethality is independent of histone mRNA misprocessing.**

(A-C) Total RNA was extracted from animals at different stages of development of the indicated genotypes and subjected to Northern analysis with a <sup>32</sup>P-labeled probe to H2B (A,C) or H3 (B). Note that the misprocessed histone mRNA is first detectable in small amounts at the second larval instar stage and that wild-type histone mRNA is absent by the third larval instar stage.





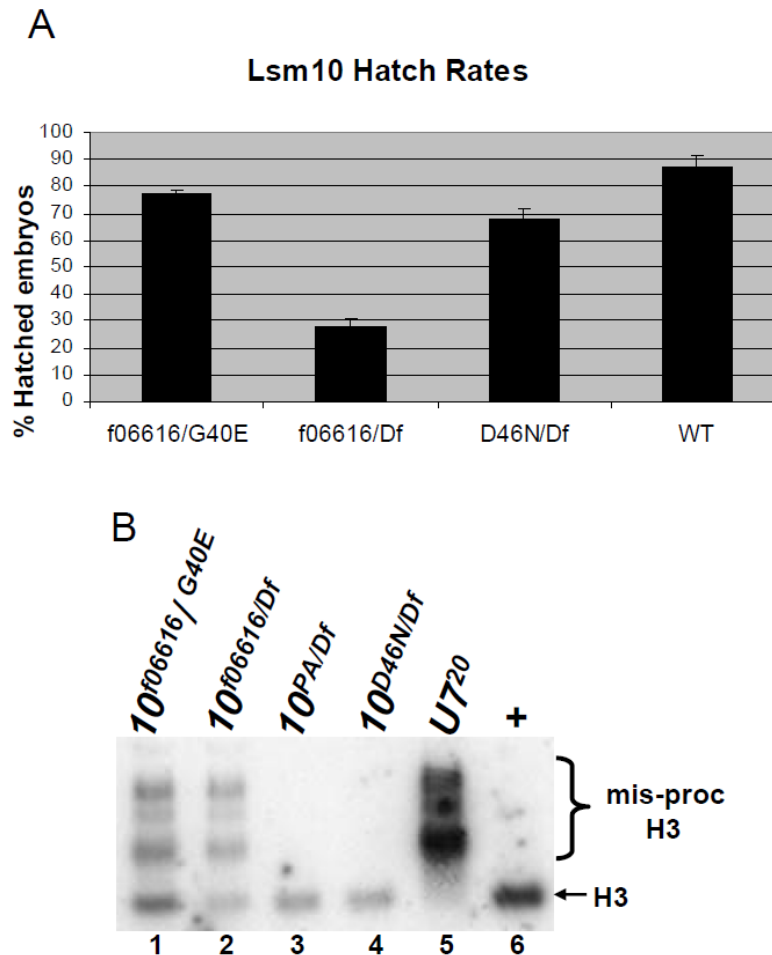
**FIGURE 3. 4. Quantitative analysis of histone mRNA levels between *U7* and *Lsm11* mutants.** (A and B) Total RNA was extracted from 1<sup>st</sup> instar larvae of the indicated genotypes and subjected to Northern analysis with a <sup>32</sup>P- labeled probe to H2B (A) or H3 (B) in titration amounts indicated. U1 is used as a loading control. Note that the level of histone mRNA is not different between *U7* and *Lsm11*.

### **Hypomorphic *Lsm10* alleles are viable with compromised fertility**

Although *U7* null mutants are viable, both males and females are completely sterile, and defects during late stages of oogenesis prevent *U7* mutant females from laying eggs (Godfrey et al., 2006). Since the hypomorphic *Lsm10*<sup>f06616</sup> and *Lsm10*<sup>D46N</sup> mutant alleles support development to adulthood when in trans to a deficiency, we assessed whether the adults were fully fertile. *Lsm10*<sup>f06616/Df</sup> and *Lsm10*<sup>D46N/Df</sup> trans-heterozygous males are fertile, and the females of the same genotype are capable of laying eggs. We then self-crossed *Lsm10*<sup>f06616/Df</sup> and *Lsm10*<sup>D46N/Df</sup> mutant males to the corresponding *Lsm10* mutant virgin females, and determined the fraction of eggs that would hatch into larvae as an assessment of the relative fecundity. We found that 30% of the progeny from the *Lsm10*<sup>f06616</sup> cross hatched, which was significantly reduced relative to the 85% hatching of wild type controls (p<0.00001) and significantly less than the 75% expected to hatch if *Lsm10* was not required for embryogenesis (p<0.005) (the *Df/Df* genotype is embryonic lethal and expected to be 25% of progeny) (Fig. 3.5A). Eggs from the *Lsm10*<sup>D46N/Df</sup> self-cross also had reduced hatching relative to wild type (70%, p<0.02), and this was slightly less than the 75% expected (p<0.05). DAPI staining of mutant embryos revealed that these decreases in hatching were not due to a failure of fertilization (not shown). These results are consistent with our Western data, which indicate that the *Lsm10*<sup>f06616</sup> mutant has a much larger reduction in *Lsm10* protein levels compared to the *Lsm10*<sup>D46N</sup> mutant (Fig. 3.1C, D). We also found that placing the *Lsm10*<sup>f06616</sup> hypomorphic allele in trans to our strongest *Lsm10* mutant allele, *Lsm10*<sup>G40E</sup>, results in viable adults, and that eggs from *Lsm10*<sup>f06616/G40E</sup> males and females

hatched less than wild type controls (75% hatching;  $p=0.048$ ). Because this hatching rate is significantly greater than that obtained from *Lsm10*<sup>f06616/Df</sup> males and females ( $p<0.00005$ ), we conclude that the *Lsm10*<sup>G40E</sup> mutation is not null and can be characterized as a strong hypomorph (i.e. *Lsm10*<sup>G40E</sup> is not equivalent to a *Df* in this assay). This is consistent with our detection of a small amount of Lsm10 protein in the *Lsm10*<sup>G40E</sup> mutant (Fig. 3.1C).

To determine whether these changes in fertility correlate with defects in histone pre-mRNA processing, we extracted total RNA from adult females of each mutant genotype and hybridized it with a histone H3 mRNA probe. The mutants of genotype *Lsm10*<sup>f06616/Df</sup> and *Lsm10*<sup>f06616/G40E</sup> contain the longer, misprocessed histone H3 mRNA that we detect in *U7* null mutants (Fig. 3.5B, lanes 1 and 2 compared to lane 5). These mutants also contain correctly processed H3 mRNA, consistent with a hypomorphic condition that is not completely defective in histone pre-mRNA processing. Interestingly, we detected only normal H3 mRNA and no misprocessed H3 mRNA in the *Lsm10*<sup>D46N/Df</sup> allele combination (Fig. 3.5B, lanes 3 and 4 compared to lane 6). This indicates that Asn substitution of the highly conserved Asp46 does not affect *U7* snRNP function. This further suggests that, any fertility defects in this mutant are likely not caused by aberrant histone pre-mRNA processing.



**FIGURE 3.5. Hypomorphic *Lsm10* alleles are viable, with some fertility defects.** (A) The average and standard deviation of the percent of hatched embryos for the indicated genotypes. Measurements were made on 6 collections of 100 eggs. (B) RNA isolated from 1-2 day old adult females of the indicated genotypes was subjected to Northern analysis with a  $^{32}$ P-labeled H3 probe. Note misprocessed H3 mRNA is detected in only two of the *Lsm10* mutant genotypes, and that these hypomorphic mutants also contain wild-type, processed mRNA.

### **U7 snRNA is present in a snRNP particle in *Lsm11* mutants**

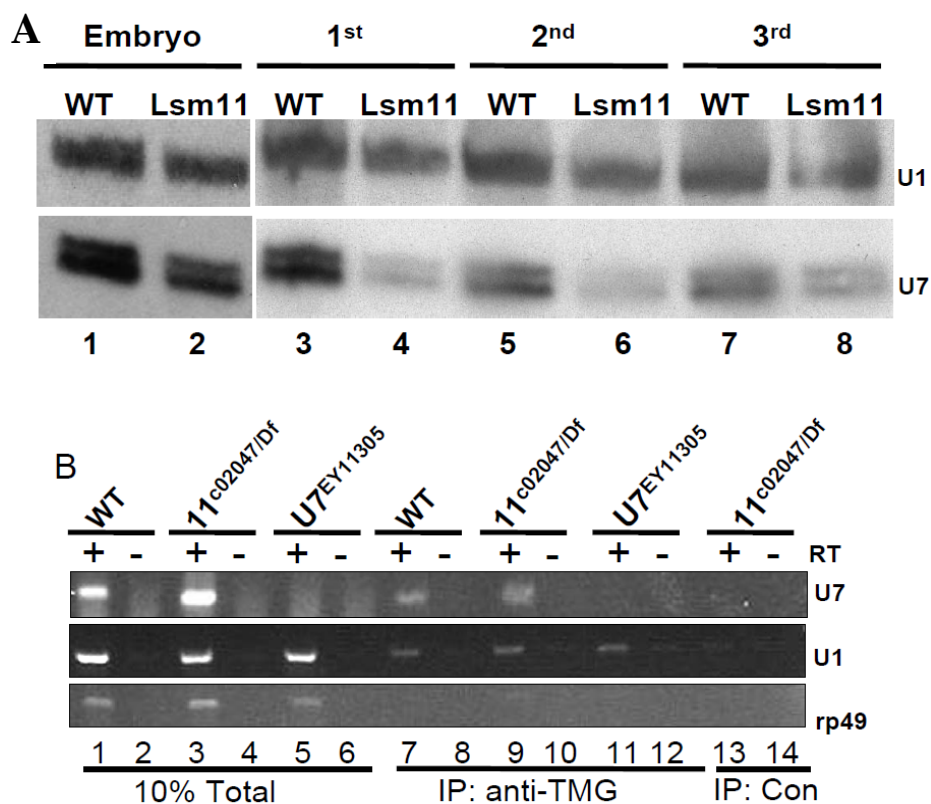
Previous studies suggest that U snRNAs, including U7, that cannot bind Sm proteins are unstable and do not accumulate in the cell (Jones & Guthrie, 1990; Grimm et al., 1993). We therefore hypothesized that a U7 snRNP would not form in the absence of Lsm10 and Lsm11,

and that we consequently would not detect any U7 snRNA in an *Lsm11* mutant. To test this we used Northern blotting to measure the accumulation of U7 snRNA in *Lsm11*<sup>c02047</sup> mutants compared to wild type throughout development. Total RNA was extracted from embryos as well as 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> instar larvae and hybridized with a U7 probe. We found that in *Lsm11*<sup>c02047</sup> mutants the U7 snRNA levels begin to drop compared to wild type at the first instar stage (Fig. 3.6A, compare lanes 3 and 4). Surprisingly, we repeatedly detected U7 snRNA in *Lsm11*<sup>c02047</sup> mutant 3<sup>rd</sup> instar larvae (Fig. 3.6A, lane 8), a stage at which all histone mRNA is misprocessed in this mutant (Fig. 3.3B-C, lane 4). Using densitometry we determined that *Lsm11*<sup>c02047</sup> mutants have 60% the amount of U7 snRNA compared to wild-type at this developmental stage (data not shown). We considered two interpretations of this result: either the U7 snRNA is stable in the absence of a bound Sm protein ring, or the U7 snRNA assembles into a snRNP particle lacking Lsm10 and Lsm11.

