A Molecular and Genetic Characterization of the Mechanisms of Histone mRNA Metabolism in *Drosophila*

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ABSTRACT

Jeremy M. Kupsco: A Molecular and Genetic Characterization of the Mechanisms of Histone mRNA metabolism in Drosophila
(Under the direction of Dr. Robert Duronio)

In metazoans the replication dependent histones are produced during S-phase of the cell cycle. The timely production of histones during S-phase is required to quickly package the newly synthesized DNA. Much of this S-phase regulation is achieved by the regulation of histone pre-mRNA processing. Histone mRNAs are the only mRNAs in metazoans that do not end in a poly(A) tail instead ending in a conserved 26 nucleotide sequence that forms a stem loop. A protein called Stem Loop Binding Protein (SLBP) binds to the stem loop which along with the U7 snRNP recruits an endonuclease complex to cleave the histone pre-mRNA to create the mature histone transcript. *Drosophila* contains a single *SLBP* gene, which is required for histone pre-mRNA processing during embryogenesis, the larval stages of development, and during oogenesis. In the absence of *SLBP*, histone mRNAs become polyadenylated by the use of cryptic polyadenylation sites downstream of the normal cleavage site. These polyadenylated transcripts perdure outside of S-phase in cells that are undergoing endocycles. In addition to *Drosophila SLBP*'s role in histone pre-mRNA processing, a 53 amino acid region of the N-terminus is required for viability of the organism but is dispensable for histone mRNA processing indicating a second function for *SLBP* in histone metabolism. At the end of S-phase histone mRNAs are rapidly destroyed. The human protein 3'hExo has been implicated in the degradation of histone mRNAs. *Drosophila* contains a single potential homologue of

3’hExo, *Snipper*, which is capable of degrading and binding to histone mRNAs *in vitro.* However, in *Snipper* mutant flies no defect in histone mRNA degradation was seen, indicating that *Snipper* is not required for the regulated destruction of histone mRNAs and has a yet to be discovered function.

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ABBREVIATIONS

AA	amino acid
CAF	Chromatin assembly Factor
cDNA	complementary DNA
CNS	Central nervous system
CPSF	Cleavage and Polyandenylation Specificity Factor
DNA	deoxynucleic acid
EDTA	Ethylenediaminetetraacetic acid
G1	Gap phase 1
G2	Gap phase 2
HDE	histone downstream element
mRNA	Messenger Ribonucleic acid
RBD	RNA Binding Domain
RNA	Ribonucleic acid
S-phase	Synthesis Phase
SL	Stem Loop
SLBP	Stem Loop Binding Protein
Snp	Snipper
snRNA	small nuclear Ribonucleic Acid
snRNP	small nuclear RiboNucleoprotein Particle

Chapter I

INTRODUCTION

In metazoans, replication-dependent histone biosynthesis is tightly coupled to S-phase to ensure rapid deposition of newly synthesized histones at the replication fork (Marzluff & Duronio, 2002). There are five types of replication dependent histone proteins, the four core histones: H2A, H2B, H3, and H4, and the linker histone H1. A dimer of each of the core histones will interact to form the core histone octamer, which is highly basic and wraps around 146 base pairs of negatively charged DNA (1.75 turns of DNA) to form the nucleosome (Moudrianakis & Arents, 1993). These nucleosomes along with other proteins compose chromatin, which is important for the compaction of DNA so that it will fit into the nucleus and for the regulation of processes such as transcription, differentiation, and cell division.

The production of histones is coordinated with S-phase of the cell cycle, so the cell can package newly synthesized DNA into chromatin. During S-phase the histone proteins that will be deposited on the newly replicated daughter strands of DNA can come from two sources, the *de novo* synthesis of histone proteins or the transfer of histone octamers from the parental strand of DNA to the daughter strands. The histone octamers are deposited on the

daughter strands within a few hundred nucleotides of the replication fork by a complex containing Chromatin Assembly Factor -1(CAF-1) along with the sliding clamp processivity factor Proliferating Cell Nuclear Antigen (PCNA) (Martini et al., 1998; Shibahara & Stillman, 1999; Krawitz et al., 2002). It is critical that the newly synthesized DNA be rapidly packaged into chromatin. The high demand for histone protein placed on a cell during S-phase is referred to as histone tension, that is, a cell is always under tension during S-phase to produce enough histone protein to quickly package the newly synthesized DNA and to limit the production of histones to S-phase (Gunjan & Verreault, 2003) Alteration of this histone S-phase coordination or of histone levels during S-phase appears to have several deleterious consequences to the cell or organism. In yeast, delays between DNA synthesis and histone deposition leads to loss of viability, while in human cells a delay leads to spontaneous DNA damage and S-phase arrest (Han et al., 1987); (Ye et al., 2003). Overexpression of the core histones *in vivo* leads to a high incidence of mitotic chromosome loss in yeast, while *in vitro* overexpression of the core histones leads to chromatin aggregation and loss of transcription (Meeks-Wagner & Hartwell, 1986; Steger & Workman, 1996). Also in yeast production of the core histones outside of S-phase causes increased DNA damage sensitivity, chromosome loss, and impaired growth (Gunjan & Verreault, 2003). Therefore the ability to achieve the appropriate levels of histone production and timing appears critical for an organism's viability.

This S-phase control of the histones operates on many levels, including transcription, pre-mRNA processing, mRNA stability, and protein stability. In yeast and Arabidopsis, which have polyadenylated histone mRNAs, this control is primarily transcriptional, while in animals, which don't have polyadenylated histone mRNAs, this control is primarily achieved

post-transcriptionaly at the level of pre-mRNA processing. Despite the different mechanisms of control, a common theme is that organisms have evolved mechanisms to ensure that histone biosynthesis is tightly coupled to S-phase.

Regulation of Histone mRNA Expression

In mammalian cells the steady state levels of histone mRNA increases approximately 30 fold as cells progress from G1-S. However, transcription initiation only accounts for a 3-5 fold increase in histone mRNA. An 8-10 fold increase in the efficiency of histone pre-mRNA processing, and an increase in the half life of the mRNA accounts for the majority of the increase of histone mRNAs during S-phase (Harris et al., 1991; Whitfield et al., 2000). The basis for the posttranscriptional regulation of the histone mRNA lies in the unique 3' end of the histone mRNA (Figure 1.1). Replication dependent histone mRNAs are the only mRNAs that do not end in a poly (A) tail, but instead end with a 26 nucleotide stem loop structure (Dominski & Marzluff, 1999). This stem loop and another cis acting sequence 10 nucleotides downstream from the stem loop termed the histone downstream element (HDE) are the critical cis acting elements for processing control of the transcripts. Production of a mature histone mRNA requires the binding of a protein called Stem Loop Binding Protein (SLBP) to the stem loop and the binding of the U7 snRNP to the HDE. These factors then recruit symplekin and at least one subunit of the cleavage and polyadenylation complex CPSF73. It's likely other subunits are also recruited that cleave the histone pre-mRNA 3-5 nucleotides after the stem loop (Figure 1.2)

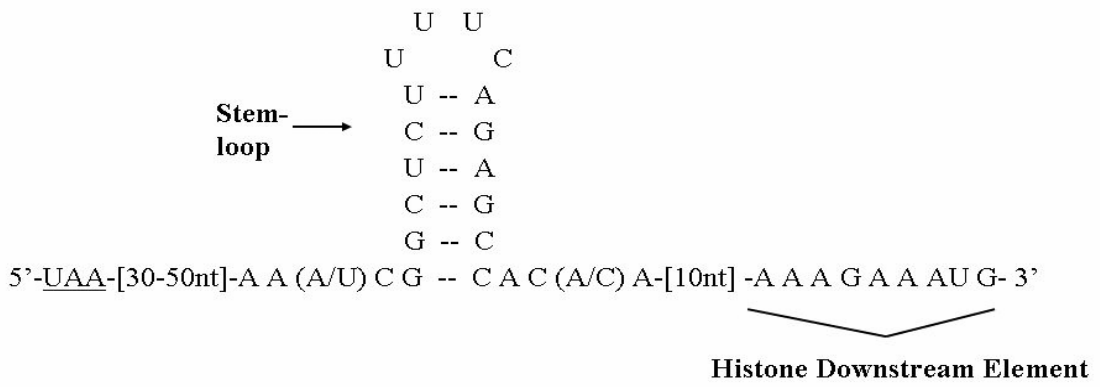


Figure 1.1 Specialized 3' end of the histone mRNA. The stem loop region is bound by SLBP, while the histone downstream element is bound by the U7 snRNP via base pairing of the U7 snRNA to this region.

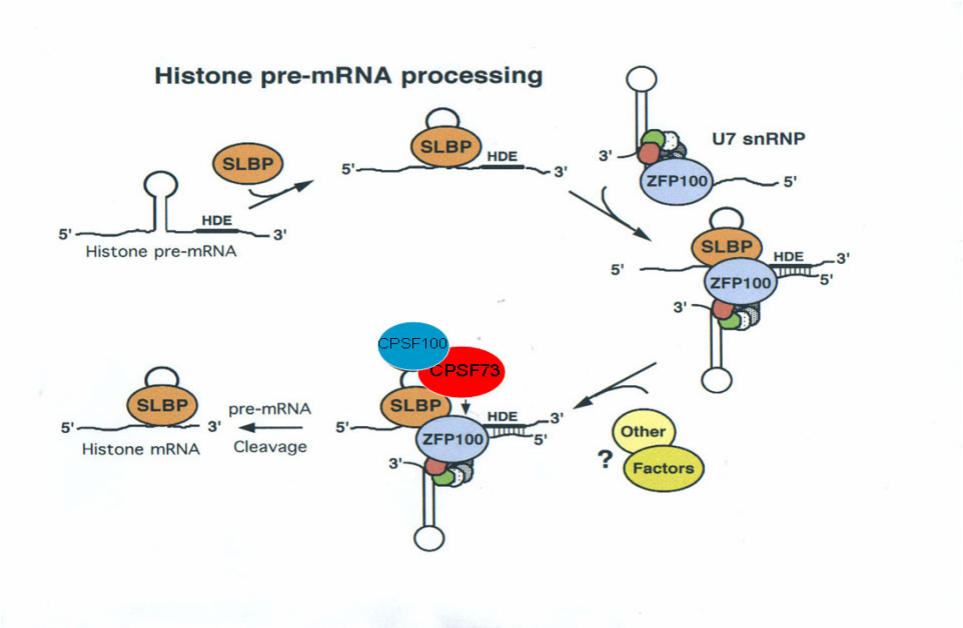


Figure 1.2 Schematic of Histone pre-mRNA processing in mammalian cells. Processing is initiated by the binding of SLBP to the stem-loop and the U7 snRNP to the HDE. These recruit members of the CPSF complex to carryout the cleavage reaction. SLBP remains associated with the mature histone mRNA and is carried out to the cytoplasm.

(Wang et al., 1996; Dominski et al., 2005b; Kolev & Steitz, 2005). Depletion of SLBP or of the U7 snRNP in mammalian cell extracts inhibits the processing of synthetic histone pre-mRNAs (Dominski et al., 1999)

Posttranscriptional Roles for SLBP

After pre-mRNA processing, SLBP remains bound to the mature histone mRNA and is shuttled out to the cytoplasm where it is thought to regulate the translation of the histone mRNA (Sanchez & Marzluff, 2002). Using a luciferase reporter assay, it was shown that the presence of an intact histone stem loop was sufficient for the stimulation of translation with the addition of *Xenopus* SLBP1 protein in both rabbit reticulocyte lysates and injected *Xenopus* oocytes (Sanchez & Marzluff, 2002). SLBP is the only identified member of the pre-mRNA processing complex that appears to be cell cycle regulated in vertebrate cells. SLBP accumulates during S-phase and then is destroyed in a phosphorylation dependent manner by the proteosome as cells exit S-phase (Whitfield et al., 2000).

The preceding models for histone mRNA metabolism and for SLBP's role in histone mRNA metabolism are largely based on *in vitro* biochemical data and experiments done in vertebrate cell lines, few studies have been performed to study the mechanisms of histone mRNA metabolism in the context of development. Therefore to take a genetic approach to study the roles of SLBP in histone mRNA metabolism throughout development we chose to study these proteins in vinegar fly *Drosophila melanogaster*.

The role of Drosophila SLBP in histone mRNA metabolism

Drosophila contains a single *SLBP* gene, which encodes a protein that is 276 amino acids in length and that contains a 72 amino acid RNA binding domain, which is 75% similar to the RNA binding domain of human SLBP. No significant homology exists outside the RNA binding domain between *Drosophila SLBP* and Human SLBP. In order to study *SLBP*'s function, mutant alleles of *SLBP* were created by imprecise excisions of a P-element in the 5'UTR of the *SLBP* locus. Three mutant alleles of *SLBP* were used for analysis, $SLBP^{10}$, $SLBP^{12}$, and $SLBP^{15}$. The $SLBP^{10}$ and $SLBP^{15}$ were formed by an internal deletion of the P-element whereas the $SLBP^{12}$ allele was formed by a deletion which removes much of the coding sequence of *SLBP* and the adjacent gene *rpn2*. Genetically both the $SLBP^{12}$ and $SLBP^{15}$ alleles act as null alleles and are larval lethal and pupal lethal depending on genetic background, while the $SLBP^{10}$ allele is a hypomorphic allele which is maternal effect lethal (Sullivan et al., 2001). When RNA extracts were analyzed from *WT* and *SLBP* null embryos by northern blot analysis, it was observed that longer mis-processed forms of histone H3 were observed in the *SLBP* mutant embryos (Figure 1.3)(Sullivan et al., 2001). Further analysis showed that the mRNAs for all the core histones and histone H1 were mis-processed in *SLBP* mutant embryos (Lanzotti et al., 2002). Furthermore it was shown that these longer mis-processed forms of the histone mRNAs were polyadenylated, and were formed by the read through of the polymerase past the normal cleavage site into cryptic polyadenylation sites which are located in the downstream regions of each of the 5 replication dependent histone genes (Lanzotti et al., 2002). To determine if these mis-processed histone mRNAs were expressed normally throughout development, *in situ* hybridizations were performed on *SLBP* mutant embryos. In late stage *SLBP* embryos it was found that the mis-processed histone mRNAs inappropriately accumulated in the endocycling cells of the anterior midgut,

hind gut, and anal pads (Figure 1.4) (Sullivan et al., 2001; Lanzotti et al., 2002). Therefore during embryogenesis *SLBP* is required for the processing of all 5 replication dependent histone mRNAs, and the mis-processed polyadenylated histone mRNAs in the endocycling cells of the gut appear to be no longer coordinated to S-phase; however work has not been done to determine *SLBP*'s role in histone mRNA processing in later developmental stages.