To distinguish between these two possibilities, we determined whether the U7 snRNA observed in the *Lsm11* mutant contains a 5' trimethylguanosine (TMG) cap. During snRNP assembly, the canonical m<sup>7</sup>G cap of newly transcribed snRNAs is hypermethylated to a TMG cap, and this requires Sm protein binding to the snRNA (Mattaj, 1986). Thus, if the U7 snRNA present in the *Lsm11* mutant has a TMG cap, we would infer that it had assembled into a snRNP particle. To test this, we developed an assay that couples immunoprecipitation with reverse transcription (RT)-PCR. Total RNA was prepared from wild type, *Lsm11* mutant or *U7* mutant 3<sup>rd</sup> instar larvae and incubated with anti-TMG antibodies that were coupled to agarose beads. Precipitated RNA was extracted from the beads and U7 snRNA was detected by RT-PCR. We found that anti-TMG antibodies could precipitate U7 snRNA from wild-type control samples, but not from *U7* mutant samples which lack U7 snRNA (Fig.

3.6B, top panel, lanes 7 and 11). The anti-TMG antibodies precipitated U1 snRNA from all samples (Fig. 3.6B, middle panel), and did not precipitate rp49 mRNA, which contains a m<sup>7</sup>G cap rather than a TMG cap, indicating that the antibody was specific (Fig. 3.6B bottom panel). No U snRNA was precipitated when a non-specific antibody was used (Fig. 3.6B, lanes 13 and 14). Thus, our assay specifically detects U7 snRNA. In *Lsm11* mutant samples we reproducibly detected U7 snRNA with the anti-TMG antibodies (Fig. 3.6B, lane 9). These data indicate that even in the absence of Lsm10 and Lsm11, U7 snRNA can assemble into a snRNP particle.

We considered the possibility that this aberrant U7 snRNP particle, which is not functional in histone pre-mRNA processing, could be toxic and thus account for the lethality of *Lsm10* and *Lsm11* mutants. This hypothesis predicts that removing U7 snRNA from an *Lsm11* mutant will suppress lethality. To test this we constructed a *U7<sup>20</sup> Lsm11<sup>c02047</sup>* double mutant strain. We found that this double mutant was still lethal, indicating that eliminating the U7 snRNP formed in an *Lsm11* mutant could not rescue the lethal phenotype, and that the non-functional U7 snRNP in *Lsm11* mutants was not the basis of *Lsm11* mutant lethality.



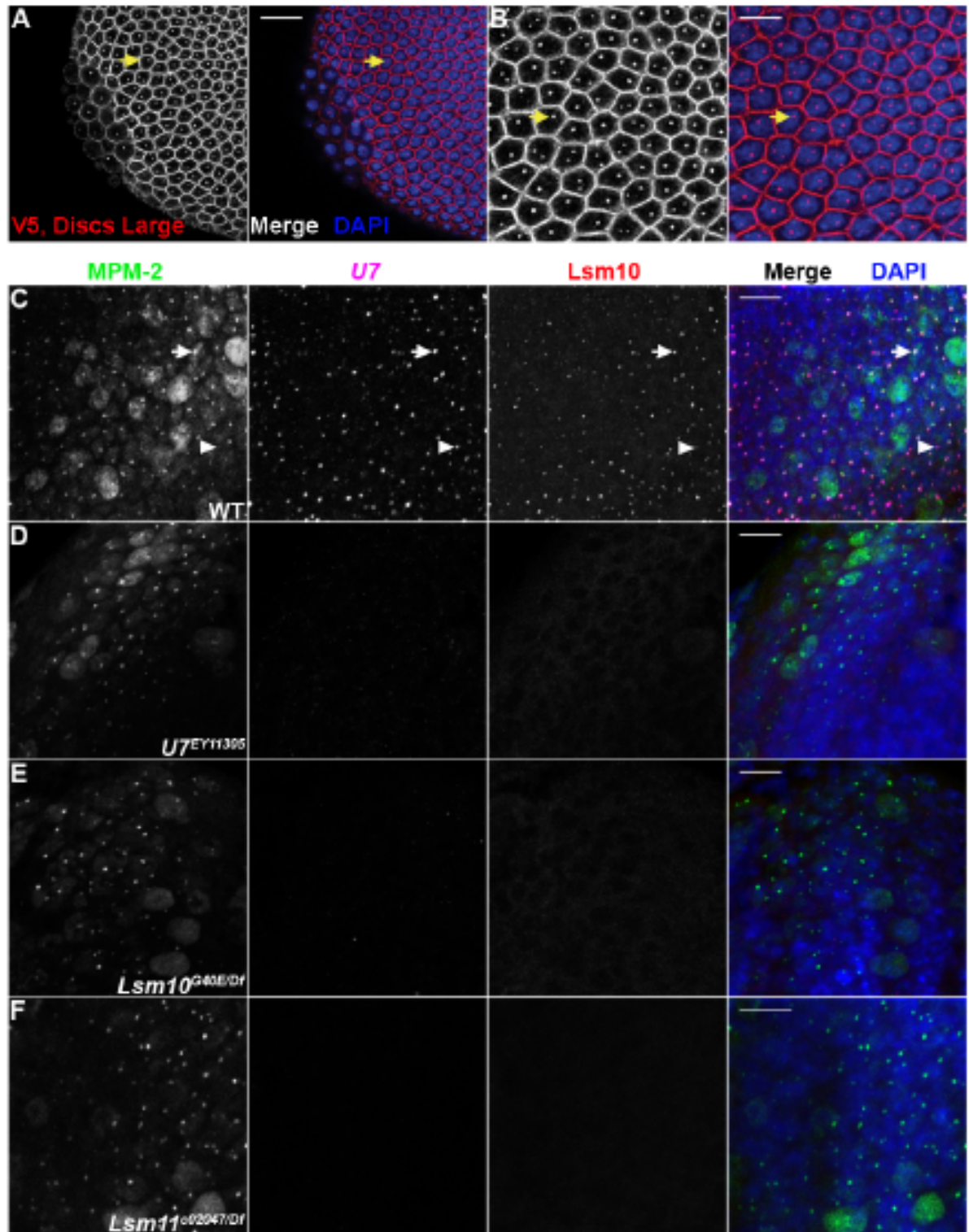
**FIGURE 3.6. U7 snRNA can form a snRNP particle in *Lsm11* mutants.** (A) U7 Northern analysis of RNA isolated from *Lsm11*<sup>c02047/Df</sup> mutant (Lsm11) or *w*<sup>1118</sup> control (WT) embryos and 1<sup>st</sup>-3<sup>rd</sup> instar larvae. Note that in the *Lsm11* mutant U7 snRNA is detected in 3<sup>rd</sup> instar larvae when all the histone mRNA is misprocessed. A U1 probe was used as a loading control. U7 snRNA migrates as a doublet as described previously (Dominski et al., 2003). (B) Reverse transcriptase (RT)-PCR analysis of RNA extracted from anti-TMG immunoprecipitates of whole third instar larvae RNA samples of the indicated genotypes. Lanes 1-6: 10% of total input RNA. Lanes 7-12: anti-TMG IP. Lanes 13 and 14: mock IP negative control. Upper panel: U7 snRNA primer pair. Note that there is no U7 present in the *U7*<sup>EY11305</sup> mutant or in the control IP lane, but U7 is detected in both WT and *Lsm11*<sup>c02047/Df</sup> TMG IP samples. Middle panel: U1 snRNA primer pair. Note that U1 is present in all three TMG IP samples, but not in the IP control. Bottom panel: rp49 (ribosomal protein 49) primer pair. Note that rp49 mRNA is not precipitated by anti-TMG antibodies because the mRNA lacks a trimethylguanosine cap.

### **The U7 snRNP formed in an *Lsm11* mutant does not localize to the histone locus body**

In mammalian cells, U7 snRNP localizes to Cajal bodies (CBs), which are nuclear structures involved in the assembly and modification of the machinery needed for pre-mRNA splicing, pre-ribosomal RNA processing, and histone pre-mRNA processing (for reviews see (Gall, 2000; Carmo-Fonseca, 2002; Gall, 2003; Matera, 2003; Cioce & Lamond, 2005). Some of these Cajal bodies are associated with histone genes, and may represent sites of histone mRNA biosynthesis. *Drosophila* cells also contain a Cajal body, but the Cajal body lacks U7 snRNP (Liu et al., 2006). *Drosophila* cells have a distinct body termed the histone locus body (HLB) which is invariably associated with the histone gene locus and where the U7 snRNP localizes (Fig. 3.7A-C) (Liu et al., 2006; White et al., 2007). HLBs likely contain all of the factors necessary for histone mRNA transcription and pre-mRNA processing (Marzluff et al., 2008). Our observation that a non-functional U7 snRNP particle forms in *Lsm11* mutants provided an opportunity to determine if U7 snRNA localization to the HLB depends on Lsm11 and Lsm10. We used fluorescent in situ hybridization (FISH) to detect U7 snRNA, and MPM-2 antibodies to detect HLBs independently from U7 snRNP. The MPM-2 monoclonal antibody recognizes a cell cycle-regulated, Cyclin E/Cdk2-dependent phospho-epitope that localizes to HLBs during S phase (White et al., 2007). We stained 3<sup>rd</sup> instar larvae brains, because HLBs are easy to identify in this tissue and the *Lsm11* mutant phenotype is fully expressed at this developmental stage. In wild-type cells, HLBs were clearly detected by the co-localization of MPM-2 staining, anti-Lsm10 staining, and U7 snRNA FISH (Fig. 3.7C). The lack of U7 FISH signal in MPM-2 positive HLBs in *U7* null mutant cells confirmed the specificity of our U7 probe (Fig 3.7D). As we previously observed for Lsm11 (White et al., 2007), we could not detect Lsm10 signal in the HLBs of



*U7* null mutants, indicating that neither *Lsm10* nor *Lsm11* accumulate in HLBs in the absence of *U7* snRNA (Fig. 3.7D). In *Lsm10* and *Lsm11* mutants we failed to detect *U7* snRNA in the HLBs, and with respect to FISH signal for the *U7* snRNA these preparations were indistinguishable from the *U7* null mutant (Fig. 3.7E, F). This indicates that in the *Lsm10* and *Lsm11* mutants, the aberrant *U7* snRNP does not localize to the HLB. We know that in these mutants other proteins can correctly localize to the HLB, because MPM-2 staining is similar to that in wild-type controls (Fig. 3.7E,F). Finally, we did not detect *Lsm10* protein in HLBs of *Lsm11* mutants, consistent with our Western blot results showing a lack of *Lsm10* protein accumulation in *Lsm11* null mutants (Fig. 3.7F). Together these results indicate that *Lsm10* and *Lsm11* are required for *U7* snRNA localization to the HLB.