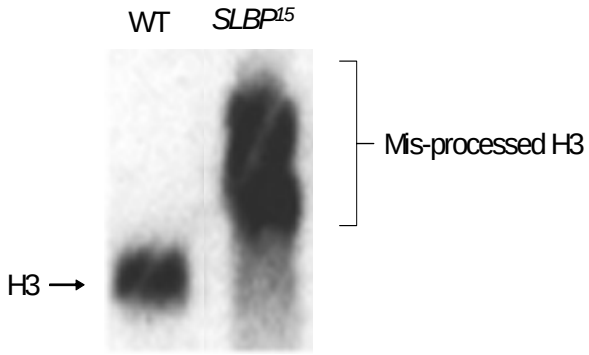


Figure 1.3 Northern Blot of histone mRNA from *SLBP* mutant embryos. RNA samples were taken form late stage Wt and *SLBP* mutant embryos and probed with a probe antisense for H3. Histone mRNAs from *SLBP* mutant embryos are mis-processed and poly-adenylated.

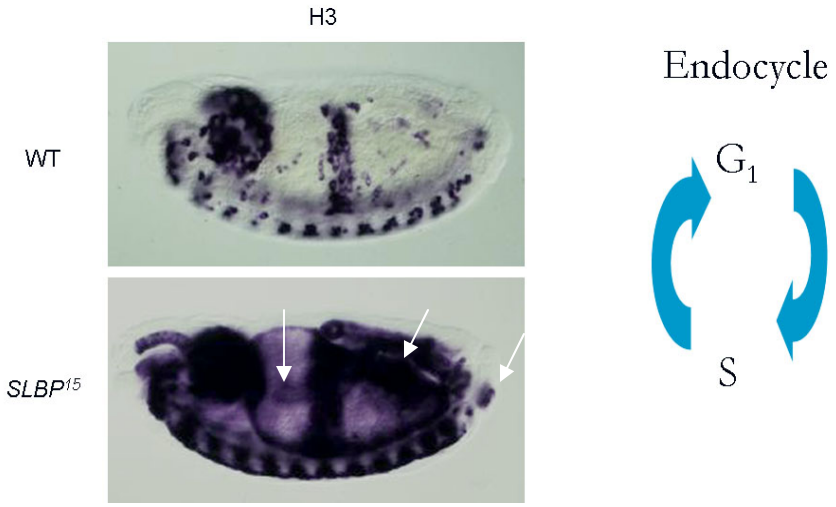


Figure 1.4 Abnormal expression of mis-processed histone mRNAs in *SLBP* mutant embryos. In situ hybridizations on WT and *SLBP*^{15} stage 14 embryos with a probe anti-sense for H3. In the *SLBP*^{15} embryo there is abnormal per durance of histone mRNAs in the endocycling cells of the anterior midgut, hindgut and anal pads (arrows left to right) compared to a WT embryo.

Regulated Destruction of Histone mRNAs

Not only is it important to have enough histones around during S-phases, studies have also shown that it is important to limit the expression of the core histones to S-phase with the perdurance of free core histones outside of S-phase causing DNA damage and genomic instability (Gunjan & Verreault, 2003). One way in which histone production is limited to S-phase is the coordinated destruction of histone mRNA as the cell exists S-phase and enters G2. In humans, 3'hExo is an exonuclease which might play a role in this coordinated destruction of histone mRNA at the end of S-phase (Dominski et al., 2003). 3'hExo belongs to family of exonucleases called the DEDD family of exonucleases, named for four invariant amino acids found in the catalytic exonuclease domain. This family includes RNA specific 3' exonucleases, such as PARN, as well as DNA specific exonucleases involved in DNA repair and the proofreading subunits of DNA polymerases (Zuo & Deutscher, 2001). 3'hExo

has been shown capable of binding to the 3' side of the stem loop on histone mRNAs and is capable of degrading the histone message in a 3'-5' manner in *in vitro* whole cell extracts (Dominski et al., 2003). This exonuclease is capable of binding to the stem loop both in the presence and absence of SLBP, however binding appears to be more efficient in the presence of SLBP. Furthermore, the presence of SLBP bound to the stem loop prevents the degradation of the histone message by 3'hExo (Dominski et al., 2003). While 3'hExo is capable of degrading histone mRNAs *in vitro*, knockdown of 3'hExo in HeLa cells by siRNAs has failed to show a defect in the regulated destruction of histone mRNAs (W.F. Marzluff personal communication). Therefore 3'hExo's role in this regulated destruction of histone mRNAs remains unclear.

Research Goals

While the early work with *Drosophila SLBP* has provided key insights to its function in histone mRNA processing, many key questions have yet to be answered. For instance, it has been shown only in the embryo that *SLBP* is required for histone mRNA processing, and that abnormal perdurance of mis-processed histone mRNAs was only seen in the endocycling cells of the gut. Is *SLBP* required through out development to process histone mRNAs? In cells under going a canonical cell division cycle, which are not found in the embryo, will the mis-processed histone mRNAs perdure abnormally? Furthermore, eggs that are laid by the hypomorphic $SLBP^{10}$ mothers have less histone mRNA deposited in them, but the transcripts that are deposited are processed normally (Lanzotti et al., 2002). Therefore is *SLBP* required for histone mRNA processing during oogenesis? To answer these questions *in situ*

hybridizations and northern blot analysis will be performed on larval 3rd instar imaginal eye discs from *SLBP* mutant larvae and on *SLBP* mutant ovaries to determine if *SLBP* is required for the processing of histone mRNAs later in development and to determine if these mis-processed histone mRNAs perdure abnormally in a mitotic cell cycle.

The second major objective is to determine if SLBP has a post processing role in histone mRNA metabolism. Both mammalian and *Drosophila* SLBP remains bound to the mature histone transcript as they are localized to the cytoplasm (Lanzotti et al., 2004). Why does SLBP get shuttled out to the cytoplasm with the mature histone transcript? *In vitro* data has shown that the C-terminal RNA binding domain is sufficient for the processing (Dominski et al., 2002), so that begs the question, does the N-terminus of SLBP have an essential function post processing? To answer these questions nested deletions of the N-terminus of SLBP were made and then assayed for their ability to rescue the lethality associated with the loss of SLBP and for their ability to rescue the defect in histone mRNA processing seen in *SLBP* mutants. If a deletion is able to rescue the defect in histone RNA processing but not the lethality associated with loss of SLBP it would suggest that the deleted region of the N-terminus has an essential role post processing.

The third and final objective is to determine if *Drosophila* has an orthologue to 3'hExo, and determine if this orthologue has a role in the regulated destruction of histone RNA at the end of S-phase as the cell enters G2, where there is about a 30 fold reduction in the amount of histone mRNA in mammalian cells. It has been shown that *in vitro* 3'hExo is capable of binding to the histone stem-loop and capable of degrading a synthetic histone mRNA; however there is no *in vivo* data on 3'hExo's role in regulating histone mRNA destruction. To examine this question, the *Drosophila* genome will be examined to

determine if there is an orthologue of 3'hExo present, then mutants of this orthologue will be examined to determine if it plays a role in the regulated destruction of histone mRNAs. This will be done by doing *in situ* hybridizations on mutant tissues to look for the abnormal perdurance of histone mRNA during development and by doing northern blot analysis to determine if there is a change in the overall levels of histone mRNAs in mutant tissues.

Chapter II

Characterization of *Drosophila SLBP*'s role in histone mRNA processing throughout development

Contributions: Portions of the figures for this chapter are taken from Godfrey et al. 2006 RNA. Ryan Zimmerman helped contribute data for figures 2.2A and figures 2.3.

Summary

Previous studies have shown that *Drosophila SLBP* is required for histone pre-mRNA processing during embryogenesis (Sullivan et al., 2001; Lanzotti et al., 2002). However, it is unknown if *SLBP* is required for histone mRNA processing throughout development. *In situ* hybridizations were performed on *SLBP* mutant third instar imaginal eye discs to determine if histone mRNAs were mis-processed and regulated normally in relation to the cell cycle. While the histone mRNAs were mis-processed, the overall expression pattern of histone mRNA was normal. Previous research has also shown that *SLBP* hypomorphic mothers lay eggs that contain normally processed histone mRNA but in reduced amount, suggesting that *SLBP* may not be needed for processing in the ovary. *In situ* hybridization in *SLBP* null ovaries shows that histone mRNAs are mis-processed; therefore it appears that throughout development *SLBP* is required for the proper processing of histone mRNAs.

Background

Metazoan replication dependent histone mRNA's are the only mRNAs that do no end in a polyA tail, instead ending with a conserved stem loop sequence (Dominski & Marzluff, 1999). In vertebrates, the 3' end of mature histone mRNA is formed by an endonucleolytic cleavage that requires both the stem loop and the histone downstream element (Strub et al., 1984; Gick et al., 1986; Mowry & Steitz, 1987). The stem loop is bound by SLBP while the HDE is bound by the U7 snRNP which together recruit endonuclease complex consisting of at least one member of the CPSF complex and symplekin (Dominski et al., 2005a; Kolev & Steitz, 2005).

In *Drosophila* there is a single *SLBP* gene and it has previously been shown that *SLBP* is required for the proper processing of all 5 replication dependent histone mRNAs during embryogenesis (Sullivan et al., 2001; Lanzotti et al., 2002). When *SLBP* is mutated the histone mRNAs are converted to poly(A) forms, using cryptic polyadenylation sites found in the downstream regions of each of the replication dependent histone genes (Lanzotti et al., 2002). Furthermore analysis of the expression of these aberrantly processed histone messages during embryogenesis has shown that in the endocycling cells of the gut these mis-processed histone mRNAs are no longer expressed in a pattern that directly corresponds with BrdU incorporation, thereby indicating that production and degradation of these mis-processed histone mRNAs may no longer be linked to S-phase of the cell cycle (Sullivan et al., 2001; Lanzotti et al., 2002). However, the requirement for SLBP for histone mRNA processing has not been looked at further in development. Also the deregulation of histone transcript accumulation from the cell cycle was only seen in endocycling cells, and cells with a canonical division cycle are not found in the embryo, therefore it is unknown if this deregulation of histone transcript expression would occur during a normal cell cycle. Finally

previous data has shown that histone transcripts deposited into eggs by *SLBP* hypomorphic mothers are normally processed, but are found in the egg in reduced amounts when compared to eggs laid by wildtype mothers (Lanzotti et al., 2002), potentially indicating that SLBP may not be required for the proper processing of histone transcripts during oogenesis. Here we show that SLBP is required throughout development for the proper processing of histone mRNAs and that in *SLBP* mutant cells undergoing mitotic division cycles histone mRNAs appear to be regulated normally in relation to S-phase, but once again it was noticed that in the endoreduplicating nurse cells of the ovary, mis-processed histone mRNAs appear to perdure outside of S-phase, suggesting a potential difference in how these mis-processed histone mRNAs are handled in mitotically dividing cells versus endocycling cells.

Experimental Procedures

In situ Hybridization

Eye discs were dissected from wandering third instar larvae and fixed with 4% formaldehyde for 15 minutes. Ovaries were dissected from 1-2 day old females and fixed with 10% formaldehyde for 20 min. In situ hybridizations were performed with digoxigenin-labeled riboprobes complementary to the coding region of H3 or to the region downstream of the normal pre-mRNA processing site (H3-ds) as described (Lanzotti et al., 2002).

Northern analysis

For northern blots total cellular RNA was isolated from wildtype, $SLBP^{10}$ and $SLBP^{15}$ ovaries with TRIzol Reagent (Gibco). For the analysis of histone mRNAs, 2 ug of RNA per lane were subjected to electrophoresis in a 1X MOPS in a 1% agarose gel containing

0.01MOPS(PH 7.0) and 6.75% Formaldehyde. Separated RNAs were transferred to a N+ nitrocellulose membrane (Amersham) using the wick method in 20X SSC. DNA containing histone H3 or rp49 coding regions were labeled with α [^{32}P]-dCTP using a random primer labeling kit (Stratagene). Hybridizations were performed at 60˚C using Quikhybe (Stratagene).

Generation of germ line clones

Mosaic *SLBP*^{15} 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 *SLBP*^{15} larvae for one hour at 37^{o} C on the third and fourth days of development. Ovaries were dissected from the result adults and fixed in 10% formaldehyde for 20 min for *in situ* hybridizations or subjected to northern analysis.