**FIGURE 3.7. The U7 snRNP formed in an *Lsm11* mutant does not localize to the histone locus body.** (A) Stage 5 *Lsm11*<sup>c02047</sup>, P[V5-*Lsm11*<sup>+</sup>] homozygous embryos were stained with anti-Discs Large antibodies, to visualize cell boundaries, and anti-V5 antibodies (left panels, both red in merge). Anti-mouse secondary antibodies were used to simultaneously detect V5-*Lsm11* and Discs Large. Embryos were also stained with DAPI (blue in merge). Note that V5-*Lsm11* localizes to one or two nuclear foci just like endogenous *Lsm11*. Arrows indicate the same cell in A (20 μm scale bar) and B (10 μm scale bar). (C-F) Brains dissected from *w*<sup>1118</sup>, *U7*<sup>EY11305</sup>, *Lsm10*<sup>G40E/Df</sup>, and *Lsm11*<sup>c02047/Df</sup> third instar larvae were stained with MPM-2 (first column; green in merge), hybridized with a fluorescent probe recognizing U7 snRNA (second column; magenta in merge), anti-*Lsm10* antibodies (third column; red in merge), and DAPI (blue in merge). Arrows indicate a histone locus body that contains MPM-2 antigen(s), U7, and *Lsm10*. Arrowheads indicate a histone locus body lacking MPM-2 staining. This nucleus is likely not actively dividing, and therefore lacks the Cyclin E/Cdk2 activity necessary to produce the MPM-2 epitope. Note that both U7 and *Lsm10* are undetectable in histone locus bodies marked by MPM-2 staining in both *Lsm10* (E) and *Lsm11* (F) mutant brains. Bar, 10 μm.

## DISCUSSION

Recent studies of snRNAs and their associated Sm proteins have revealed new snRNP particles and novel functions for existing particles (Beggs, 2005). Here we report the first genetic analysis of *Lsm10* and *Lsm11*, which are both components of the Sm protein ring of the U7 snRNP particle. Our data indicate that, like U7 snRNA, *Lsm10* and *Lsm11* are essential for histone pre-mRNA processing *in vivo*. Surprisingly, our data also suggest that these *Lsm* proteins play an essential role in development that is likely independent of U7 snRNA and histone mRNA metabolism.

## Genetic evidence for a novel function for Lsm10 and Lsm11

As predicted from previous studies of U7 snRNP (Pillai et al., 2001; Azzouz & Schumperli, 2003; Pillai et al., 2003; Azzouz et al., 2005; Wagner et al., 2006), including our phenotypic analysis of U7 snRNA mutations in *Drosophila* (Godfrey et al., 2006), we find that *Drosophila* Lsm10 and Lsm11 are both essential for normal histone mRNA biosynthesis during development. The longer, aberrant histone mRNAs produced in *Lsm10* and *Lsm11* mutants are identical to those we previously described in mutants of U7 snRNA and other components of the histone pre-mRNA processing machinery, and arise from the use of cryptic downstream polyadenylation signals located within each histone gene (Sullivan et al., 2001; Lanzotti et al., 2002; Godfrey et al., 2006; Wagner et al., 2007). Thus, as expected, loss of Lsm10 and Lsm11 results in the same molecular phenotype as loss of U7 snRNA. However, unlike viable U7 snRNA mutants, both *Lsm10* and *Lsm11* mutants die as non-pharate pupae. This is reminiscent of *Slbp* mutants, which also die as larvae or pupae (Lanzotti et al., 2002). We attribute the lethality of *Slbp* mutants to an earlier onset of histone pre-mRNA misprocessing during development (i.e. embryonic stage in *Slbp* mutants versus 3<sup>rd</sup> instar larval stage in U7 mutants) resulting from the lack of maternal SLBP and a large store of maternal U7 snRNA. However, this model cannot explain the lethality of *Lsm11* and *Lsm10* mutants, since the timing of onset of histone misprocessing during development in *Lsm11* and U7 mutants is identical for each of the five replication-dependent histone mRNAs and there is no difference in the amount of histone mRNA present between the two mutants.

What might cause the *Lsm10* and *Lsm11* mutants to die? Surprisingly, there is still U7 snRNA present in *Lsm10* and *Lsm11* mutant third instar larvae. One possibility is that an aberrant U7 snRNP particle assembles in the absence of Lsm10 and Lsm11, perhaps with SmD1 and SmD2 replacing Lsm10 and Lsm11 in the Sm protein ring that binds U7 snRNA. This aberrant U7 snRNP particle may be detrimental and result in a dominant negative effect on some essential process. However, this model is not supported by our observation that *Lsm11 U7* double mutants are not viable, because it predicts that removing U7 snRNA should suppress the lethality of Lsm11 mutants. Therefore, we interpret our data as an indication that Lsm10 and Lsm11 are involved in a U7-independent function that is required for viability.

Since Lsm10 and Lsm11 are binding partners in the U7 Sm ring, it is possible that they could both be part of another Sm or Lsm ring. Lsm proteins participate in many aspects of RNA metabolism, and the full repertoire of Lsm complexes that exists *in vivo* is not known (Beggs, 2005). The best understood Lsm complexes are the heptameric Lsm2-8 complex, which binds U6 and functions in the nucleus during pre-mRNA splicing, and the heptameric Lsm1-7 complex, which functions in cytoplasmic mRNA decay (Beggs, 2005). There is evidence for other novel Lsm complexes whose composition remains incompletely understood. For instance, in *Saccharomyces cerevisiae* a complex containing Lsm2-7 that resides in nucleoli associates with the small nucleolar RNA (snoRNA) snR5, which is a member of the box H/ACA class of snoRNAs that function in pseudouridylation of rRNA (Fernandez et al., 2004). This Lsm complex is likely distinct from the Lsm2-8 complex (Fernandez et al., 2004). In *Xenopus* a complex containing Lsm2, -3, -4, -6, -7, and -8 associates with the U8 snoRNA, which is a member of the box C/D class of snoRNAs

(Tomasevic & Peculis, 2002). It is unclear whether this is the same Lsm2-8 complex that binds the U6 snRNA or whether an unidentified Lsm protein binds only to U8 (Tomasevic & Peculis, 2002). In neither the yeast nor the frog complex have all the Lsm components been identified. Thus, in principle, Lsm10 and Lsm11 could be part of an uncharacterized Lsm ring that can transiently bind to any of the many snRNAs or snoRNAs.

There is recent evidence that Lsm10 and Lsm11 could function in aspects of RNA metabolism other than histone pre-mRNA processing. Park et al. (2004) reported that RNAi-mediated depletion of Lsm11 in *Drosophila* S2 cells causes a shift from one alternatively spliced variant of the *Drosophila paralytic* gene to another (Park et al., 2004). *paralytic* encodes a neuronal sodium channel that is essential for development (Loughney et al., 1989). Therefore, in *Lsm11* mutants a shift in *paralytic* splice variants may disrupt expression of the sodium channel in a way that causes lethality. However, we were unable to detect any significant difference in accumulation of paralytic splice variants between wild-type and *Lsm11* mutants (data not shown). Nevertheless, there are many alternatively spliced, essential genes in *Drosophila*. Disruption of normal expression of even a single one of these by loss of Lsm10 or Lsm11 may be sufficient to cause the lethality of *Lsm10* and *Lsm11* mutants. Our future work will involve testing whether we can identify a novel function for Lsm10 and Lsm11.

### **U7 snRNA assembles into a snRNP without Lsm10 and Lsm11**

During the biogenesis of snRNP particles, newly transcribed snRNA is exported to the cytoplasm where it is bound by the Survival Motor Neuron (SMN) Complex, which then assembles the Sm ring onto the snRNA (Matera et al., 2007). Based on previous work

indicating that snRNAs which are incapable of binding Sm proteins do not accumulate (Jones & Guthrie, 1990; Grimm et al., 1993), we did not expect to detect U7 snRNA in *Lsm11* mutants (which also lack Lsm10). Instead, we readily detected trimethylguanosine cap-modified U7 snRNA in *Lsm11* mutant larvae when histone pre-mRNA processing was completely defective. Because the 5' cap of snRNA is hyper-methylated only after assembly into a snRNP particle (Mattaj, 1986), we infer that the U7 snRNA present in an *Lsm11* mutant is part of an intact snRNP. The composition of such an aberrant U7 snRNP is not known. However, we speculate that SmD1 and SmD2 inappropriately bind U7 snRNA in the absence of Lsm10 and Lsm11. There is some precedence for this possibility. Changing the U7 Sm binding site to the canonical site found in spliceosomal snRNPs results in U7 snRNP particles that can not function in histone 3' end processing and that contain SmD1 and SmD2 instead of Lsm10 and Lsm11 (Stefanovic et al., 1995; Pillai et al., 2003). In the protozoan parasite *Trypanosoma brucei* two novel Sm proteins, Sm15K and Sm16.5K, replace SmB and SmD3 in the U2 snRNP. The Sm site in *Trypanosoma* U2 snRNA differs by one base pair from the consensus Sm site in other *Trypanosoma* U snRNAs, and a single base pair change in the U2 Sm site can convert the special Sm ring containing Sm15K and Sm16.5K to the canonical one containing SmB and SmD3 (Wang et al., 2006). Since the SmD1/SmD2 heterodimer is very similar in structure to the Lsm10/Lsm11 heterodimer, perhaps, in the absence of competing Lsm10 and Lsm11 protein, SmD1 and SmD2 bind to the non-consensus Sm site in wild type U7 snRNA. Further studies are needed to test this hypothesis directly. An alternative possibility is that in the absence of Lsm10 and Lsm11 an intermediate in the U7 snRNP assembly pathway accumulates sufficiently to be detected.

The aberrant U7 snRNP we detect in *Lsm11* mutants is non-functional in histone pre-mRNA processing, presumably because it lacks both Lsm10 and Lsm11 proteins, which contribute to U7 snRNP function in at least two ways. First, we cannot detect U7 snRNA, and by inference the aberrant U7 snRNP particle, at the histone locus in *Lsm11* or *Lsm10* mutants. Thus, Lsm10 and/or Lsm11 are required for the proper localization of U7 snRNP to the sites of histone mRNA biosynthesis. Second, Lsm11 plays a direct role in histone pre-mRNA processing by interacting with other components of the processing machinery. For instance, in human cells Lsm11 interacts with ZFP100, a zinc finger protein that helps coordinate the processing machinery on nascent histone mRNA (Dominski et al., 2002; Pillai et al., 2003; Wagner et al., 2006). This interaction requires a unique NH<sub>2</sub>-terminal domain of Lsm11 that is not found in other members of the Sm/Lsm protein family. Consequently, even if the aberrant U7 snRNP localized correctly to the HLB, histone pre-mRNA processing would likely remain defective.

In summary, our genetic analysis of Lsm10 and Lsm11 provide the groundwork for exploring novel roles for these two proteins in both histone pre-mRNA processing and other aspects of RNA metabolism.

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## CHAPTER IV

### STRUCTURE FUNCTION ANALYSIS OF LSM11

#### **Preface**

This chapter presents work I did to try to create a separation of function allele of *Drosophila* Lsm11 by making and analyzing N-terminal deletions of the protein. This work did not lead to a publication, but did create reagents that will be used by other members of the lab. Robert J. Duronio, my thesis advisor, and I conceived this project and I did most of the experimental work described in this chapter. Jeff Simms, a former research technician in the lab, taught me how to inject cloned constructs to make transgenic fly lines. Anne White, a graduate student in the lab, did the confocal imaging of my deletion lines looking for V5 expression in third instar larval brains (data not shown).

## **ABSTRACT**

Both Lsm10 and Lsm11 are only known to bind to the U7 snRNA and there is no evidence that the U7 snRNP is involved in any process other than histone pre-mRNA processing. In spite of this, *Lsm11* null mutants do not survive to adulthood while *U7* null mutants do. Both mutations however, result in the production of Poly A<sup>+</sup> histone mRNA. Lsm11 has a long N-terminal domain that is not present in other Sm or Lsm proteins and contains regions of high conservation from vertebrates to invertebrates. I have created N-terminal deletions in this region to try and separate Lsm11's two functions: histone processing and viability. I have created transgenic flies with these deletions in order to ask if I could rescue the lethality of Lsm11 mutants or the misprocessed histone mRNA phenotype of Lsm11 mutants. I was able to rescue both the lethality and misprocessed mRNA phenotype with a wild-type copy of Lsm11, but was unable to rescue either the lethality or the misprocessed mRNA phenotype in my three deletion lines. I have found that this is due to the inability of these lines to accumulate truncated versions of Lsm11 protein to any measurable level in the fly.

## **INTRODUCTION**

The U7 snRNP is required for histone pre-mRNA processing and is related to the spliceosomal snRNPs, which cleave out the introns of mRNAs. The U7 snRNP has been shown to contain two unique proteins, Lsm10 and Lsm11, named for being Like-Sm proteins, which contain the two Sm binding domains and are therefore similar in structure to Sm proteins. These two proteins replace the Sm proteins D1 and D2, respectively, in the 7 member Sm protein ring which binds to the U7 snRNA. Both Lsm10 and Lsm11 are only

known to be part of the U7 snRNP and are not known to bind to any other snRNA or other Lsm proteins.

Lsm10 is very closely related to Sm D1 while Lsm11 is much more divergent from Sm D2 (Pillai et al., 2003) (Pillai et al., 2001). Lsm11 also contains a unique structure that is not present in any other Sm or Lsm protein. The two Sm motifs of Lsm11 are separated by a long linker sequence, 138 amino acids in human and 49 in *Drosophila*. The Lsm11 protein also contains a long N-terminal extension that is not seen in any other Sm motif bearing protein, and Lsm11 does not contain a C-terminal tail after Sm motif 2 (Pillai et al., 2001; Pillai et al., 2003). It is known in mammalian cells that the unusually long N-terminus of Lsm11 is directly required for histone pre-mRNA processing and when the N-terminus is deleted, cleavage of a synthetic histone pre-mRNA injected into *Xenopus* oocytes is abolished completely (Pillai et al., 2003). A likely candidate for binding to Lsm11's N-terminus in histone pre-mRNA processing in mammals was ZFP100, a novel 100-kDa Zn finger protein which bound to the SLBP/SL complex. Antibodies to ZFP100 were found to precipitate U7 snRNA from nuclear extracts (Dominski et al., 2002). It was later shown that ZFP100 binds directly to Lsm11 (Azzouz et al., 2005) (Pillai et al., 2003) and it is believed that this binding helps to stabilize the complex on the histone pre-mRNA and recruit the cleavage endonuclease, CPSF73 (Dominski et al., 2005).

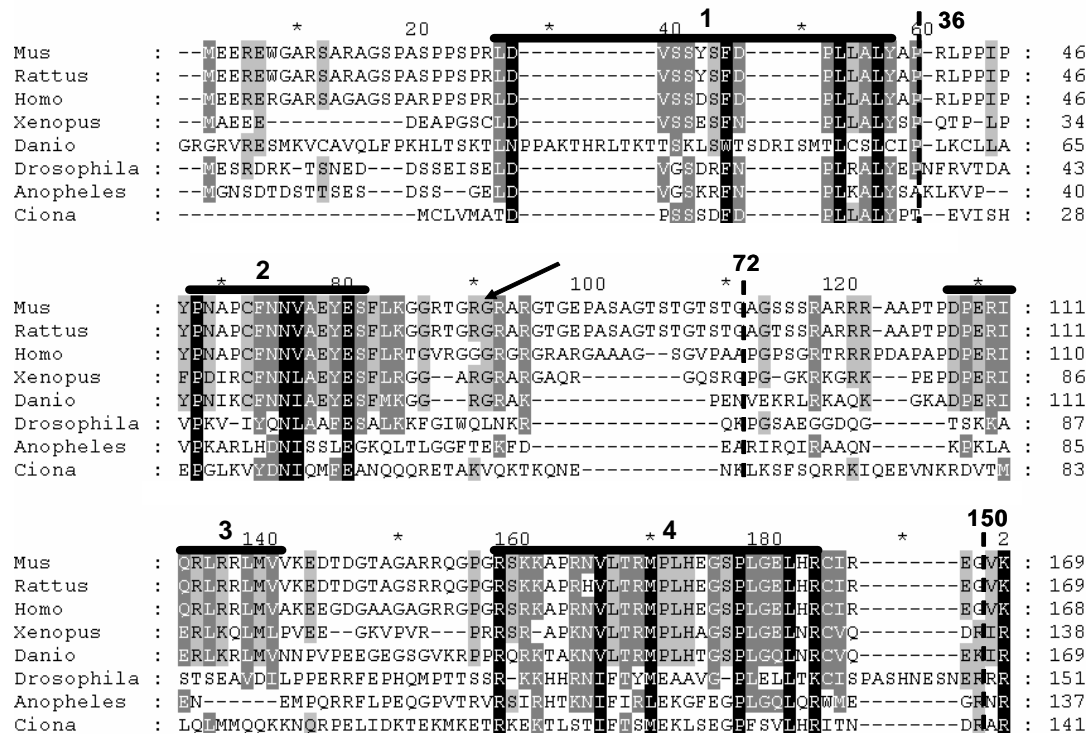
Lsm11's N-terminal domain contains four regions of amino acids which are conserved from vertebrates to invertebrates (Azzouz & Schumperli, 2003). Figure 4.1 shows an alignment of Lsm11's N-terminal region. The black lines numbered 1-4 show the four regions of conserved amino acids. The specific function of these regions is not known although the binding of human Lsm11 to ZFP 100 occurs via a specific region which is not



conserved in invertebrates (Wagner et al., 2006) (Fig 4.1 black arrow). It has been shown that some amino acids of these conserved regions are necessary for Lsm11's function in histone pre-mRNA processing, since these point mutations impair processing but not Lsm11's binding to ZFP100 (Azzouz et al., 2005), but exactly how these regions mediate the function of Lsm11 is currently unknown. It is very interesting to note that the long linker sequence in Lsm11 does not contain any regions with such high conservation from vertebrates to invertebrates, but instead is only highly conserved in vertebrates (Azzouz et al., 2005).

Our results indicate that the *Lsm11* null mutant flies do not survive to adulthood, whereas homozygous *U7* mutants are viable, but male and female sterile (chapter III). However, the developmental onset of misprocessed histone mRNA is the same for both mutants as measured by northern blots of all five cell cycle regulated histone genes (Fig 3.2, 3.3, 3.4 and data not shown). This suggests that Lsm11 is participating in a process outside of histone pre-mRNA processing, and this process is essential for *Drosophila* viability. If this is the case, then we might be able to separate those two distinct functions of Lsm11. We hypothesize that one or more of these regions might be necessary for Lsm11's involvement in histone pre-mRNA processing while another region might be involved in a different, essential process. To test this, I have made three V5 N-terminal tagged deletion constructs as well as a wild-type V5 N-terminal tagged construct of Lsm11 protein and made transgenic fly lines carrying these constructs. I then tested each transgene to see if it could rescue the misprocessed histone mRNA phenotype and the lethality caused by the loss of Lsm11. I found that the wild-type copy of Lsm11 could rescue both phenotypes, but the other three deletions lines could not rescue either phenotype. This is due to the fact that the three N-

terminal deletion lines were not stably expressed in both the fly and in S2 cells and therefore they were not able to rescue either phenotype caused by loss of *Lsm11*.



**Figure 4.1. *Lsm11* N-terminal alignment.** The species used are named on the left. The numbered black lines show the conserved regions named region 1-4 respectively. The black arrow points to the conserved region in mammals that lies within the ZFP 100 binding site (Wagner 2007). Note this area is also part of region 2. The dashed vertical lines are the amino acids numbered above in *Drosophila melanogaster*. These amino acids show the boundaries that outline the three deletion constructs described in figure 4.2.

## Material and Methods

### Western Blots and Immunoprecipitations

Protein lysates were prepared in NET buffer (.05 M Tris pH 7.5, .4 M NaCl, .005 M EDTA, and 1% NP40) with 100mM PMSF, 1mg/ml Leupeptin, and .5mg/ml Pepstatin at 1-100 and 1-1,000 final concentrations respectively. Homozygous mutant larvae were

identified as the GFP negative class from heterozygous parents containing *CYO P[twi-GFP]* balancer chromosomes. Whole third instar homozygous mutant larvae were collected and homogenized using a tissue homogenizer or transiently transfected S2 cells were collected and lysed in NET buffer. The lysates were cleared by centrifugation at 10,000g for 10 min at 4°C. For the V5 IP the lysates were incubated with 1° antibody, mouse anti-V5 (1:500 from Invitrogen), at 4°C for 2 hours on a nutator. 40µL of washed and resuspended protein G beads were then added and samples were incubated at 4°C on a nutator overnight. The samples were then spun at 10,000 rpm for 10 sec and allowed to sit on ice for 5 min. The supernatant was then removed and the beads were washed 3X with 1mL of NET buffer. 40µL of SDS loading buffer was then added and samples were boiled for 5 min. and then loaded onto a 15% Tris-HCl gel (BIO-RAD Ready Gels). Samples were run in 1X TRIS GLYCINE and transferred to a .45 µm Pure Nitrocellulose Membrane (BIO-RAD Trans-Blot Transfer Medium) in 1X Transfer Buffer (2 M Glycine, .125 M Tris Base, and .2% Methanol). Membranes were then probed with mouse anti-V5 (1:1,000 from Invitrogen). Horseradish Peroxidase linked secondary (Amersham Biosciences) was used, (1:1,000), with ECL (Amersham Biosciences) to visualize. For straight V5 and GFP Western lysates were run on the same % gel and blotted the same as above. Rabbit anti-GFP (Abcam) was used at 1:10,000.

### **Northern Analysis**

For Northern blots, total cellular RNA was isolated using TRIzol Reagent (Gibco). For the analysis of Histone H3 mRNA, 2 µg/lane of RNA were subjected to electrophoresis in 1XTBE through a 1% agarose gel containing 0.01 M MOPS (pH 7.0) and 6.75%

formaldehyde. Separated RNAs were transferred via the wick method in 20X SSC. DNA containing the histone H3 coding region was labeled with  $\alpha$ [<sup>32</sup>P]-dCTP using a random primer labeling kit (Stratagene). Hybridizations were performed at 60° C.