Results

SLBP is required for histone pre-mRNA processing in larval imaginal discs

To examine whether histone mRNAs are processed and expressed properly in larval tissues we examined histone H3 expression in the highly regulated tissues of the developing eye. To visualize histone mRNA expression, two *in situ* hybridization probes for histone H3 were used. One probe is anti-sense to the coding region of histone H3, while the second probe is anti-sense to a region downstream of the pre-mRNA processing site called the H3-ds probe. Previous work with the H3-ds probe has shown that this probe will only hybridize to H3 transcripts that are mis-processed and will not hybridize to normally processed messages creating a powerful tool to detect the expression of mis-processed histone transcripts

(Lanzotti et al., 2002). During early larval stages asynchronous cell proliferation occurs in the eye imaginal discs, in the late third instar larvae a wave of differentiation called the morphogenetic furrow (MF)(Figure 2.1 asterisk) sweeps across the disc from posterior to anterior. As cells enter the MF they arrest in G1 phase. Some of these arrested cells will differentiate, while a population will remain undifferentiated and undergo a final cell division called the 2nd mitotic wave, which can be visualized with the coding probe for H3 (Arrow Figure 2.1) before terminally differentiating. The ds-H3 probe shows that mis-processed histone mRNAs are not found in WT discs (Figure 2.1B), but stains *SLBP* mutant discs (Figure 2.1C). The expression pattern of histone H3 appears normal when compared to WT eye discs. Mis-processed H3 did not accumulate in the cells arrested in G1 phase in the furrow, and the width of the second mitotic wave was not noticeably larger in the *SLBP* mutant discs, a larger 2nd mitotic wave would indicate cells were unable or slow to degrade H3 as they exit S-phase for the last time and terminally differentiate. This normal expression pattern of mis-processed H3 suggests that in mitotically dividing cells the aberrantly processed histones maintain their S-phase regulation. This is in contrast to what was seen in the endocycling cells of the embryonic gut where there was an aberrant perdurance of H3 outside of S-phase.

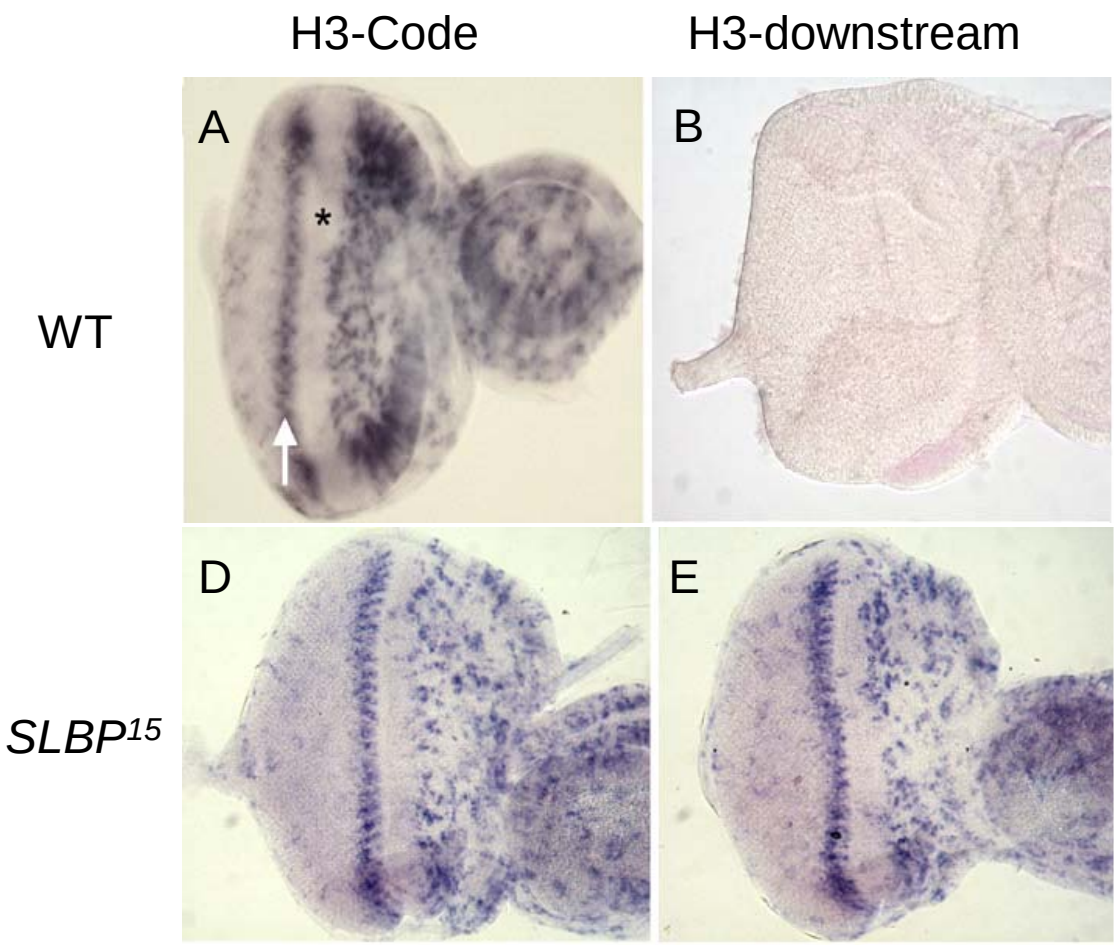


Figure 2.1 *SLBP* mutant eye imaginal discs display a wild-type pattern of mis-processed histone H3. Eye imaginal discs were dissected from w^{1118} control (A,B) and $SLBP^{15}$ larve (C,D) and probed with H3 coding (A,C) and H3 ds probes (B,D). The asterisk indicates the morphogenetic furrow, which contains cells arrested in G1, while the arrow shows the 2nd mitotic wave, in which cells are in S-phase. Cells to the left of the arrow are terminally differentiated, while cells to the right of the asterisk are undifferentiated cells dividing asynchronously.

SLBP is required for Histone pre-mRNA processing in the Ovary

The basic unit of *Drosophila* oogeneis is the egg chamber which consists of 16 interconnected germ cells surrounded by a single layer of somatic follicle cells(Spradling, 1993). Fifteen of these germ cells will become nurse cells, which endoreduplicate to become highly polyploid. The function of the nurse cells is to produce the maternal stores of mRNA and protein to be loaded into the oocyte needed for early development. The follicle cells contribute to oogenesis by producing the proteins needed for the eggshell and by participating in the patterning of the dorsal/ventral and posterior/anterior axis. Histone mRNA expression during oogenesis in the nurse cells and follicle cells correlates with S-phase. Since the nurse cells replicate asynchronously, only a portion will stain for H3 at one time. Late in oogenesis, there is a final burst of histone mRNA expression that is not associated with active DNA replication, these mRNAs are the ones that will be transported in to the oocyte and will be used to support early embryonic development (Ambrosio & Schedl, 1985; Ruddell & Jacobs-Lorena, 1985). *SLBP* hypomorphic female flies are maternal effect lethal and lay normal numbers of eggs but these eggs cannot hatch because of severe mitotic defects during the syncytial cycles (Sullivan et al., 2001). In these eggs there is a decreased maternal deposition of both histone mRNAs and histone protein (Lanzotti et al., 2002)(W.F. Marluff unpublished). Despite being approximately 10 fold less histone mRNA in the eggs laid by *SLBP* hypomorphic mothers, all the histone transcripts that are deposited are processed normally, potentially indicating that SLBP may not be required for histone mRNA processing in the germline. To test whether SLBP is required for histone mRNA processing in the germline, northern blots and S1 nuclease protection assays were performed on ovaries dissected from $SLBP^{10}$ females. These results showed reduced amounts of histone H3 and H2a in the ovary, but no mis-processed histone H3 or H2a (Figure 2.2 A & B). Similar to

what was seen in the eggs deposited by these mothers. To analyze this in more detail *in situ* hybridizations with the coding and ds probes for H3 were performed on $SLBP^{10}$ mutant egg chambers. $SLBP^{10}$ mutant egg chambers stained with H3 coding probe appear similar to WT egg chambers (Figure 2.3 A, C, E). H3 expression can be seen in both the nurse cells and follicles cells, however when $SLBP^{10}$ egg chambers were stained with the ds-probe, strong staining was only seen in very early egg chambers (stages 2-3), while the nurse cells in older egg chambers did not stain, and sporadic staining was seen in the somatic follicle cells (Figure 2.3 B, D F). The *in situ* data shows that only a small fraction of the total H3 in $SLBP^{10}$ ovaries is mis-processed, which is likely the reason why no mis-processed RNAs were seen in the northern and S1 assays. Overall it appears that there is a reduced amount of processed histone mRNAs in the *SLBP* hypomorphic mothers indicating some role for *SLBP* in histone biosynthesis.

To further test *SLBP*s role in histone biosynthesis in the ovary we generated $SLBP^{15}$ null egg chambers using FLP/FRT mediated recombination. In contrast to what was seen with $SLBP^{10}$, northern analysis of RNA from *SLBP* mosaic ovaries showed that the majority of H3 was now in the polyadenylated form (Figure 2.2 lane 3). This result was confirmed by *in situ* hybridizations with the ds-probe and H3 coding probe on the *SLBP* mosaic egg chambers which showed the ds-probe and coding probes now had similar staining patterns suggesting a large portion of the H3 in the germline is now mis-processed (Figure 2.3 G, H). This data shows that *SLBP* is required for histone mRNA processing in the germline and that the cryptic polyadenylation sites can also be used in the germline just like in somatic tissues.

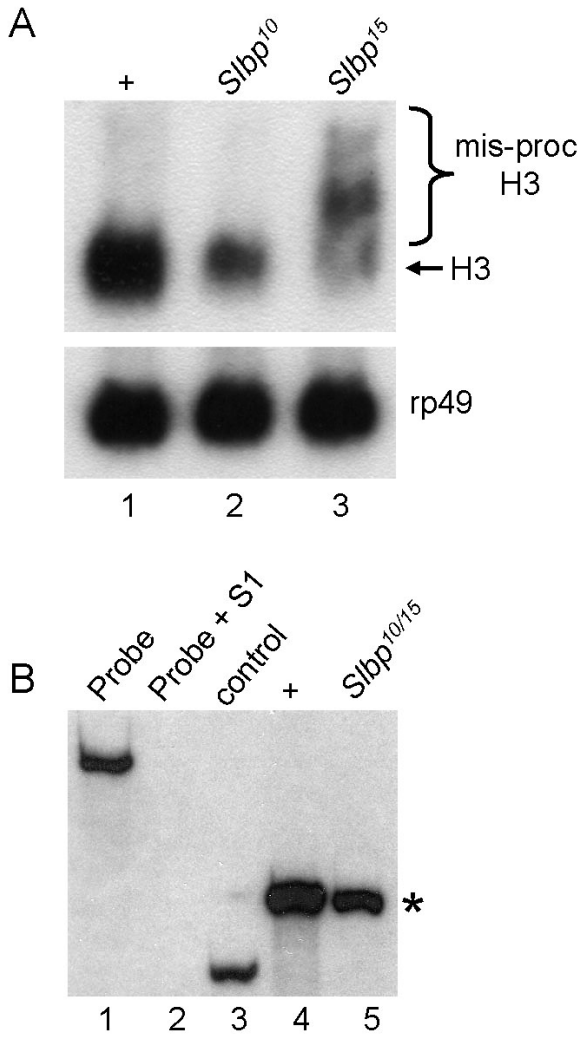


Figure 2.2 *SLBP* is required for histone pre-mRNA processing during oogenesis. A) Northern analysis of total RNA isolated from ovaries collected from adult female flies of the indicated genotype, the blot was probed with P^{32} labeled DNA probes for H3 and rp49. B) Detection of H2a mRNA by S1 nuclease protection. (Lane 1) 5' end labeled 650 nt H2a probe. (Lane 2) H2a probe incubated with S1 nuclease (Lane 3) H2a probe with S1 nuclease + synthetic partial H2a fragment that yields a 265 nt protected fragment. (Lane 4) H2a probe + S1 and total RNA isolated from wild-type females, wild type probe protects a fragment of 340 nt (asterisk). (Lane 5) H2a probe + S1 and total RNA isolated from ovaries dissected from $SLPB^{10}/SLBP^{15}$ mutant females.

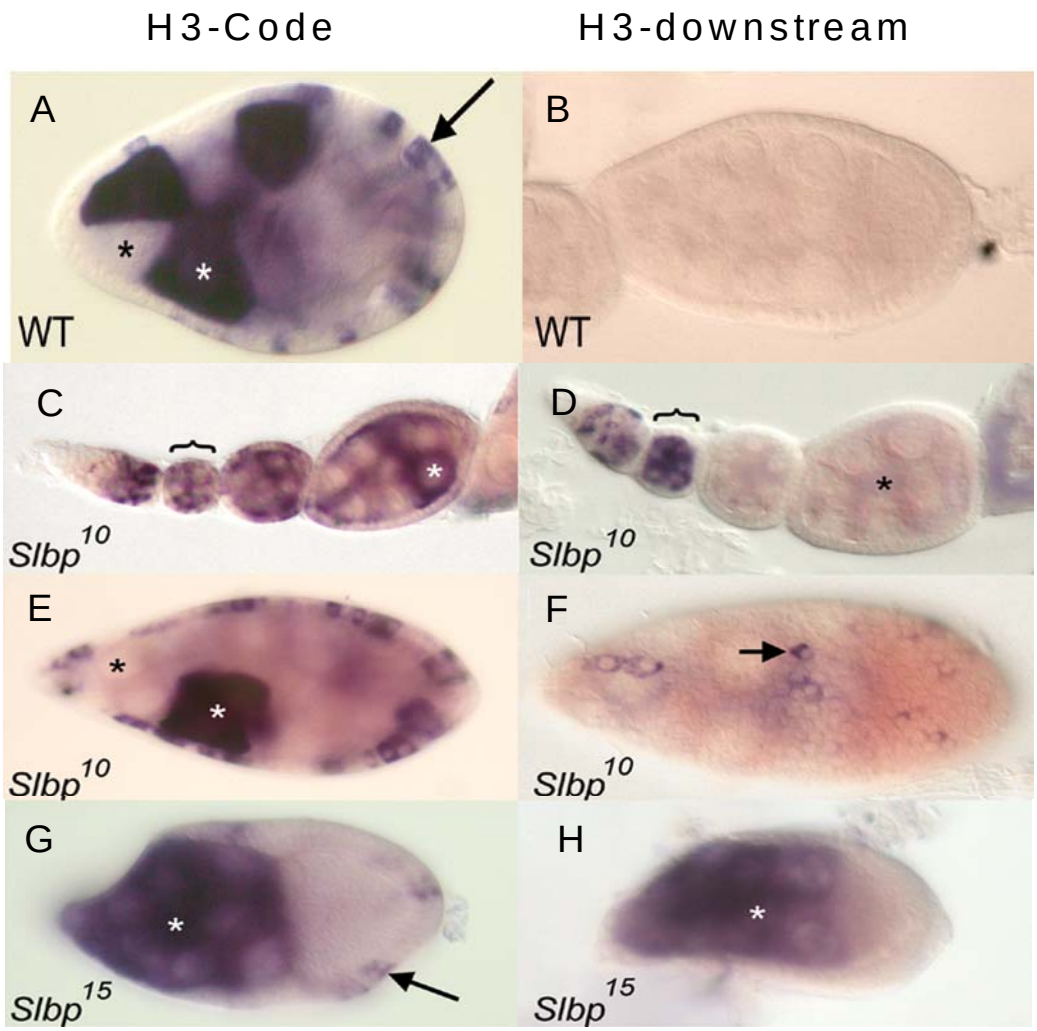


Figure 2.3 *SLBP* is required for histone pre-mRNA processing during oogenesis. (A) w^{1118} control egg chamber hybridized with histone H3 coding probe. In this and subsequent panels white and black asterisks indicate individual nurse cells with and without H3 staining respectively, and arrows indicate follicle cells. (B) w^{1118} control egg chamber hybridized with the H3-ds probe, note the lack of staining indicating the lack of poly-adenylated histone RNAs in wt tissues. (C, D) 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 endoreduplication cycles. Note the lack of H3-ds staining in the later egg chambers (asterisk in D). (E) $SLBP^{10}$ egg chamber hybridized with the H3 coding probe indicating the production of H3 mRNA in both nurse and follicle cells. (F) $SLBP^{10}$ mutant egg chamber hybridized with H3-ds probe. The arrow indicates the follicle cells expressing the aberrant poly-A form of H3. (G, H) Mosaic egg chambers containing $SLBP^{15}$ mutant germ cells hybridized with the H3 coding and H3-ds probes respectively. Because this is a mosaic egg chamber, the follicle cells are phenotypically WT, and do not stain with the ds-probe because they only express wt H3 mRNA (arrow in G). All egg chambers except C, D are stage 9. All egg chambers are oriented anterior at left and posterior at right.

Discussion

This report demonstrates that *Drosophila SLBP* is required for the proper processing of replication dependent histone mRNAs during larval development and during oogeneis. The loss of SLBP during larval development and oogeneis leads to the accumulation of polyadeynlated histone transcripts as was reported for loss of SLBP during embryogenesis (Sullivan et al., 2001; Lanzotti et al., 2002). However there appears to be a difference in how these mis-processed histone transcripts are regulated between cells undergoing a proliferation cycle versus cells that are undergoing an endocycle.

As seen with embryonic tissues, the replication dependent histone mRNAs were mis-processed in *SLBP* mutant larval eye discs. In *SLBP* mutant 3rd instar eye discs the histone H3 message was mis-processed, indicating that SLBP is required for histone pre-mRNA processing in the larval eye discs. Furthermore, it appeared that the expression pattern of histone H3 was normal with respect to the cell cycle in the eye discs. No apparent perdurance of H3 was seen as cells arrested in G1 in the morphogenetic furrow, and no perdurance of H3 was seen as cells exited the cell cycle for the final time as they exited the 2nd mitotic wave. While this result is different from what was seen with the embryonic gut, where the mis-processed RNAs appear to perdure outside of S-phase, it should be noted that all other cell types in the embryo appear to be able to regulate these mis-processed histone mRNAs normally, and most of these cells are undergoing variant proliferation cycles (Lanzotti et al., 2002). Therefore the result that these polyadenylated histone mRNAs are regulated normally with respect to the cell cycle is not that surprising. However it does set up a very interesting question as to if there is a difference between how cells undergoing proliferation cycles and cells undergoing endocycles degrade these mis-processed histone

transcripts. So far the perdurance of the poly(A) histone transcripts has only been seen in endocycling cells, not in proliferating cells, it would be very interesting to examine a third set of endocycling cells, the cells of the larval salivary gland, in *SLBP* mutants to see if these mis-processed transcripts are regulated normally or not. If the poly (A) transcripts are mis-regulated in these cells it would suggest that there is a difference in mechanisms to degrade these transcripts in endocycling cells versus proliferating cells.

While very little mis-processing of histone mRNA was seen in the hypomorphic *SLBP* egg chambers, virtually all of H3 found in *SLBP* null mutant egg chambers was mis-processed, indicating that SLBP is indeed required for proper histone pre-mRNA processing throughout development and during oogenesis. This result suggests that in the hypomorphic ovaries the amount of SLBP present is enough to handle the demands of processing. This potentially indicates that SLBP acts catalytically for processing, that is, one molecule of SLBP is able to process more than one histone mRNA before being carried out to the cytoplasm. Another potential explanation for the hypomorphic phenotype is that these cells are endocycling and with each cell cycle there is an increase in the copy number of the *SLBP* locus, this increase in copy number might produce enough SLBP to meet the demands of processing. This would potentially explain why mis-processing is seen in the first stages of oogenesis but not seen in the later stages off oogenesis because the increase in *SLBP* copy number allows for the production of enough SLBP to handle processing in the later stage egg chambers. However while no mis-processing is seen in the later staged egg chambers, there is still a decrease in the total amount of histone mRNA produced in the later stage egg chambers and there is a large decrease in the amount of maternal histone mRNA deposited into the embryo. One potential explanation for the large decrease in the amount of maternal

histone mRNA deposited into the egg despite the histone mRNA being processed normally is that during the final stages of oogenesis there is a large final rapid burst of histone mRNA transcription that is not associated with active DNA replication, and the function of this last burst is to produce the maternally loaded histone mRNAs to be loaded into the oocyte to support the first two hours of development for the embryo (Ambrosio & Schedl, 1985; Ruddell & Jacobs-Lorena, 1985). Our data suggests that when the nurse cells are endocycling there is enough SLBP to support the proper production of histone mRNA, however during this final burst there is not enough SLBP present to produce enough histone mRNA to be loaded into the egg to support the early hours of development. Since no mis-processing of the histone transcripts are seen during this phase it could possibly suggest that due to the limited amount of SLBP the rate of pre-mRNAs that can be cleaved into mature histone mRNAs is less and because of this not enough mature histone mRNA can be produced during this final quick burst to support the early stages of embryogenesis.

Acknowledgements

I would like to thank David Lanzotti for technical help with the experiments performed in this chapter. I would also like to thank Ryan Zimmerman for his work in making the *SLBP* germline clones and for the northern and *in situ* analysis of these ovaries.

Chapter III

An Essential Post-Processing role for SLBP in Histone Metabolism

Contributions:

The WT SLBP construct along with the Δ179 construct were made by David Lanzotti. The Δ35, 186 and 179 constructs were made by Nihal Cackmacki. The constructs were injected into flies by members of the Duronio lab. I made stocks for each transgene, crossed transgenes into *SLBP* mutant background and scored viability along with Andy Courson. I generated all data for the *in situ* hybridizations, western blot and northern blots.

Summary

In vitro studies have indicated that only the C-terminus of *Drosophila* SLBP is required for its activity to process histone pre-mRNAs (Dominski et al., 2002). While studies in vertebrate systems have shown that the N-terminus of SLBP is required for the efficient translation of histone transcripts *in vitro*, no function has been ascribed to the N-terminus of *Drosophila SLBP* or to the N-terminus *in vivo*. To determine if the N-terminus of *SLBP* is required for viability, nested deletions of the N-terminus were made and assayed for their ability to rescue viability in an *SLBP* mutant background and for their ability to rescue the loss of histone pre-mRNA processing in an *SLBP* mutant background. A 53

amino acid region between amino acids 86 and 139 was found to be essential to rescue viability but dispensable for rescuing histone pre-mRNA processing, strongly suggesting that this region of SLBP is involved in an aspect of histone metabolism other than pre-mRNA processing that is required for viability of the organism.

Background

Metazoan replication dependent histone mRNA's are the only mRNAs that do no end in a poly(A) tail, instead ending with a conserved stem loop sequence (Dominski & Marzluff, 1999). In vertebrates, the 3' end of mature histone mRNA is formed by an endonucleolytic cleavage that requires both the stem loop and the histone downstream element (Strub et al., 1984; Gick et al., 1986; Mowry & Steitz, 1987). The stem loop is bound by SLBP while the HDE is bound by the U7 snRNP which together recruit an endonuclease complex consisting of members of the CPSF complex and symplekin (Dominski et al., 2005a; Kolev & Steitz, 2005). Once the mature histone mRNA is produced SLBP remains bound to the stem loop and is carried out to the cytoplasm, where in vertebrate systems it appears that SLBP is required for the efficient translation of the histone message (Sanchez & Marzluff, 2002).

In *Xenopus* there are two SLBPs, SLBP1 and SLBP2. Studies have shown that SLBP1 is capable of supporting translation while SLBP2 is an oocyte specific form of SLBP that binds to the stem loop of histone messages that are stored during early oogenesis and prevents translation of the message until it gets degraded as the oocyte matures and allows activation of translation by SLBP1 (Sanchez & Marzluff, 2002). Previous studies have identified a 15 amino acid region of SLBP1 that is required for the efficient translation of histone transcripts; deletion or mutation of this sequence completely abolishes translation of

histone reporter transcripts *in vitro* (Sanchez & Marzluff, 2002). Furthermore recent unpublished studies have shown that an 8 amino acid motif found within the 15 amino acid region of xSLBP, **DW**X_3**E**E, which is conserved in many species including Human, Ciona, and Sea Urchin SLBP, found that mutation of the bold amino acids abolishes translation of histone reporter transcripts *in vitro* (Cakmakci et al. unpublished data). It was also found that in both Human and *Xenopus* a novel protein termed SLIP-1 (Slbp Interacting Protein) is also required for the efficient translation of histone reporter mRNAs *in vitro*, and was shown by yeast two hybrid as well as by pull down assays in HeLa cell extracts to bind to SLBP. Furthermore it was shown that if the conserved **DW**X_3**E**E motif was mutated by deletion or by mutating the bold motifs to alanine that SLIP-1 is no longer able to bind to SLBP in a yeast two hybrid assay. SLIP-1 was also shown both *in vitro* and by *in vivo* pull down assays to be able to bind to eIF4G, suggesting that the function of SLIP-1 is to bring the 3'end of the histone transcript to the 5' end of the transcript by serving as a bridge between SLBP and eIF4G to allow the transcript to be circularized for efficient translation (Cakmackci et al. unpublished data). Therefore it appears that SLBP acts analogously on histone messages to how Poly (A) Binding Protein (PABP) acts on polyadenylated messages to allow for the circularization of the transcript. Therefore it appears in vertebrate systems that the N-terminus of SLBP is required for the efficient translation of histone transcripts.

As mentioned previously there is little conservation between *Drosophila SLBP* and human SLBP except for the RNA binding domain and studies in *Drosophila* have yet to establish any functional role for the N-terminus of *SLBP*. Based on the recent data showing that a region of the N-terminus of Human and *Xenopus* SLBP is required for translation of the histone transcripts it is intriguing to think that *Drosophila SLBP* may also contain a

region in its N-terminus that potentially plays a role in the translation of histone transcripts in *Drosophila*. However since again there is no significant homology between the N-terminus of *Drosophila* SLBP and human SLBP it is not possible to identify a clearly conserved translation activation domain in *Drosophila*. We therefore hypothesized that if the N-terminus provided an essential function to histone metabolism, such as regulating translation, then removal of this function should be lethal to an organism. Therefore to try and find regions of the N-terminus of *Drosophila* SLBP that are essential for viability, nested deletions of the N-terminus of *Drosophila* SLBP were made and injected into flies. These deletions were assayed for their ability to rescue the lethality associated with mutation of *SLBP* and for their ability to rescue histone pre-mRNA processing. A deletion that fails to complement lethality in a *SLBP* mutant fly but is able to rescue histone pre-mRNA processing would suggest that the deleted region plays an essential role other than in histone pre-mRNA processing, such as stimulating translation of histone transcripts or some other novel role for *SLBP* in histone metabolism during the development of the fly.