Homozygous mutant larvae were identified as the GFP negative class from heterozygous parents containing *CYO* P[*twist*-GFP] balancer chromosomes. For the analysis of the WT V5 construct homozygous *11*<sup>50-3</sup> third instar larvae mutants were collected from homozygous parent since the flies are carried as a healthy stock.

### **Cell Culture and Transfection**

Schneider S2 cells were provided by Shusaku Shibutani. Cells were routinely grown at room temperature in Schneider's *Drosophila* medium (Gibco). For transfection, S2 cells diluted to  $5 \times 10^5$  cells/ml were plated in 6 well plates (1.6 ml culture per well) and grown for a day until transfection. For transient transfection with pCaSpeR 4 constructs or a GFP expressing construct (provided by Shusaku Shibutani), cells were transfected with 0.4  $\mu$ g per well of plasmid using Effectene<sup>®</sup> (Qiagen), and subjected to analyses at 2 or 3 days after transfection.

### **Transgenic Flies**

Cloning of Lsm11's entire genomic locus including its promoter and 3' end is described in chapter III. This clone in Pcasper4 was used as a template for PCR amplification of the four tagged lines. The V5 epitope was added to the 5' end of each construct by PRC primer so that it was in frame with Lsm11's ORF and ended before the first codon in Lsm11. The deletions were made in the same way except primers specific for

each deletion were generated to make the desired PCR product and then the PCR product was subcloned into the existing Lsm11 genomic construct in Pcasper4.

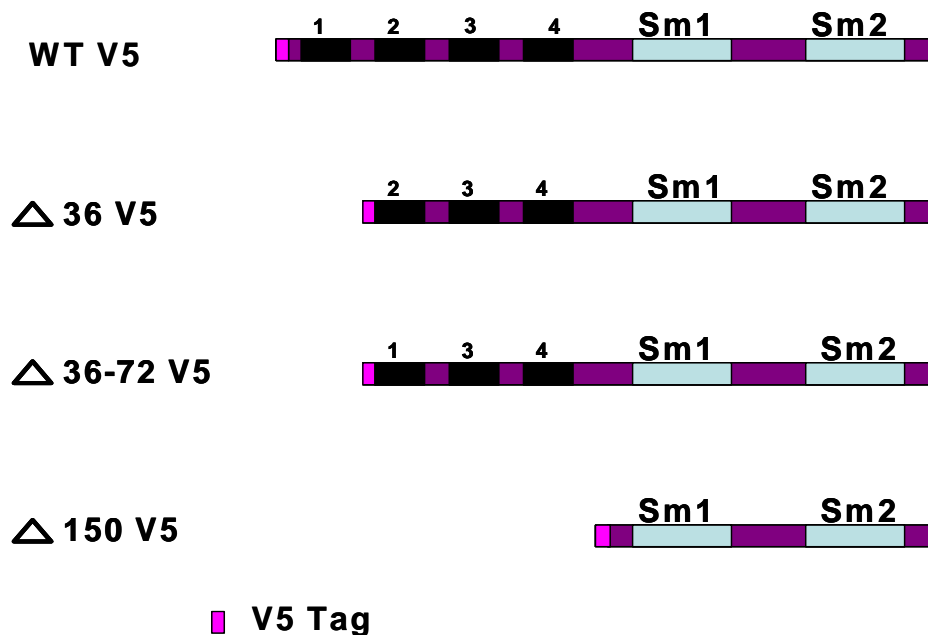
Transgenic fly lines were generated from each construct and for each construct different insertions on different chromosomes were mapped and kept as a balanced stock. Lsm11 mutant/V5 construct stocks were made from insertions on the third chromosome by crossing [Lsm11 V5]/[Lsm11 V5] to  $II^{c02047}/CYO$  P[*twist*-GFP] and from here males that were  $II^{c02047}/+; [Lsm11 V5]/+$  were crossed to  $Wg^{Sp-1}/CYO$  P[*twist*-GFP] virgins to get  $II^{c02047}/CYO$  P[*twist*-GFP]; [Lsm11 V5]/+ males and virgin females that were then crossed to each other to get a balanced stock of  $II^{c02047}/CYO$  P[*twist*-GFP]; [Lsm11 V5]/[Lsm11 V5]. The presence of both the Lsm11 P-bac and the V5 construct were checked by PCR primers specific to each. For the rescue of lethality males that were  $II^{c02047}/CYO$  P[*twist*-GFP];[Lsm11 V5]/+ were crossed to virgins that were  $Df2RMO73/CYO$  P[*twist*-GFP] and from here the progeny were checked for the presence of non-CYO wings meaning  $II^{c02047}/Df2RMO73; [Lsm11 V5]/+$  flies were able to survive to adulthood. Making of the WT V5 second chromosome recombinant was described in Chapter III.  $W^{118}$  flies were used as a positive control throughout and the  $II^{c02047/Df}$  allele was described in chapter III.

## Results

### **WT V5 tagged Lsm11 constructs rescue both histone mRNA misprocessing and lethality**

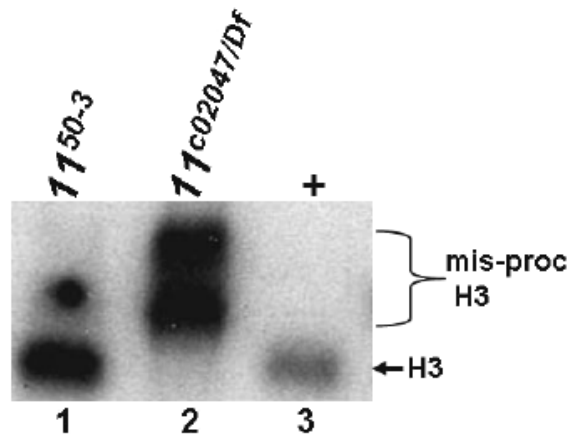
In order to try to create a separation of function mutant and to study the function of Lsm11's uniquely long N-terminus I made four constructs, three which delete different regions of Lsm11's N-terminal region and one encodes wild-type Lsm11 protein. The first was a V5 tagged wild-type copy of the Lsm11 genomic locus including its own endogenous

promoter and 3' UTR (Fig. 4.2). This serves as our positive control. The second and third were both tagged with the same V5 epitope tag as the first and included the same 5' and 3' sequences as the first, but each one deletes a different region of conserved amino acids. The first deletes the first 36 amino acids which includes region 1 of conserved amino acids (Fig. 4.2). The second deletes amino acids 36-72, which is region 2 of conserved amino acids (Fig. 4.2). We chose only these two regions to start with because region 1 has the highest amount of conservation among invertebrates of the 4 regions, and region 2 is next to the area of conserved amino acids in vertebrates that has been shown to bind ZFP 100 in vitro (Wagner 2007) (Fig. 4.1). The fourth construct deletes the first 150 amino acids which is the entire N-terminus up to the beginning of the first Sm domain (Fig 4.2) and it serves as our negative control since the N-terminus is known to be required for histone pre-mRNA processing. I generated transgenic lines of flies carrying each one of these constructs and used these flies to test for rescue of the misprocessed histone mRNA phenotype and rescue of lethality.



**Figure 4.2 V5 tagged Lsm11 constructs.** Representation of the four Lsm11 constructs made. The black boxes represent the four regions of amino acids conserved from vertebrates to invertebrates and the blue boxes represent the two Sm domains. The pink box at the beginning of each construct represents the V5 tag put on the beginning of the N-terminus right before the ATG start site. The triangle and numbered name of each construct represents the number of deleted amino acids.

I first checked the WT V5 line for rescue of the misprocessed histone mRNA phenotype. I made a second chromosome recombinant between a WT V5 construct that was inserted onto the second chromosome and the *Lsm11* null mutant, *l1<sup>c02047</sup>*, (chapter III) which was also located on the second chromosome. I then did an H3 northern blot on this recombinant line compared to my null mutant and a wild-type positive control. I was able to see complete rescue in the recombinant lane with none of the longer, misprocessed bands that are present in the null mutant lane (Fig 3.4 A, lane 1 compared to lane 3). I also crossed one of my WT V5 constructs inserted on the X-chromosome to my *Lsm11* null mutant line and did an H3 northern blot to make sure that the rescue was not specific to one transgenic insertion line. I found that again I got rescue of the misprocessed histone mRNA phenotype (Fig 4.3). This shows that my WT V5 construct is able to fully rescue the misprocessed histone mRNA phenotype caused by the loss of Lsm11. Both the second chromosome and the X-chromosome construct were also able to fully rescue the lethality as well, and are both able to be carried as a stable stock in the *Lsm11* null mutant background, meaning that they can not only rescue the lethality all the way to adulthood, but both WT V5 constructs rescue fertility enough to produce healthy progeny (data not shown).



**Figure 4.3. H3 Northern on WT V5 construct.** RNA isolated from whole third instar larvae was subjected to Northern analysis with  $^{32}\text{P}$ -labeled H3 probe. (Lanes 1 and 2) indicated genotypes.  $11^{50-3}$  is an X-chromosome line carrying a V5 N-terminal tagged wild-type copy of Lsm11 under its endogenous promoter that has been crossed to  $11^{c02047}$  flies. (Lane 3)  $W^{118}$  positive control. Homozygous  $11^{50-3}$  mutants collected from homozygous parents since the flies are viable and can be carried as a healthy stock. Homozygous  $11^{c02047/Df}$  mutants collected from  $11^{c02047}/\text{CYO P}[\text{twist-GFP}] \times \text{Df}2\text{RM}073/\text{CYO P}[\text{twist-GFP}]$  parents. Note that there is almost no detectable misprocessed H3 in lane 1. The dark spot in lane 1 is not a misprocessed band, but a smudge presumably from the hybridization process.