Experimental Methods

Northern analysis

For northern blots total cellular RNA was isolated from wildtype, SLBP15, Δ35, Δ86, Δ139, Δ179 homozygous mutant larve with TRIzol Reagent (Gibco). For the analysis of histone mRNAs, 2 ug of RNA per lane were subjected to electrophoresis in a 1X MOPS in a 1% agarose gel containing 0.01MOPS(PH 7.0) and 6.75% Formaldehyde. Separated RNAs

were transferred to an N+ nitrocellulose membrane (Amersham) using the wick method in 20X SSC. DNA containing histone H3 or rp49 coding regions were labeled with $\alpha[^{32}P]$-dCTP using a random primer labeling kit (Stratagene). Hybridizations were performed at 60 °C using Quikhybe (Stratagene).

Western analysis

For protein extraction, adult female flies were collected from homozygous transgenic lines. The flies were ground in a solution of lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, 50mM NaF) and protease inhibitors (1:1000 leupeptin, 1:100 pepsin, and 1:100 PMSF). Sodium dodecyl sulphate (SDS) containing sample buffer was then added. One hundred micrograms of protein from each extract were loaded into each lane and resolved on a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane and blocked with 5% milk for 1 hour at RT, then probed with 1:1000 mouse monoclonal anti-HA(Covance, Denver CO) and 1:1000 sheep anti-mouse horseradish peroxidase antibody(Amersham, Piscataway NJ) for 1 hour at room temp. For loading control, blots were probed with 1:2000 mouse anti-beta tubulin (Amersham Biosciences).

In situ hybridizations

Embryos were collected and aged at 18 °C until the appropriate stage and were dechorionated in 50% bleach and fixed in a 1:1 mixture of Heptane and 4% Formaldehyde for 15 minutes. *In situ* hybridization was performed using digoxigen-labeled riboprobes complimentary to the ds- region of H3 as previously described. (Lanzotti et al., 2002).

Fly Stocks and Crosses

All deletion constructs are N-terminal HA tagged, the following constructs have three HA tags on the N-terminus FL-SLBP and Δ179.

The following cross is a representative cross as to how the deletion transgene were crossed in an SLBP background.

Cross 1: ♀ p[Δ *Slbp*]/p[Δ *Slbp*] ; +/+ X ♂ +/+ ; *D*/TM3 *Sb, Ser*

↓

Cross 2: ♀ p[Δ *Slbp*]/+ ; +/ TM3 *Sb, Ser* X ♂ +/+ ; *Slbp* ^{15}/TM3 *Sb [act-GFP]*

↓

Cross 3: ♀ p[Δ *Slbp*]/+ ; *Slbp* ^{15}/TM3 *Sb, Ser* X ♂ p[Δ *Slbp*]/+ *Slbp* ^{15}/TM3 *Sb Ser*

↓

Products: ♀ and ♂ p[Δ *Slbp*]/ p[Δ *Slbp*] ; *Slbp* ^{15}/ *Slbp* ^{15}

Results

A 53 Amino acid region of the N-terminus of SLBP is required for viability

To determine if a region of SLBP's N-terminus is required for viability, nested deletions of the N-terminus were made that removed the first 35 amino acids (Δ35), the first 86 amino acids (Δ86), the first 139 amino acids (Δ139) and the first 179 amino acids (Δ179) (Figure 3.1). To determine if these deletions removed a region of the N-terminus that is required for viability these deletion constructs and a wild type transgene for SLBP were injected into flies and then crossed into an *SLBP* ^{15} null background, except for the Δ86 transgene which is in a background of *SLBP* ^{15}/*SLBP* ^{12} (the Δ86 transgene exists as a recombinant chromosome with the *SLBP* ^{12} , a null deletion allele of *SLBP*) and the ability for each deletion to complement the lethality associated with loss of SLBP was measured.

The wild type transgene along with two deletions, Δ35 and Δ86 were able to fully rescue viability in an *SLBP* mutant background, while the Δ139 and Δ179 deletions were not able to rescue viability in an *SLBP* mutant background to any extent (Table 3.1). This result suggests that there is a region between amino acids 86 and 136 in the N-terminus of SLBP that is required for viability. To make sure that the inability of Δ139 and Δ179 to rescue viability in a *SLBP* mutant background was not due to the transgenes not being expressed western analysis was performed to determine expression. Expression of all the transgenes could be detected by probing for the HA tag on the N-terminus of all the transgenes except for the Δ86 deletion (Figure 3.2) However since this transgene is able to genetically rescue viability in an *SLBP* mutant background it suggests that this transgene is being expressed, but at levels below detection by western blot, but more importantly the transgenes that failed to rescue viability were expressed at levels similar to that of the wild type transgene, indicating that the inability of these transgenes to rescue viability in an *SLBP* mutant background is not due to the transgenes not being expressed.

In vitro studies have shown that the RNA binding domain of SLBP is sufficient to carry out histone pre-mRNA processing in *in vitro* processing extracts, (Dominski et al., 2002). However in an *in vivo* setting it is not known how much of the SLBP protein is required to process histone pre-mRNAs or if the N-terminus plays any role in pre-mRNA processing. One potential reason that the Δ139 and Δ179 deletions may not be able to rescue viability is that the deletion of large sections of the N-terminus renders the protein incapable of functioning in histone pre-mRNA processing. Therefore to determine if all the deletion constructs are able to rescue histone pre-mRNA processing in an *SLBP* mutant background,

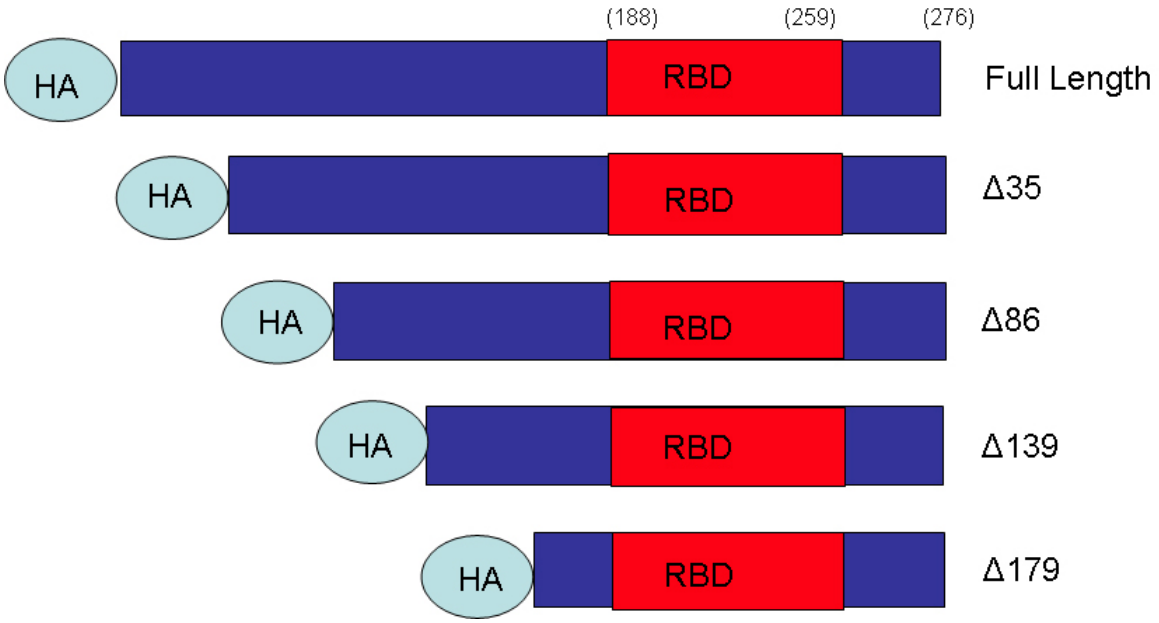


Figure 3.1 Schematic showing N-terminal deletions of SLBP. All deletion constructs have an N-terminal HA tag. The RBD box delineates the RNA binding domain of SLBP starting at amino acid 188 and ending at amino acid 259.

Table 3.1 Ability of N-terminal deletions to rescue viability in an *SLBP* mutant background.

Transgene	Rescues Viability?
WT-SLBP	YES
Δ35	YES
Δ86	YES
Δ139	NO
Δ179	NO

in situ hybridizations with the H3-ds probe, which only recognizes mis-processed H3, were performed on $SLBP^{15}$ homozygous embryos that carry two copies of each transgene, except for the Δ86 transgene which is in an $SLBP^{15}/SLBP^{12}$ trans heterozygous background and only carries one copy of the Δ86 transgene due presence of the transgene on a recombinant chromosome with $SLBP^{12}$. The WT transgene appears to significantly rescue histone pre-mRNA processing in the central nervous system. However a fair amount of mis-processed H3 is still seen in the gut (Figure 3.3 C) while the Δ86 line appears to rescue processing with virtually no mis-processing seen in the Δ86 embryos (Figure 3.3 D). In the Δ35 embryos no distinguishable *in situ* signal was seen compared to background (data not shown) indicating that processing might be fully rescued with this deletion. In both Δ139 and Δ179 embryos a staining pattern similar to that of the WT-transgene was seen with apparent rescue of processing in the CNS, but with a fair amount of mis-processing seen in the gut (Figure 3.3 E, F). To more precisely examine the amount of rescue of histone pre-mRNA processing by the deletion constructs, northern analysis was performed on RNA extracted from SLBP mutant 3rd instar larvae containing each of the transgenes. Surprisingly, all the deletion constructs were able to significantly rescue the levels of processing of H3 to levels that were equal or better to the amount of processing seen by the WT SLBP transgene (Figure 3.4). Both the Δ139 and Δ179 constructs appear to rescue processing just as well as the WT transgene, but these transgenes still fail to rescue viability. This result strongly suggests that the inability of these two deletions to rescue viability is not due to the constructs' inability to participate in processing, but some other essential function. Furthermore Δ179's ability to rescue processing confirms the *in vitro* result that only the C-terminus, which contains the

RNA binding domain, of SLBP is required for SLBP's ability to process histone pre-mRNAs.

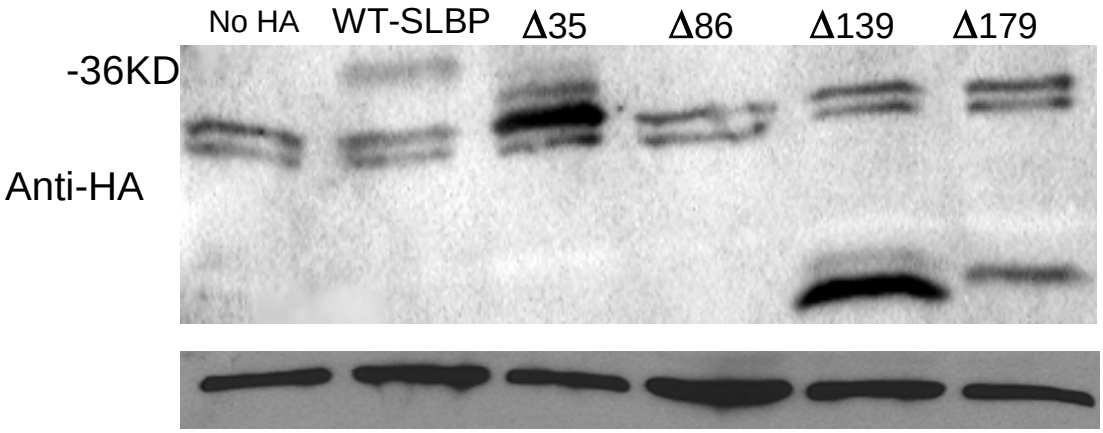


Figure 3.2 Western blot showing expression of N-terminal deletion constructs in 3rd instar larvae. Protein extracts were taken from 3rd instar larvae and blotted and probed with anti-HA. Lane one is an extract from wt larvae that have no transgene. Asterisks in all lanes denote the tagged protein. The Δ179 deletion in lane 6 runs at a higher mobility than the Δ139 deletion in lane 5 due to Δ179 construct containing 3 HA tags on the N-terminus versus a single HA tag for Δ139. Expression of the Δ86 construct could not be detected in lane 4, despite being able to rescue viability. Beta-tubulin was probed as a loading control. Bands seen in all lanes including the non HA lanes are nonspecific bands.

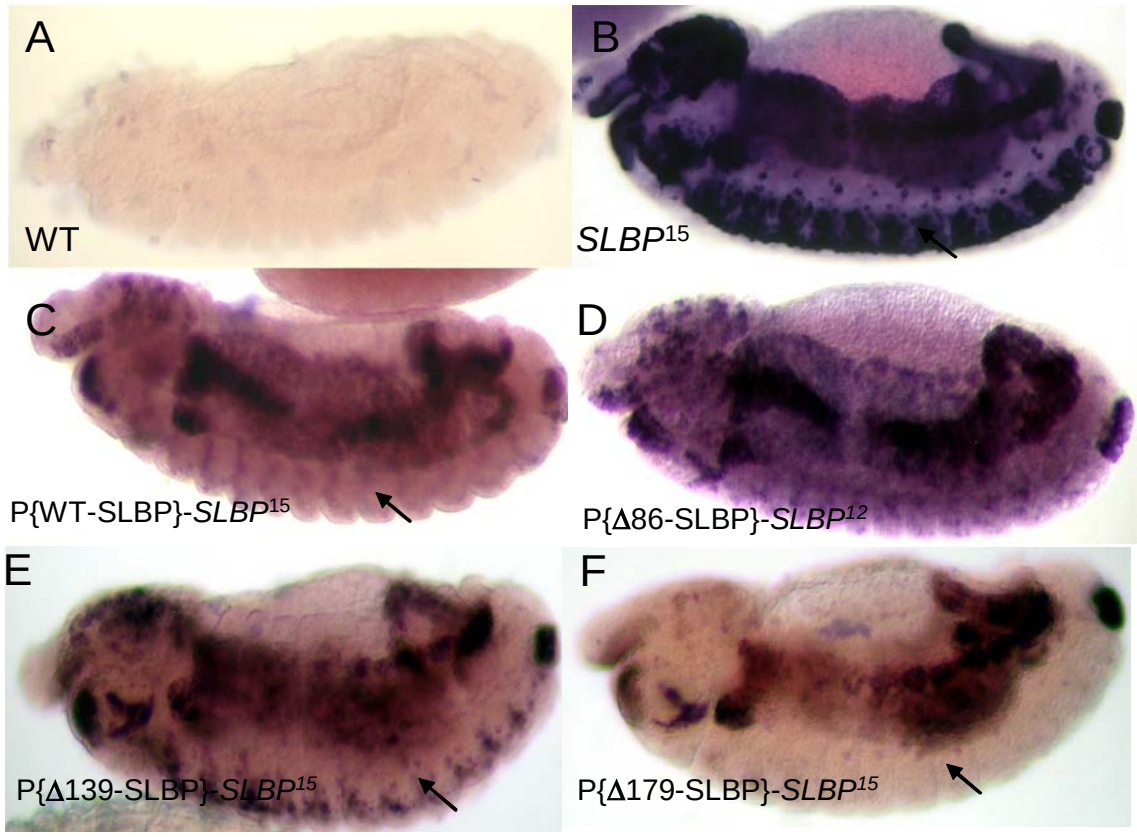


Figure 3.3 *In situ* hybridizations on stage 13 embryos with the H3-ds probe. (A) No mis-processed H3 is detected in wt embryo. (B) Large amounts of mis-processed H3 can be detected in an *SLBP*^{15} mutant embryo. (C) The full length SLBP transgene is able to rescue processing of H3 in the central nervous system when crossed into an *SLBP*^{15} mutant background. (D, E, F) The Δ86, Δ139, Δ179 constructs all appear to rescue some mis-processing of H3 when crossed into an SLBP mutant background particularly in the CNS. Arrows in (C, D, E, F) show the central nervous system

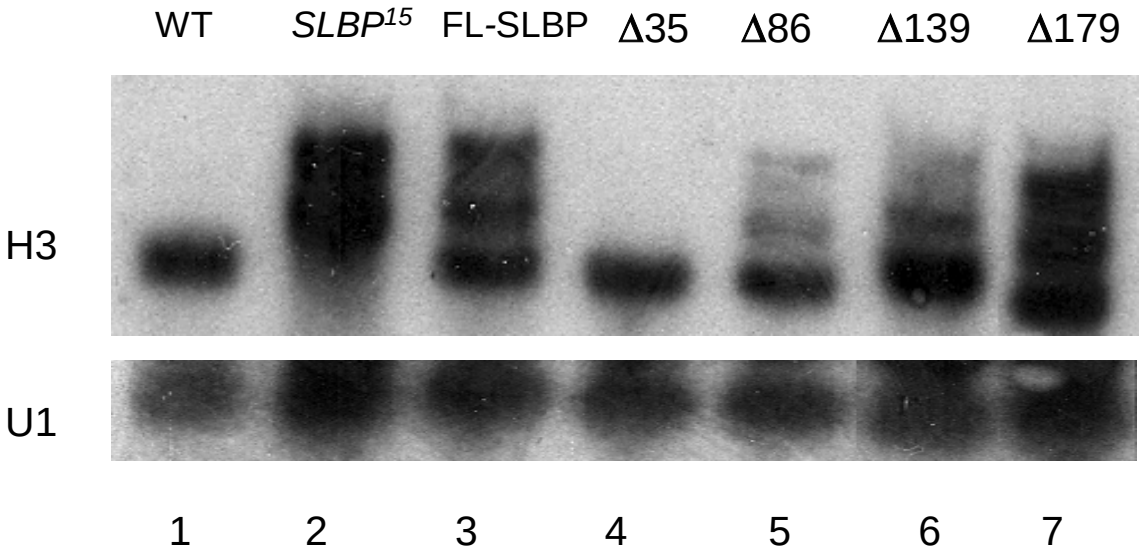


Figure 3.4 Northern analysis showing rescue of histone pre-mRNA processing in deletion constructs. RNA was extracted from *SLBP* mutant 3rd instar larvae that contained two copies of the indicated deletion transgene, except for Δ86 where only one copy of the transgene is present. The blot was probed with H3. Notice that all deletion transgenes are able to rescue processing as well as the Full length SLBP transgene in lane 3, even the Δ139 and Δ179 transgenes in lanes 6 and 7 which fail to rescue viability. The U1 snRNA was probed for a loading control.

Discussion

While in vertebrates recent studies have shown that a region of the SLBP's N-terminus is required for the efficient translation of histone transcripts, currently in *Drosophila* there is no known function for the N-terminus of SLBP with *in vitro* studies indicating that only the C-terminal RNA binding domain of *Drosophila* SLBP is required to process histone pre-mRNAs. This report demonstrates for the first time that a 53 amino acid region of SLBP's N-terminus is required for viability, and that loss of this 53 amino acid region does not disrupt *SLBP*'s ability to process histone pre-mRNAs *in vivo,* demonstrating that this region of the N-terminus has an essential function for viability in the fly that is not related to *SLBP*'s role in processing.

A region of SLBP's N-terminus is required for viability

We find in this study that a region of *Drosophila* SLBP's N-terminus is required for viability of the organism. This region lies within a 53 amino acid stretch between amino acids 86 and 139. Furthermore this region required for viability is dispensable for SLBP's role in histone pre-mRNA processing indicating that this region is involved in some other process that is required for the viability of the organism. It is highly likely that this region of the N-terminus of SLBP essential for viability indicates that SLBP does function in more than its role in processing and that this new role for SLBP is essential to the organism. Since SLBP has not been found to bind any RNAs except the replication dependent histone mRNAs it is very likely that we have uncovered a new role for *Drosophila* SLBP in histone metabolism, however we can't formally exclude a potential role for SLBP outside of histone metabolism (Townley-Tilson et al., 2006). Based on the data from mammalian systems it is

intriguing to think that this 53 amino acid region of SLBP might also contain a motif that is required for the efficient translation of histone transcripts. There is an apparent homologue of SLIP-1 in the Drosophila genome (CG13124) so it is possible that *Drosophila* could use the same machinery as in higher organisms to make sure histone mRNA transcripts are translated efficiently. Studies are currently in progress to investigate if this 53 amino acid region of SLBP interacts with the apparent *Drosophila* homologue of SLIP-1.

Acknowledgements

I would like to thank Dave Lanzotti for making the WT and Δ179 deletions, and Nichal Cackmacki for making the Δ 35, 86, and 139 deletions. I would also like to thank Andy Courson for performing the complementation assays with the nested deletions.

CHAPTER IV

Genetic Characterization of Drosophila Snipper: a member of the 3'hExo/ERI-1 Family of Nucleases

Contributions: The figures for this paper are representations from Kupsco et al. (2006) RNA. Roopa Thapar and Ming Wu performed the Exonuclease assay and Gel Shift experiments in figures 4.2 and 4.3. I performed all *in vivo* characterizations of Snipper found in subsequent figures.

SUMMARY

The DnaQ-H family exonuclease Snipper (Snp), encodes a 33 kDa *Drosophila melanogaster* homologue of 3'hExo and ERI-1, exoribonucleases implicated in the degradation of histone mRNA in mammals and in the negative regulation of RNA interference (RNAi) in *C. elegans*, respectively. In metazoans, Snp, 3'hExo, and ERI-1 define a new sub-class of structure-specific 3'-5' exonucleases that bind and degrade double stranded RNA and/or DNA substrates with 3' overhangs of 2-5 nucleotides in the presence of Mg^{2+} with no apparent sequence specificity. These nucleases are also capable of degrading linear substrates. We identified a *Snp* mutant and used it to test whether Snp plays a role in regulating histone mRNA degradation or RNAi *in vivo*. *Snp* mutant flies are viable and display no obvious developmental abnormalities. The expression pattern and level of histone

H3 mRNA in *Snp* mutant embryos and 3rd instar imaginal eye discs was indistinguishable from wild type, suggesting that Snp does not play a significant role in the turnover of histone mRNA at the end of S-phase. The loss of *Snp* was also unable to enhance the silencing capability of two different RNAi transgenes targeting the *white* and *yellow* genes, suggesting that Snp does not negatively modulate RNAi. Therefore, Snp is a nonessential exonuclease that is not a functional orthologue of either 3'hExo or ERI-1.

Background

Replication-dependent histone biosynthesis is tightly coupled to DNA replication during S-phase to ensure the rapid deposition of newly synthesized histones at the replication fork (Marzluff & Duronio, 2002). Cells cannot tolerate breakdowns in this coupling. For example, a delay between DNA synthesis and histone deposition in yeast leads to loss of viability, while in human cells a delay leads to spontaneous DNA damage and S-phase arrest (Han et al., 1987; Nelson et al., 2002). Furthermore, the production of the core histones outside of S-phase causes increased DNA damage sensitivity, chromosome loss, and impaired growth in yeast (Gunjan & Verreault, 2003).

The regulated destruction of histone mRNA is an important aspect of the coupling of histone mRNA abundance to both the cell cycle and the rate of DNA synthesis. In mammalian cells the steady state level of histone mRNA increases approximately 35-fold as cells progress from G1-S. This results from an increase in both the synthesis and half life of histone mRNA (Harris et al., 1991). As cells exit S phase, histone mRNA synthesis terminates and existing mRNAs are rapidly degraded. Histone mRNAs are also rapidly

destroyed after treating cells with inhibitors of DNA replication such as hydroxyurea (Sittman et al., 1983).

While the precise mechanism of histone mRNA degradation is unknown, the 3' end of histone mRNA is the cis element responsible for regulating histone mRNA degradation (Pandey & Marzluff, 1987). Metazoan replication dependent histone mRNAs are the only mRNAs that do not end in a polyA tail, but instead end in a 26 nucleotide stem loop structure (Dominski & Marzluff, 1999). The stem loop binds Stem Loop Binding Protein (SLBP), and the SLBP-histone mRNA complex plays an important role in the coordinate regulation of histone mRNA processing, translation, and stability. Ongoing translation is required for the regulated destruction of histone mRNA (Graves et al., 1987; Kaygun & Marzluff, 2005a, 2005b). SLBP may also participate in the destruction of histone mRNAs in response to replication inhibitors by recruiting the nonsense mediated decay factor UPF1 in response to ATR checkpoint kinase activation (Kaygun & Marzluff, 2005a).

Recently, an exoribonuclease termed 3'hExo was identified as a candidate regulator of histone mRNA degradation in mammalian cells. 3'hExo binds the histone mRNA stem loop, and removes nucleotides in the 3' flanking region of histone mRNA (Dominski et al., 2003). The 3'hExo is also capable of forming a ternary complex with stem loop histone mRNA and human SLBP. However, as yet there is no evidence that 3'hExo participates in histone mRNA destruction *in vivo*. Intriguingly, the closest *C. elegans* homologue of 3'hExo, ERI-1, has been implicated as a negative regulator of RNAi (Kennedy et al., 2004), and loss of ERI-1 leads to enhancement of gene silencing by exogenous dsRNAs. However, the precise role of ERI-1 in the RNAi pathway (Duchaine et al., 2006) or whether it has a role in histone mRNA metabolism is not clear.

Experimental Methods

Alignments and Sequences

To obtain potential homologues of Snipper, the Snipper protein sequence (NP_611632) was subjected to the NCBI BLASTp program (http://www.ncbi.nlm.nih.gov/BLAST), and the following sequences were obtained from the BLAST search: ERI-1 Ce(O44406), 3'hExo (NP_699163), Crn-4(NP_508415), AAH10503, Prion Protein interacting Protein Hs (NP_076971), Prion Protein interacting Protein Ce (AAB94148), RNaseT (AAC37008). To determine the closest homologs of Snp in the DnaQ-H superfamily, a multiple sequence alignment was generated using ClustalX 1.83 (Jeanmougin et al., 1998) and a phylogenetic tree was produced using ClustalX1.83 NJ-Bootstrap Tree option with a Bootstrap of 1000, the tree was viewed and edited using the NJ Plot program (M. Gouy). Members of the DEDDh family of exonucleases have a low level of sequence identity, therefore the reliability of the sequence alignment was tested by generating a multiple structure alignment using the program Combinatorial Extension (CE) (Shindyalov & Bourne, 2001) using the structures of the nuclease domains of 3'hExo (PDB code 1W0H), epsilon (PDB code 1J53), and oligoribonuclease (PDB code 1J9A) as templates. This structure-based alignment was used as the input template to generate a multiple sequence alignment in the program T-coffee (Notredame et al., 2000). All approaches used produced similar dendograms suggesting that Snp is most closely related to the hypothetical human exonuclease AAH10503, followed by members of the 3'hExo subfamily.

Exonuclease activity assays.