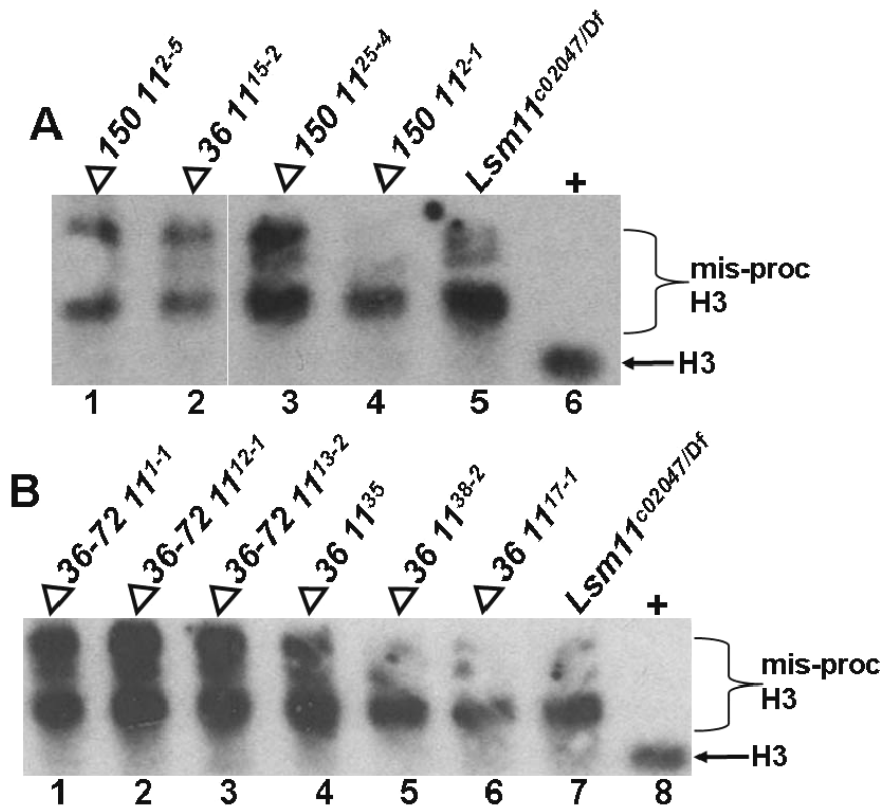
### **V5 tagged Lsm11 deletion lines do not rescue histone mRNA misprocessing or lethality**

The WT V5 construct was acting as expected for my positive control so I next tested whether the three deletion lines could also rescue the misprocessed histone mRNA phenotype. If my hypothesis is correct then I expected to see that the entire N-terminal deletion construct,  $\Delta 150$  V5, would not rescue the misprocessed histone mRNA phenotype since it is known that



the N-terminus of Lsm11 is required for this function (Pillai et al., 2003; Azzouz et al., 2005). If either region 1 or region 2 of conservation was required for histone pre-mRNA processing then I would expect the construct which deletes the necessary region to not rescue the misprocessed histone mRNA phenotype while the other construct which contains the region required for histone pre-mRNA processing should be able to rescue. I chose several different insertion lines that were mapped to the third chromosome for each of the remaining three constructs,  $\Delta 150$  V5,  $\Delta 36$  V5, and  $\Delta 36-72$  V5, and crossed them all to my *Lsm11* null mutant, *l1<sup>c02047</sup>/CYO P[*twist-GFP*]*, and made a stock for each one that was *Lsm11<sup>c02047</sup>/CYO P[*twist-GFP*];V5 construct/V5*. I then took RNA from third instar larvae that had two copies of the V5 construct and were null for the wild-type Lsm11 gene and did H3 Northern blots to look for rescue of the misprocessed histone mRNA phenotype. I found that all three of the  $\Delta 150$  insertion lines failed to rescue the misprocessed histone mRNA phenotype (Fig 4.4 A, lanes 1, 3, and 4 compared to lane 6). This was what I expected and suggests that the entire N-terminal extension of Lsm11 is necessary for histone pre-mRNA processing in *Drosophila*. The other two deletion constructs,  $\Delta 36$  and  $\Delta 36-72$ , which delete regions 1 and 2 respectively, also did not rescue the misprocessed histone mRNA phenotype. For both constructs I tested several different insertion lines, all on the third chromosome; none of them were able to produce any correctly processed histone H3 as measured by Northern blot (Fig. 4.4 A, lane 2 compared to lane 6 and B, lanes 1-6 compared to lane 8). None of the different third chromosome insertion lines for  $\Delta 36$ ,  $\Delta 36-72$ , and  $\Delta 150$  rescued the lethality of Lsm11 null mutant, *Lsm11<sup>c02047/Df</sup>*. This means that all three deletion lines did not rescue the lethality caused by loss of Lsm11. Since the  $\Delta 150$  V5 construct could not rescue the lethality seen in *Lsm11* null mutants, this supports the idea that the entire N-terminal region of Lsm11 is

necessary for its essential function as well as its function in histone pre-mRNA processing. In addition neither the  $\Delta 36$  or  $\Delta 36-72$  V5 construct was able to rescue either the lethality or the misprocessed histone mRNA phenotype seen in *Lsm11* null mutants. This could be due to two things. One is that both region 1 and region 2 are required for both functions of *Lsm11* protein, and the second is that both of these mutant proteins do not stably accumulate and are therefore unable to rescue either phenotype.



**Figure 4.4. V5 tagged Lsm11 deletion lines do not rescue histone mRNA misprocessing or lethality.**

RNA isolated from whole third instar larvae was subjected to Northern analysis with <sup>32</sup>P-labeled H3 probe. (A) (Lanes 1-5) indicated genotypes. Each number represents a different third chromosome inserted line for each V5 construct. (Lane 6) *W<sup>118</sup>* positive control. Each transgenic line was crossed to *Lsm11<sup>c02047</sup>/CYO P[*twist*-GFP]* and a stock was made carrying two copies of V5 construct. Homozygous Larvae that were *Lsm11<sup>c02047</sup>/Lsm11<sup>c02047</sup>*; V5 construct/V5 construct were selected from heterozygous *Lsm11<sup>c02047</sup>/CYO P[*twist*-GFP]*; V5 construct/V5 construct parents. Homozygous *11<sup>c02047/Df</sup>* mutants collected from *11<sup>c02047</sup>/CYO P[*twist*-GFP]* x *Df2RM073/CYO P[*twist*-GFP]* parents. Note that none of the different V5 constructs contain any correctly processed histone H3. (B) (Lanes 1-7) indicated genotypes. (Lane 8) *W<sup>118</sup>* positive control. GFP negative larvae for each construct genotype was collected the same as in A. Homozygous *11<sup>c02047/Df</sup>* mutants also collected the same as in A. Again note that none of the different V5 constructs contain any correctly processed histone H3.

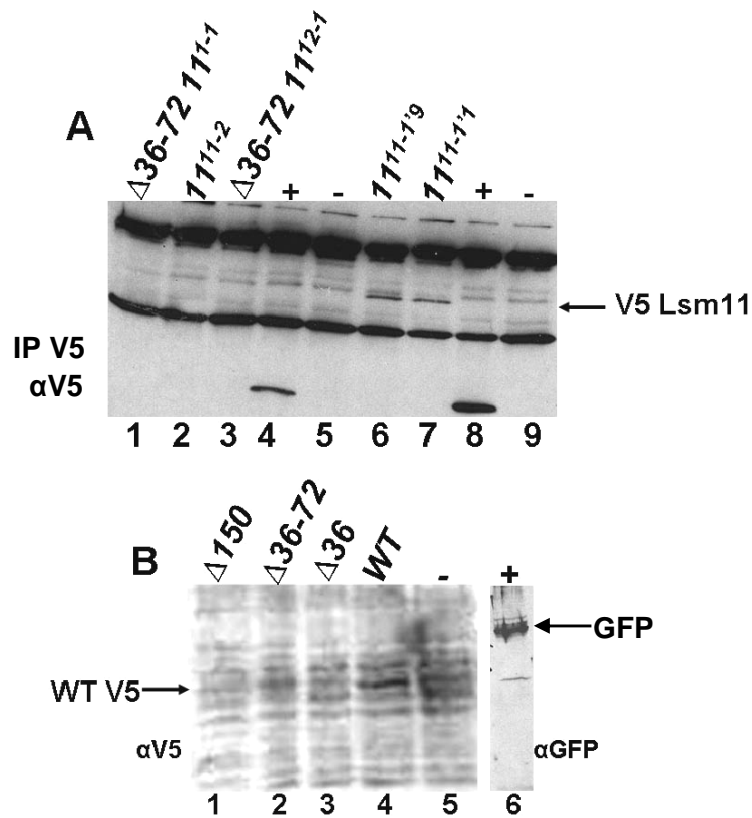
**The three V5 deletion constructs did not accumulate in either whole flies or S2 cells**

To make sure that the lack of rescue was not due to a lack of expression in the fly, I first measured the expression of the V5 tag in all of my transgenic fly lines. I did western blots using the V5 antibody to detect the N-terminal tag, in protein from embryo collections taken from my transgenic fly lines which had two copies of each construct in an otherwise wild-type background. I was unable to see any expression of the V5 tagged protein for any of my four constructs (data not shown). This included the two WT V5 lines that I rescued the misprocessed mRNA phenotype and lethality (Fig 3.4 A and Fig 4.3). I also tried re-blotting my western blots using an antibody for Lsm11 to try and see the larger running band for Lsm11 due to the V5 N-terminal tag and was again unable to see anything for all of my constructs (data not shown). This could mean that the four V5 constructs do not stably accumulate in the fly. However, at least for the WT lines I know that they must be expressed

since they can rescue both phenotypes caused by loss of Lsm11 protein. Since I was able to make a stable stock of flies that were carrying two copies of the WT V5 construct in my *Lsm11* null mutant background I took embryos from this stock and did a V5 Western blot on that sample compared to the WT V5 construct alone in an otherwise wild-type background. I was able to see expression of the V5 tag from the stable stock carrying two copies of the WT V5 construct in an *Lsm11* null mutant background compared with almost none from the construct alone in a wild-type background (Fig 3.4 B, lane 1 compared to lane 2 and 3). This suggests that the WT V5 construct only stably accumulated in an *Lsm11* null mutant background where the V5 construct is the only copy of Lsm11 protein. This could be the reason why I was unable to see any V5 expression for the deletion constructs since they were not in an *Lsm11* null mutant background. Since I had already made stocks carrying two copies of each V5 construct on the third chromosome with my Lsm11 mutant on the second chromosome for my northern blot analysis, I took protein samples from third instar larvae and used the V5 in immunoprecipitation down with and then blotted for V5 with a Western blot. Again I was able to see the WT V5 construct in my second chromosome recombinants, *11<sup>11-1'9</sup>* and *11<sup>11-1'1</sup>* (Fig. 4.5 A, lanes 6 and 7 compared to lane 9), but was unable to see any accumulation of the two  $\Delta 36-72$  V5 constructs,  $\Delta 36-72$  *11<sup>1-1</sup>* and  $\Delta 36-72$  *11<sup>12-1</sup>* compared to my non-transgenic, negative control (Fig. 4.5 A, lanes 1 and 3 compared to lane 5). I was also unable to see any accumulation of the second chromosome WT V5 line in a wild-type background (Fig. 4.5 A, lane 2). I performed the same experiment on all the rest of my third chromosome V5 deletion constructs that I had crossed into my *Lsm11* null mutant background and was unable to see any accumulation of any of my three V5 tagged deletion constructs,  $\Delta 36$ ,  $\Delta 36-72$  and  $\Delta 150$  (data not shown). I expected once I had crossed all of my

constructs into an *Lsm11* null mutant background I would be able to finally see accumulation of the V5 tagged deletion lines, but this was not the case. We also stained third instar larval brains from the three deletion lines in an *Lsm11* mutant background to visualize V5 tagged protein and were also unable to see anything above background for all three deletion lines (data not shown). This result does however explain why only the WT V5 construct was able to rescue both the misprocessed histone mRNA phenotype and lethality caused by loss of *Lsm11* and the three V5 deletion constructs were not able to rescue either phenotype.