Nuclease reactions (10 μl each) contained 20 mTris pH 7.5, 5 mM $MgCl_2$, 2 mM DTT, 100 mM KCl, 1 μl of a 10 mg/ml stock solution of bovine serum albumin, 5 pmol of 5'-^{32}P probe and between 5-100 pmol 3'hExo or Snp or buffer for the control reaction. The reactions were incubated at 37^{o}C for 30 min and stopped by the addition of 20 μl of stop/quench dye containing formamide to give a total volume of 30 μl. The reactions were heated at 95^{o}C for 5 min and 5 μl was loaded onto a pre-warmed 10% denaturing polyacrylamide gel.

Electrophoretic mobility shifts assays (EMSA)

Snp and 3'hExo, with or without SLBP proteins, were incubated with 28 nucleotide 5'end labeled ^{32}P stem-loop probe ($SL_{28,R}$) for 30 min on ice in binding buffer (20 mM Tris pH 7.9, 20 % glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.1 μg/μl BSA) for 10 min. The total reaction volume (10 μl) was analyzed on an 8% native polyacrylamide gel in Tris-borate buffer at 125 V for 1.5 hr. The gel was dried at 80^{o}C for 2 hrs and then exposed to film.

Fly Stocks and Characterization of CG6393 Expression

Fly stocks EP(2)2457, and EY84235, along with *da*-Gal4 and DNaseII^{lo} flies were obtained from the Bloomington Stock center, while Pbacc00465 and Df(2R)Excel7170 was obtained from the Exelixis collection of insertions at the Bloomington Stock Center. Dicer-2 stocks and GMR-wIR were kind gifts of Richard Carthew (Northwestern U.), and yIR transgenes were acquired from (Piccin et al., 2001), via Jeff Sekelsky (UNC-CH). First strand synthesis was performed on duplicate samples one containing RT (NEB), one without RT. RT-PCR was performed on embryos, 3rd instar eye discs and adult females for all fly

lines with an annealing temperature of 62°C for 30 cycles. RNA was isolated using TRIzol Reagent (Gibco). The following primers were used to amplify the Snp transcript: Primer A: 5' GGGCCATGGCTGCCTTGATAAAACTA3', Primer B: 5'GCGCGCTTAGCAGTAAACTTC 3', Primer C: 5' TCCAGCGAGCAGGGGATCCGT 3' Primer D: 5'CAACACAAGCCTGGGATTAAG 3'. Primers for the coding region of rp49 were as follows Forward primer: 5'ATCCGCCCAGCATACAGG 3', Reverse primer: 5'CTCGTTCTCTTGAGAACGCAG.

Northern Analysis

For northern blots total cellular RNA was isolated from third instar eye discs with TRIzol Reagent (Gibco). For the analysis of histone mRNAs, 2 ug of RNA per lane were subjected to electrophoresis in a 1X MOPS in a 1% agarose gel containing 0.01MOPS(PH 7.0) and 6.75% Formaldehyde. Separated RNAs were transferred to a N+ nitrocellulose membrane (Amersham) using the wick method in 20X SSC. DNA containing histone H3 or rp49 coding regions were labeled with α[^{32}P]-dCTP using a random primer labeling kit (Stratagene). Hybridizations were performed at 60 °C using Quikhybe (Stratagene).

In Situ hybridization and TUNEL labeling

Eye discs were dissected from wandering third instar larvae and were fixed in 4% formaldehyde. Embryos were collected and aged at 18° C until the appropriate stage and were dechorionated in 50% bleach and fixed in a 1:1 mixture of Heptane and 4% Formaldehyde for 15 minutes. *In situ* hybridization was performed using digoxigen-labeled riboprobes complimentary to the coding region of H3 as previously described. (Lanzotti et

al., 2002). For TUNEL labeling, embryos were fixed in a 1:1 mixture of paraformaldehyde and heptane for 20 min, then devitilineized by shaking in a 1:1 mixture of heptane and methanol. Embryos were then incubated in 100mM Sodium Citrate for 30 min at 60 ˚C for 30 min, then TUNEL labeling was performed using an In situ Cell Death Kit (Martini et al.).

RNAi Transgenes

For RNAi analysis the following fly crosses were performed. ♀GMRwIR/GMRwIR X ♂+/Y; $Snp^{c00465}/Snp^{c00465}$. Male off spring of the following genotype ♂GMRwIR/Y; Snp^{c00465}/+, were then crossed to ♀ $Snp^{c00465}/Snp^{c00465}$. The eye colors of the resulting progeny were analyzed and photographed with a Nikon digital camera. An identical crossing scheme was used to analyze Dicer2's function on the GMRwIR, since Dcr2 is also on the 2nd chromosome. To analyze Snps possible function in RNAi using a yIR transgene the following crosses were performed. ♀$Snp^{c00465}/Snp^{c00465}$ X ♂+/+: yIR/yIR and ♀$Snp^{c00465}/Snp^{c00465}$ X ♂ +/+, da-GAL4/da-GAL4. The using the progeny from each class the following crosses were performed. ♀ Snp^{c00465}/+, yIR/+ X ♂ Snp^{c00465}/+, daGAL4/+. The resulting offspring were analyzed and photographed with a Nikon Digital camera.

RESULTS

A family of closely related metazoan exonucleases, including 3'hExo/ERI-1 and Drosophila Snp.

Nucleases have traditionally been classified based on the substrate hydrolyzed (DNA vs RNA), the mechanism of nucleolytic attack (endo vs exo), the hydrolytic products formed (oligonucleotides terminating in a 3'- or 5' phosphate), and the nature of the bond

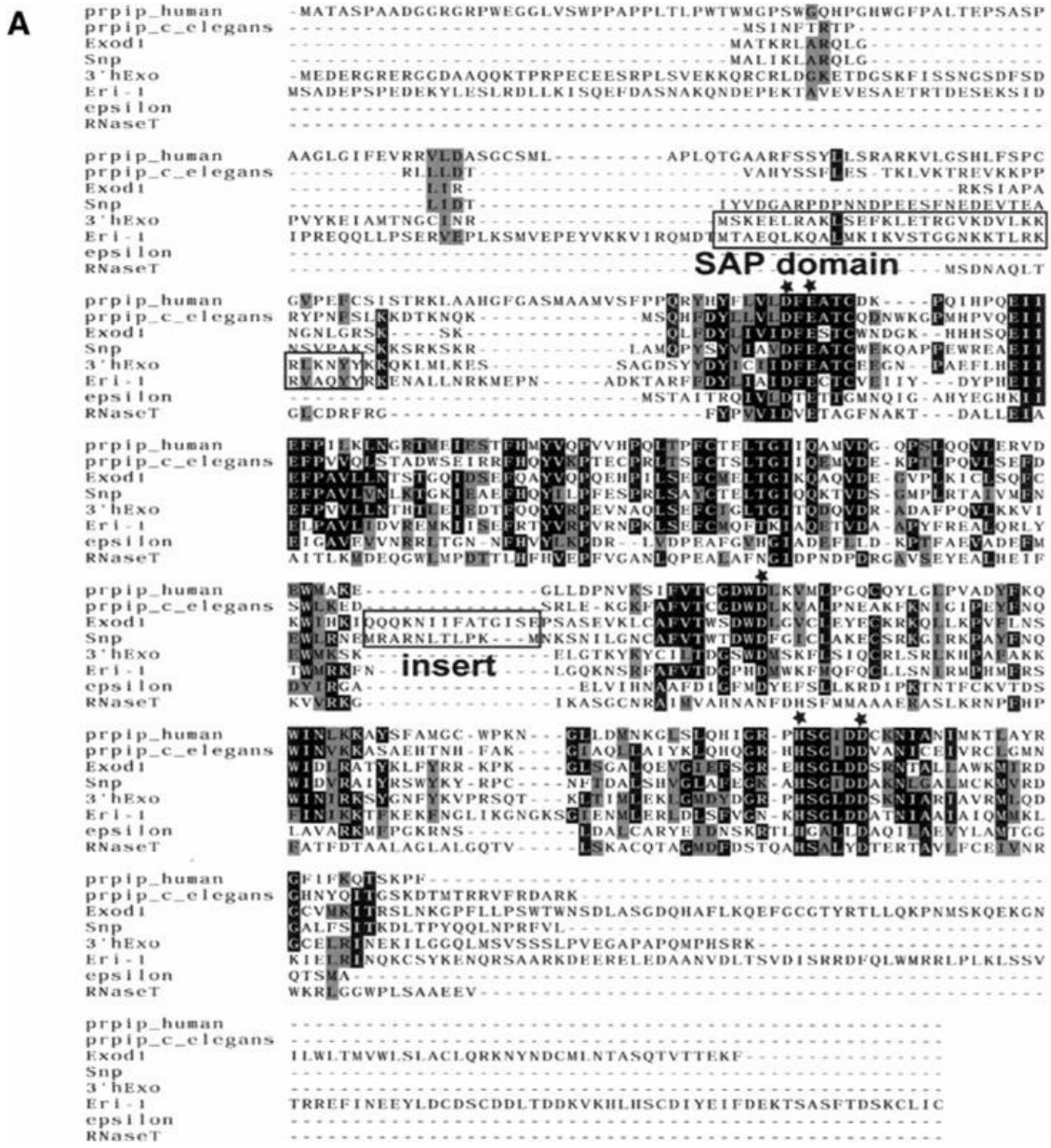
hydrolyzed. Based on these criteria, 3'hExo and ERI-1 belong to the DnaQ-H family (Viswanathan & Lovett, 1999) of 3' to 5' exoribonucleases that produce hydrolytic products releasing a nucleotide 5'monophosphate and leaving a 3'OH on the penultimate nucleotide. 3'hExo and ERI-1 share 38% sequence identity and 60% sequence similarity over all residues (and the same degree of identity and similarity between the respective nuclease domains). There are no other proteins that are homologous to ERI-1 that shows a similar tandem arrangement of an N-terminal SAP domain followed by an ExoIII domain in either the *C. elegans* or the human genome. The *Drosophila* genome contains only one exonuclease in the DnaQ-H superfamily homologous with ERI-1 and 3' hExo. This protein (Flybase ID CG6393) shares 31% sequence identity with 3'hExo as well as ERI-1 (Figure 4.1A, B). We named CG6393 "Snipper (Snp)" based on its potent exonucleolytic activity *in vitro* (see below). We also identified several other putative exonucleases in *C. elegans* and humans (Under "Alignments and Sequences" in Methods) that are closely related to 3'hExo, ERI-1 and Snp. These proteins comprise a metazoan-specific subfamily of exonucleases, which we call the 3'hExo/ERI-1 family.

A structure-based sequence alignment (Figure 4.1A, B) of members of the DnaQ-H superfamily of 3'-5' exonucleases (Viswanathan & Lovett, 1999) that includes replicative proofreading DNases such as Exo1 (Breyer & Matthews, 2000), epsilon (Hamdan et al., 2002) and Klenow (Ollis et al., 1985), RNases such as oligoribonuclease (Yu & Deutscher, 1995), 3'hExo (Dominski et al., 2003), and ERI-1 (Kennedy et al., 2004), and dual specificity nucleases such as RNase T (Deutscher, 2006) shows that Snp has a characteristic DEDDh motif that is essential for the catalytic activity of all these DnaQ-H family members.

This group of metazoan proteins share extensive sequence similarity and conservation of active site residues that are unique to this group of exonucleases.

Snipper is an Exonuclease that can bind the histone stem loop

Before we undertook the biological characterization of Snp, we sought to determine (i) whether Snp was indeed a 3'-5' exonuclease as predicted from its amino acid sequence, and if Snp was capable of interacting with the histone stem loop. Recombinant Snp was expressed in bacteria and purified to homogeneity. A RNA probe corresponding to the last 28 nucleotides of the histone stem loop was 5' labeled with P^{32} and incubated in buffer and in buffer plus recombinant Snp for 30 minutes (Figure 4.2). In the control buffer lane, there is very little background degradation of the stem loop probe, however in the Snp lane the probe is degraded in a 3'-5' manner and degradation intermediates of one nucleotide difference are seen. This indicates that *in vitro* Snp is a 3'-5' exonuclease and is capable of degrading the 3' end of histone mRNA. Since 3'hExo binds to the histone stem loop and forms a ternary couples with SLBP and the RNA, we asked whether Snp could form a complex with the histone stem loop in the presence of EDTA and whether Snp could form a ternary complex with *Drosophila* SLBP and the stem loop. When the stem loop probe was incubated with just buffer, no shift was seen (Figure 4.3 first lane), however when the stem loop probe was incubated with Snp, a shift was seen in the electrophoretic mobility of the probe, indicating that Snp is capable of binding to the stem loop (Figure 4.3 second lane). When Snp was incubated with the stem loop probe and SLBP no supershift was seen indicating that Snp does not form a ternary complex with SLBP and the histone stem loop (data not shown).