Just to be sure that I was really unable to get stable expression of my three V5 deletion constructs I transiently transfected all four V5 constructs along with empty vector (negative control) and a GFP expressing vector (positive transfection control) into *Drosophila* S2 cells and did a Western blot to look at both V5 expression and GFP expression. I was able to see a band that ran at the correct size for the WT V5 construct in that sample that was not present in my empty vector negative control (Fig. 4.5 B, lane 4 compared to lane 5). I was unable to see any bands of the correct size for each deletion that were not also present in the negative control (Fig. 4.5 B, lanes 1, 2, and 3 compared to lane 5). So in both the whole animal and in cell culture, I was unable to see stable accumulation of the three V5 deletion constructs  $\Delta 36$ ,  $\Delta 36-72$  and  $\Delta 150$ .



**Figure 4.5. The three V5 deletion constructs did not accumulate in either whole flies or S2 cells.** (A) Protein lysates isolated from whole third instar larvae was subjected to immunoprecipitation then Western blot analysis with a V5 antibody. (Lanes 1-3 and 5-7) indicated genotypes. *11<sup>11-2</sup>* is a second chromosome insertion of WT V5 not crossed into the *11<sup>c02047</sup>* background and *11<sup>11-19</sup>* and *11<sup>11-11</sup>* are two second chromosome recombinants made by crossing *11<sup>11-1</sup>* transgenic flies to *11<sup>c02047</sup>* mutant flies. The two  $\Delta 36-72$  stocks are the same as used in figure 4.4 B. (Lanes 4 and 8) V5 positive control. (Lanes 5 and 9) Negative, non transgenic control. Homozygous *11<sup>11-19</sup>* and *11<sup>11-11</sup>* mutants collected from homozygous parents since the flies are viable and can be carried as a healthy stock. Homozygous Larvae that were *Lsm11<sup>c02047</sup>/Lsm11<sup>c02047</sup>*;  $\Delta 36-72$  V5 construct/ $\Delta 36-72$  V5 construct were selected from heterozygous *Lsm11<sup>c02047</sup>/CYO* P[*twist-GFP*];  $\Delta 36-72$  V5 construct/ $\Delta 36-72$  V5 construct parents. Note that there is only expression of the WT V5 second chromosome recombinant lines and none for *11<sup>11-2</sup>* or two  $\Delta 36-72$  stocks. (B) Protein lysates were prepared from *Drosophila* S2 cells that had been transiently transfected and subjected to Western blot analysis using indicated antibodies. (Lanes 1-4) indicated V5 construct. (Lane 5) empty vector negative control. (Lane 6) GFP expressing vector, positive transfection control. Note that only the WT V5 construct shows any expression over the empty vector background and that the GFP expressing vector shows good expression of GFP.

## Discussion

Our analysis of *Lsm11* null mutants in *Drosophila* compared to null mutants in the U7 snRNA has revealed a surprising difference in their terminal phenotype. We have found that while both mutants disrupt the normal processing of histone mRNA at the same time during development (Fig 3.3, 3.5 and data not shown), *Lsm11* null mutants do not survive to adulthood and U7 null mutants do. We hypothesize this is evidence for a novel function of Lsm11 that is independent of the U7 snRNP and is required for viability. We hypothesize that regions of high conservation in the unique N-terminal extension of Lsm11 (Fig 4.1) (Pillai et al., 2003) are required for its novel essential function and so we made V5 tagged deletion constructs to try and separate Lsm11's histone pre-mRNA processing function from its essential function. However all of the deletion constructs we made were defective in both functions of Lsm11 and we were even more surprised to find that the three deletion lines were not stably expressed in both the fly and in *Drosophila* S2 cells.

We have found that our positive control, which is a V5 N-terminally tagged copy of full length Lsm11 protein under its endogenous promoter, is able to fully rescue both the production of Poly A+ histone mRNA and the lethality caused by the loss of Lsm11 (Fig. 3.4 A, 4.3 and data not shown). This result was not surprising however we were surprised to find that the WT V5 tagged protein did not accumulate in a background that is wild-type for Lsm11 protein (Fig. 3.4 B and 4.5 A). Instead we had to put the WT V5 constructs in a background that was null for Lsm11 protein before we could see any measurable accumulation (Fig. 3.4 B and 4.5 A). We were also surprised to find that none of our three of our deletion lines,  $\Delta 36$ ,  $\Delta 36-72$  and  $\Delta 150$ , were able to rescue the misprocessed histone mRNA phenotype or the lethality caused by the loss of Lsm11 ( Fig. 4.4 and data not shown).

This is likely due to the inability of the three deletion constructs to accumulate at any measurable level, even in a background null for Lsm11 protein where we were able to see accumulation of our WT V5 construct (Fig. 4.5 A and data not shown). This was true even when we transiently transfected the three deletion constructs into S2 cells and looked for accumulation of the V5 tagged protein. We were able to see accumulation of the WT V5 construct and a positive transfection control (Fig. 4.5 B).

We did not expect to find that an N-terminally tagged wild-type copy of Lsm11 under its endogenous promoter could only accumulate to measurable levels when put into a background that is null for Lsm11. We know from previous data that an N-terminally tagged YFP-Lsm11 accumulates in the fly in an otherwise wild-type background and can be seen by staining for YFP, but this construct is not under the control of Lsm11's endogenous promoter but is driven by a constitutively active driver. This means that in this particular transgenic line the YFP-Lsm11 protein is being over expressed (Liu et al., 2006). This is a key difference between the two constructs and this could explain why we have to get rid of the endogenous Lsm11 before our WT V5 construct can stably accumulate to measurable levels. It is possible that the N-terminal V5 tag somehow makes the Lsm11 protein less stable or, reduces its affinity for binding to its partners in the Sm ring. If this is the case then the cell will preferentially use the endogenous untagged Lsm11 protein first in assembly of the U7 snRNPs, any leftover V5 tagged Lsm11 protein may then be degraded by the cell because it is not stably bound by the Sm ring or Lsm10 protein. When you remove the endogenous Lsm11 protein the cell has no choice and incorporates the tagged protein into all the U7 snRNPs. When the tagged Lsm11 protein is over expressed it is likely that it can then outcompete the endogenous protein and will get incorporated into the Sm ring of most U7



snRNPs first and that is why stable expression can be seen. As far as we know the V5 tagged Lsm11 protein functions as well as wild-type protein once it is in the U7 snRNP since both *Lsm11* null phenotypes are rescued fully by this construct.

Even in a background that is null for Lsm11 protein, all three of our deletion lines could not accumulate to high enough levels to detect by western blots or by immunostaining (Fig. 4.5 and data not shown). After transient transfection into *Drosophila* S2 cells we were only able to detect the WT V5 construct by V5 Western blot and none of our deletion constructs. This could mean that any deletion mutant of Lsm11's N-terminus in *Drosophila* is unstable and is degraded by the cell. This could be due to the inability of these deletions to stably bind to Lsm10 and the rest of the Sm ring or it could be due to their inability to fold correctly, but we favor the former idea. We hypothesize that the four regions of high conservation in Lsm11's N-terminus are necessary for its function in histone pre-mRNA processing. We also hypothesize in *Drosophila* that region 1 and region 2 as well as all four regions together are necessary for its stability and losing either region 1 and 2 separately or all four together destabilize the protein. Because we only deleted region 1 and region 2 separately we can not say if this is true as well for deleting region 3 and region 4 separately or any combination of two or three regions at the same time, but we think it is likely that if made those proteins would be unstable as well. While this work did not lead to a separation of function mutant for Lsm11, it did give us the WT V5 tagged transgenic lines which are a very powerful reagent for looking at Lsm11 protein under its endogenous promoter by use of the V5 tag. In the future those transgenic lines could be used as tools for isolation of protein or RNA complexes that are binding to Lsm11.

## **Acknowledgements**

We would like to thank Eric J. Wagner for help with the design and cloning of the N-terminal deletion constructs, and Jeff Simms for help with the injection of the cloned constructs to make the transgenic flies. We would also like to thank Anne E. White for the staining and confocal imaging of third instar larval brains from the deletion constructs and the rest of the Duronio lab for helpful discussion.

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## CHAPTER V

### DISCUSSION AND FUTURE DIRECTIONS

Histone biosynthesis is an important and necessary process in all cells. However, the study of this process and the unique machinery that is needed for it has been done mostly through the use of nuclear extract systems that support the processing of synthetic histone pre-mRNAs and by examining the processing of histone pre-mRNAs injected into *Xenopus* oocytes (reviewed in Dominski & Marzluff, 1999). In this thesis, we started with the question of what role does U7 snRNA play in histone pre-mRNA processing during *Drosophila* development. We found the U7 snRNA is required for correct processing of all five replication dependent histone mRNAs. We also found that the U7 snRNA is not required for viability in *Drosophila*, but is required for oogenesis (Chapter II). We then focused on studying the two proteins which are unique to the U7 snRNP, Lsm10 and Lsm11, and what role they play in histone pre-mRNA processing during development in *Drosophila*. During the course of our study of these two proteins we found that unlike the U7 snRNA, they are both required for viability in *Drosophila*, and this in turn led us to the idea that they may have a function that is independent of the U7 snRNA. We further showed that in the absence of both Lsm10 and Lsm11 the U7 snRNA can form a snRNP particle which is non-functional and does not localize correctly (Chapter III). In addition, we created transgenic flies carrying tagged constructs which deleted parts of Lsm11's N-terminal domain in an effort to create

separation of function mutants in this protein. This revealed that the N-terminal domain of Lsm11 is essential for protein stability. We have also created transgenic flies carrying a tagged wild-type copy of Lsm11 protein which has become a very useful research tool in my laboratory (Chapter IV).

### **The U7 snRNA is not required for viability in *Drosophila***

It has been shown through mutations in the HDE and compensatory mutations in the U7 snRNA that in mammals the U7 snRNA acts as a molecular ruler guiding the correct site of cleavage in the histone pre-mRNA (Schaufele et al., 1986; Bond et al., 1991). There is also much evidence showing that in mammalian cells the U7 snRNP is essential in histone pre-mRNA processing while SLBP is only essential in processing the pre-mRNAs that do not form stable duplexes with the U7 snRNA (Streit et al., 1993; Spycher et al., 1994). Despite everything that is known about the U7 snRNA from extracts and mammalian cells, no one had examined what will happen to the whole animal when the U7 snRNA is knocked out. We hypothesized that the U7 snRNA is also required for histone pre-mRNA processing in *Drosophila*, as it is in mammals. To test this hypothesis, we created null alleles of the U7 snRNA in *Drosophila* and analyzed the effect this mutation had on development of the whole organism (Chapter II).

Based on U7 snRNA's known role in histone pre-mRNA processing and previous data from our lab showing loss of SLBP caused lethality in *Drosophila* (Lanzotti et al., 2002), we expected to find the loss of U7 snRNA to also cause lethality. This however, is not the case as, U7 snRNA null mutants survive to adulthood (Chapter II results). How are these flies surviving all of development through to adulthood without any U7 snRNA? The answer

could simply be that during *Drosophila* development U7 snRNA is not required for proper histone pre-mRNA processing. This however, is not the answer, since we have shown that loss of U7 snRNA causes all five of the replication dependent histone mRNAs to be improperly processed (Fig. 2.3) consistent with our previous data on *Slbp* null mutants where polyadenylation of the histone pre-mRNAs occurs through the use of cryptic downstream polyadenylation signals present in the 3' region of each histone gene (Sullivan et al., 2001) (Lanzotti et al., 2002). Since we see the same molecular phenotype in our *U7* null mutants as we see in *Slbp* null mutants, what then accounts for the different terminal phenotype seen in those two mutants, i.e. death in *Slbp* mutants compared with survival to adulthood in *U7* mutants? We have shown that unlike SLBP, which has little to no maternal supply of the protein (Lanzotti et al., 2002), U7 snRNA has a large maternal store which leads to a developmental delay in the onset of the misprocessed mRNA phenotype (Fig. 2.4 B) compared to *Slbp*. This data led us to the idea that although the U7 snRNA is essential in *Drosophila* for histone pre-mRNA processing, it is not required for viability due to the overwhelming amount of maternal U7 snRNA (Fig. 2.4) which allows the animal to make it through embryogenesis, 1<sup>st</sup> and 2<sup>nd</sup> instar larval stages with little to no defect in histone pre-mRNA processing (Fig. 2.4). Based on our double mutant synthetic lethality between U7 null mutants and a hypomorphic SLBP allele (Fig 2.6) this idea seems to fit with our data very well. The best way to really test this idea would be to make U7 snRNA null mutant germ cells that do not have any maternal supply of U7 by using the FLP/FRT-mediated mitotic recombination (explained in Chapter II material and methods). We know that the U7 snRNA is necessary for oogenesis (Chapter II) and when we make *Slbp* mutant germ cells there are no eggs produced and the few clones that are made contain completely

misprocessed histone mRNA and do not make it through oogenesis (Figure 2.7 and 2.8). *U7* null mutant germ cells would probably also be unable to produce any eggs due to their inability to make it through embryogenesis, and this would preclude our ability to take away the maternal supply of U7 snRNA. However, this experiment would still be useful, since some germ line clone cells might be able to make it through embryogenesis and lay eggs devoid of any U7 snRNA. It would be interesting to then look and see if those eggs can survive through embryogenesis. Our data would predict they would not assuming there will be no U7 snRNA present and complete misprocessing of the histone mRNAs, but it would be very exciting if they did because this would point to another idea as to why our *U7* null mutants survive to adulthood while *Slbp* null mutants do not.

### **SLBP may be required for an essential function along with its function in histone pre-mRNA processing**

As discussed in Chapter II, even though all of our data strongly support the idea that the difference in terminal phenotypes between *U7* null mutants and *Slbp* null mutants is simply due to differences in maternal supply of each gene product and therefore differences in the developmental onset of the misprocessed histone mRNA phenotype, we can not rule out the idea that SLBP is necessary for another function in *Drosophila* which is essential for development, while the U7 snRNA is only required for histone pre-mRNA processing which is not essential for viability in *Drosophila*. This also would explain why *Slbp* null mutants die, but *U7* null mutants do not. We know that SLBP stays bound to the mature histone mRNA and accompanies it to the cytoplasm (Erkman et al., 2005) where it promotes the translation of histone mRNA (Sanchez & Marzluff, 2002) (Whitfield et al., 2004), and there

is recent evidence from mammalian cells that a 15 amino acid region in the amino-terminal portion of SLBP is required for translation by binding directly to SLIP1 (SLBP-interacting protein 1) a protein shown to stimulate translation of histone mRNAs in coordination with SLBP (Cakmakci et al., 2008). It would make sense then, if *Drosophila* SLBP is required for both the processing of histone mRNAs and for their efficient translation, but the U7 snRNA is only required for the correct processing of histone pre-mRNAs, that SLBP might be essential for viability while the U7 snRNA is not. Of course, more evidence is needed to show that the presumed homologue of SLIP1 in *Drosophila* also binds to SLBP and that this interaction is necessary for translation of histone mRNAs. Our lab, in collaboration with the Marzluff lab, is working on separating the two functions of SLBP and it will be very interesting to see the results.

### ***Drosophila* Lsm10 and Lsm11 may have a novel, essential function independent of histone pre-mRNA Processing**

In mammals the two U7 snRNP specific proteins, Lsm10 and Lsm11, are also necessary for histone pre-mRNA processing, and the Lsm11 N-terminal region is required for this reaction to occur (Pillai et al., 2003; Schumperli & Pillai, 2004). From this data and our previous analysis of *U7* null mutants in *Drosophila*, it was no surprise to us that both of these proteins are also required to correctly process histone pre-mRNAs in *Drosophila* (Chapter III results). What did surprise us was our finding that despite having the same degree of misprocessed histone H3 mRNA as *U7* null mutants at the third instar larval stage



of development, *Lsm10* and *Lsm11* mutants do not survive to adulthood (Fig. 3.2 A). We next tested whether this could simply be explained by a difference in the maternal supply of U7 versus Lsm10 and Lsm11, causing an earlier onset of the misprocessed mRNA phenotype. However, this is not the case since we are unable to detect any difference between *U7* and *Lsm11* mutants in the timing or degree of misprocessed histone mRNA (Fig. 3.3, 3.4, and data not shown). This data led us to the tantalizing idea that Lsm10 and Lsm11 perform a function outside of the U7 snRNP, which is essential for development in *Drosophila*. We hypothesize that Lsm10 and Lsm11 may be part of another snRNP particle or Lsm complex; this would fit well with the recent evidence showing that Lsm proteins can perform a wide range of functions in RNA processing. New complexes and new roles for these complexes are being discovered at a rapid rate (reviewed in Matera et al., 2007) and (Beggs, 2005).

We have begun to search for this novel role of Lsm10 and Lsm11. A recent report in *Drosophila* S2 cells revealed that RNAi knockdown of Lsm11 affects the ratio of alternative splice variants of the *Drosophila* paralytic gene (Park et al., 2004). Although we were unable to verify this result, we have used deep sequencing technology through the use of an Illumina sequencing facility on our campus to look for any differences between *Lsm11* mutants and Wild Type. We used RNA from whole third instar larvae from *Lsm11* null mutants versus Wild Type, and also RNA from RNAi knockdown of Lsm11 and control *Drosophila* S2 cells, and made cDNA from all the poly A+ transcripts, ensuring that we are sequencing only transcribed regions of the genome. We now have the entire transcribed genome from each sample ready to be mined for data. It will be very interesting to see what we can learn from this vast amount of data.

In an effort to further explore the hypothesis that Lsm11 may perform a novel function outside of the U7 snRNP, we tried to create a separation of function allele of Lsm11 by deleting parts of its N-terminal region and assaying each deletion's ability to rescue histone mRNA misprocessing and lethality in Lsm11 mutants (Chapter IV). While this data did not give us a clear separation of function mutant (data and conclusions discussed in Chapter IV), it did reveal an unexpected role for the N-terminal domain in Lsm11 stability. It also gave us another useful tool for helping us discover Lsm11's novel role in *Drosophila*: a transgenic line of flies where the only available copies of the Lsm11 gene are tagged with the V5 epitope which we can easily detect by immunoprecipitation, western blot, and immunofluorescence (Fig. 3.2, 3.6 and 4.5). This tagged Lsm11 functions well enough to rescue both misprocessing and lethality in *Lsm11* null mutants (Fig 3.2 and Chapter IV results). Our dream experiment would be to use this tagged Lsm11 to pull down any interacting proteins or RNAs that bind to Lsm11 independently of the U7 snRNA. Since we also have *U7* snRNA null mutants we can enrich for U7 snRNA independent proteins and RNAs. It will be interesting to see what data we can get from this and also what evidence we can gather in the future which helps support our idea that Lsm10 and Lsm11 may be part of another Lsm or snRNP complex.

### **Does Polyadenylated histone mRNA directly cause lethality in *Drosophila*?**

It was previously shown in our lab that mutation of *Drosophila Slbp* causes a unique phenotype where histone pre-mRNAs, which are not normally polyadenylated, have a poly A<sup>+</sup> tail added in lieu of not making any histone mRNA at all (Sullivan et al., 2001; Lanzotti et al., 2002). We have further shown that this interesting phenotype is not limited to *Slbp*

mutants, and that loss of any part of the processing machinery in *Drosophila* leads to polyadenylation of histone mRNAs (Chapter II and Chapter III results). This observation was used to design a screen in *Drosophila* S2 cells to search for novel factors involved in histone pre-mRNA processing (Wagner et al., 2007), but it has also left us with the question as to why this might happen in *Drosophila* when it has not been seen in other species? There is evidence that a low level of polyadenylation of histone genes occurs normally in *Drosophila*, specifically in the male testis, but the purpose of these transcripts is unclear (Akhmanova et al., 1997). This low level of polyadenylation could be the result of selection in *Drosophila* for some function, possibly specifically in the testis, but it could also be the result of a low level of inefficient processing in *Drosophila* in that specific tissue. Our data does not show a direct connection between polyadenylation of histone pre-mRNAs and lethality in *Drosophila*. Since the loss of correctly processed histone mRNA is always connected with the gain of misprocessed histone mRNA (Chapter II and Chapter III results), we can not say that one or the other is the reason for lethality in *Slbp*, *Lsm10* and *Lsm11* mutants. Harmony Salzler, a graduate student in my lab, has examined both *Slbp* and *U7* mutants and found that while histone proteins appear to be made at normal levels in these mutants, there are chromatin defects which are seen in both mutants and in *Slbp* mutants there is a slowed growth rate (Salzler et. al., unpublished results). These phenotypes, which are presumed to be caused by the polyadenylation of all five replication dependent histone genes, could account for the lethality seen in *Slbp* mutants, but in order for us to really be able to conclude that polyadenylation of histone mRNA directly causes lethality we need to conduct an experiment where we introduce the polyadenylated transcripts into an otherwise wild type animal and examine the consequences this has on development. It would be

interesting to examine whether *Drosophila* can actually tolerate the misprocessing of its replication dependent histone mRNAs, especially since this does not seem to affect the production of histone proteins. This would lend further evidence to the idea that *Slbp*, *Lsm10* and *Lsm11*'s null mutant lethality is due to each proteins role in an essential process outside of histone pre-mRNA processing and not directly linked to the misprocessed mRNA phenotype seen in each mutant.

### **Concluding remarks**

The work presented in this thesis has helped to further our understanding of the processing of replication dependent histone mRNAs. We have found that polyadenylation of histone mRNAs is a phenotype that occurs from the mutation of any factor that is necessary for the unique process of histone pre-mRNA processing in *Drosophila*. This observation was used to design a screen that will further advance our understanding of histone pre-mRNA processing by finding novel factors involved in this process. Our data has also shown that there are different outcomes seen from mutating separate parts of the U7 snRNP particle, and we have used this evidence to validate searching for a novel function for its two unique proteins, Lsm10 and Lsm11. It should also be noted that we have created a very useful tool, V5 tagged Lsm11 transgenic flies, for both general use in studying the structure and function of HLB's in *Drosophila* and also for use in searching for Lsm11's novel function in *Drosophila*. Our data has also shown that we can gain powerful new insight into the function of a gene, such as U7, that has been studied previously but not knocked out and studied in a whole organism such as *Drosophila*.

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