A
prpip_human
prpip_c_elegans
Exod1
Snp
3'hExo
Eri-1
epsilon
RNaseT
SAP domain
insert

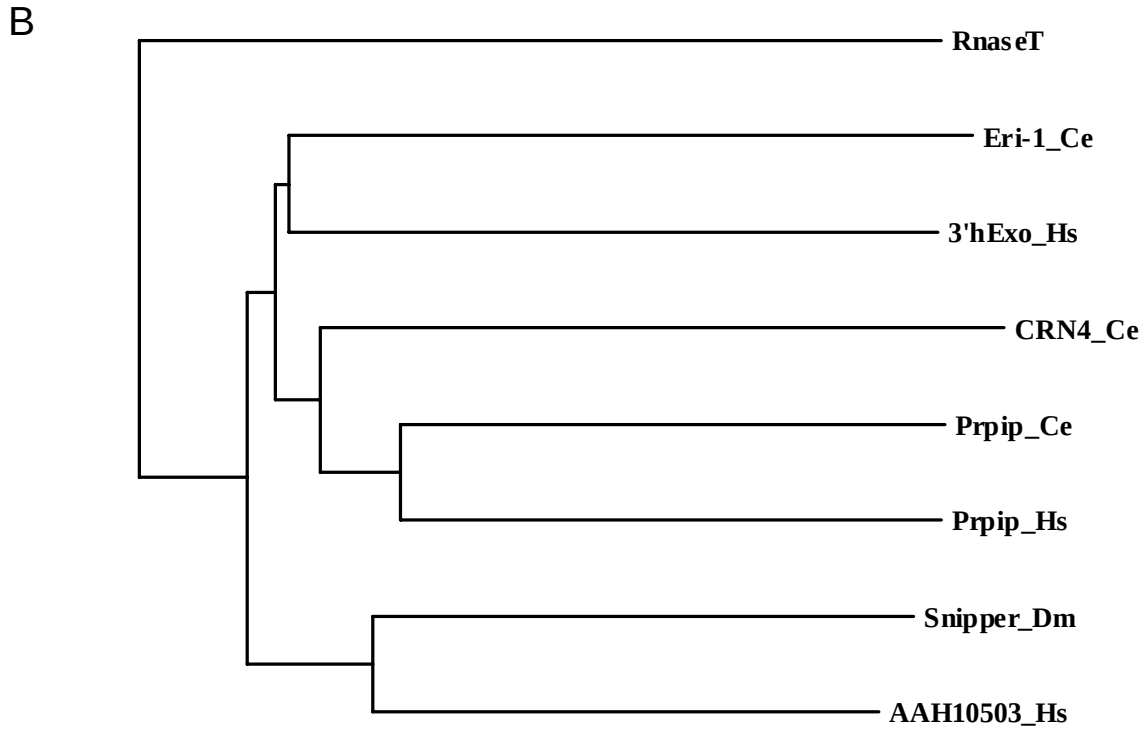


Figure 4.1. A) A structure-based alignment of 3'hExo/ERI-1 family of Exonucleases was generated using the programs CE and T-Coffee. Identical conserved residues are shown in black boxshade, similar conserved residues in grayshade and non-identical residues are left with a white background. The location of the SAP domain (present only in 3'hExo and ERI-1) and the 13 residue insert (present only in Snp and Exod1) are boxed. The conserved DEDDh residues that coordinate the divalent metal ion cluster are highlighted with black stars. There are several residues that are conserved in Snp, Exod1, prpip, 3'hExo, and ERI-1 but not in epsilon or RnaseT. B) Rooted Phylogenetic tree produced from ClustalW and NJPlot, showing that Snp is closely related to the uncharacterized human protein, Exod1 (AAH10503) followed by prpip nucleases and then 3'hExo and ERI-1.

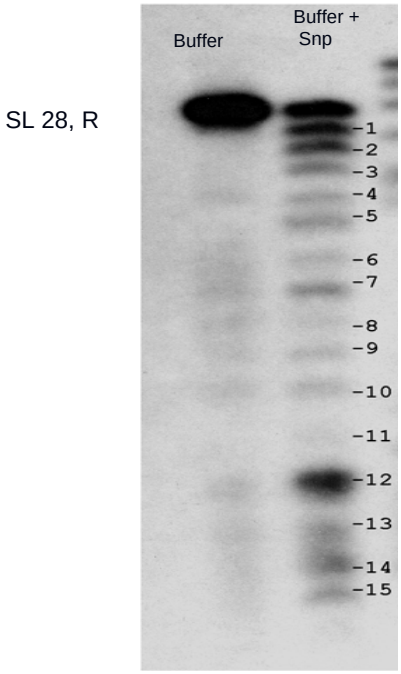


Figure 4.2. Snp acts as a 3-5' exonuclease. A synthetic RNA probe consisting of the last 28 nucleotides of the histone stem loop that is 5' labeled was incubated with buffer alone or in the presence of recombinant Snp protein for 30 min. In the buffer +Snp lane a single nucleotide laddering is seen indicating Snp is a 3-5' exonuclease.

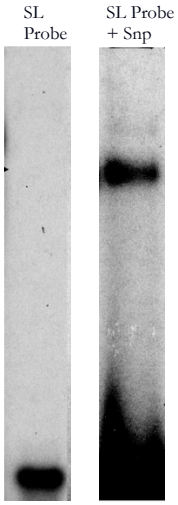


Figure 4.3 Snp is capable of binding to the 3' end of histone mRNAs. A synthetic RNA probe that corresponds to the last 28 nucleotides of a histone mRNA was 5' labeled and incubated in buffer alone or with recombinant Snp and buffer.

Characterization of the Snp gene and mutant alleles

To examine whether Snp plays a role in any of these processes *in vivo*, we characterized a mutant allele of *Snp* that was present in the public collection of transposon insertion alleles. We obtained two P-element transposon insertions in the *Snp* 5' UTR, EP2457 and EY08423, and one PiggyBac transposon insertion in the large first intron, c00465 (Figure 4.4A). All 3 insertions are viable and fertile as homozygotes or when placed in trans to Df(2R)Excel7170**,** which deletes the entire *Snp* locus (data not shown). RT-PCR was performed to determine if any of these transposable elements affected the expression of *Snp* (Figure 4.4B). A *Snp* RT-PCR product was detected using RNA isolated from wild type control and homozygous EP2457 and EY08423 adult female flies (Figure 4.4B, lanes 1-6). In contrast, this RT-PCR product was not detected in homozygous c00465 flies (Figure 4.4B, lanes 7-8). Similar data was obtained with RNA isolated from embryos or third larval instar imaginal discs (not shown). These data indicate that c00465 is a mutant allele of *Snp*, and that EP2457 and EY08423 affect *Snp* expression very little if at all. The absence of an RT-PCR product in c00465 samples suggests that this insertion may represent a null allele of *Snp*.

The c00465 PBac insertion is located in the first intron of *Snp*, 614 bp upstream of an open reading frame annotated as a distinct gene called CG30327 (Figure 4.4A). CG30327 is an intron-less open reading frame that has the potential to encode a protein of 407 amino acids that contains an RNA binding RRM domain. Because of this, and because 3'hExo contains a SAP domain upstream of the exonuclease domain, we hypothesized that CG30327 may be a differentially spliced exon of *Snp*, even though none of the known cDNAs of Snp include CG30327 sequence. To determine if CG30327 was an exon of *Snp*, RT-PCR was

performed with RNA isolated from wild type adult flies. We were unable to amplify a *Snp* transcript containing CG30327 from adult female RNA using two different primer pairings (Figure 4.4B, lanes 9-12). Thus, it appears that CG30327 is not an exon of *Snp,* and that *Snp* does not contain a RNA binding domain. In addition, we were unable to directly amplify a CG30327 mRNA using primers b and c, and there are no cDNA or EST sequences in current databases containing CG30327 sequence. These data suggest that, if transcribed, CG30327 mRNA accumulates to very low abundance or in very few tissues**.**

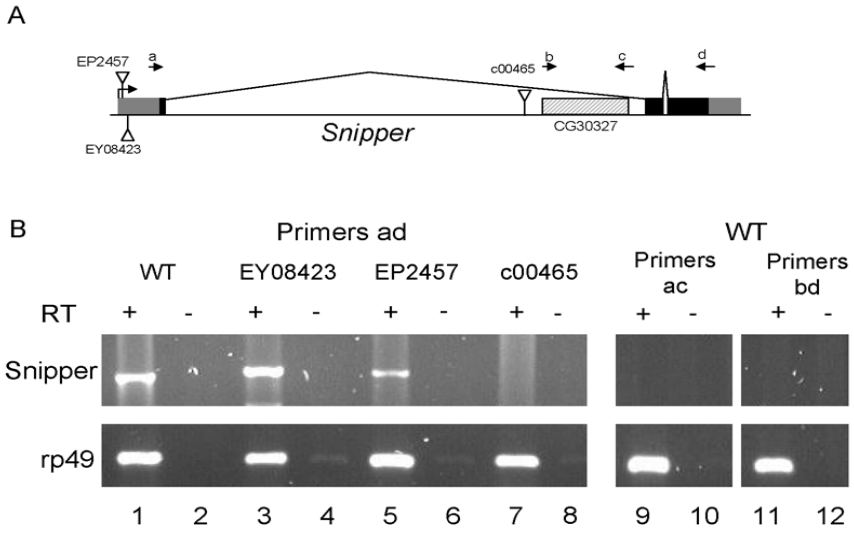


Figure 4.4. *Snp* expression in transposon insertion lines. A) Genomic locus of Snipper showing the intron and exons and the insertion sites of the three transposable elements characterized in this study. Arrows a through d indicate primers used for RT-PCR. B) Snp RT-PCR of total RNA isolated from homozygous adult females of the indicated genotypes using the indicated primers. Reverse transcriptase was omitted from the reaction in even numbered lanes.

Snp does not control histone mRNA degradation in vivo

Since Snp is capable of degrading histone mRNA *in vitro*, we examined histone mRNA expression in Snp^{c00465} mutants by *in situ* hybridization to determine whether Snp

affects histone mRNA accumulation *in vivo*. In endocycling cells of the embryonic midgut, histone mRNAs accumulate only during S phase, resulting in a dynamic but stereotypical pattern (Figure 4.5A). We have previously shown that in *Slbp* mutants histone mRNAs are not properly processed and become polyadenylated via the use of cryptic downstream polyadenylation signals (Lanzotti et al., 2002). In the embryonic midgut, these polyadenylated histone mRNAs are not rapidly destroyed at the end of endo S phase and inappropriately accumulate throughout the midgut in stage 14 embryos (compare Figures 4.5B and 4.5C) (Sullivan et al., 2001). This aberrant pattern provides a diagnostic for mis-regulation of histone mRNA destruction at the end of S phase. *In situ* hybridization of *Snp* mutant embryos with an H3 probe results in a wild type pattern of expression at stage 14 (Figure 4.5D), indicating that loss of *Snp* expression does not affect the accumulation of histone H3 mRNA in endocycling embryonic cells (Figure 4.5B, 4.5D).

To examine if mutation of *Snp* affects histone mRNA expression in a mitotic cell population, *in situ* hybridizations were performed with eye imaginal discs of third instar larvae. In eye discs there is a stereotyped pattern of development in which a subset of G1-arrested retinal precursor cells undergo a synchronous round of S-phase followed by cell division before terminally differentiating (Figure 4.5E). This synchronous S phase is part of a wave of differentiation called the morphogenetic furrow that sweeps across the eye disc epithelium, and can be easily visualized as a stripe of accumulation of histone H3 mRNA (Figure 4.5E). As in the embryo, the pattern of expression of H3 mRNA in *Snp* mutant eye discs appears identical to wild type, indicating that H3 mRNA accumulates only in replicating cells (Figure 4.5F). Consistent with this, northern blot analysis indicates that the total amount of H3 mRNA is similar between wild type and *Snp* mutant eye discs (Figure

4.5G). Based on the pattern of accumulation of H3 mRNA in *Snp* mutant embryos and eye discs, we conclude that Snp does not play a major role in the degradation of histone mRNA at the end of S-phase.

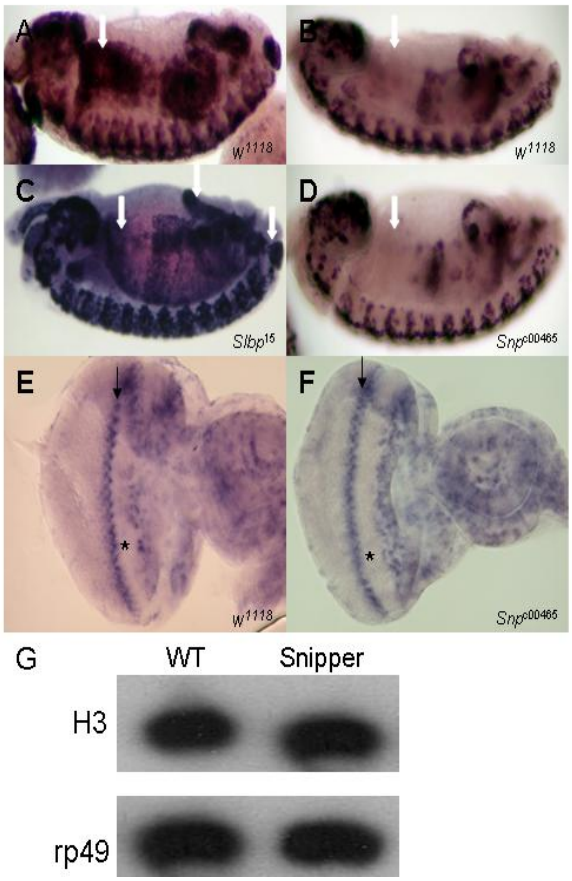


Figure 4.5. Histone mRNA expression is not affected in *Snp* mutants. A-B) In situ hybridization of w^{1118} stage 13 (A) and stage 14 (B) control embryos showing the wild type pattern of histone H3 expression. The arrows indicate the anterior midgut, which down regulates H3 expression by stage 14. C) Stage 14 $Slbp^{15}$ homozygous null mutant embryos with inappropriate H3 expression in the anterior midgut, hind gut and anal pads (left to right arrows, respectively). D) Stage 14 Snp^{c00465} homozygous mutant embryo obtained from homozygous mutant parents showing a normal pattern of H3 expression. Arrow as in (B). (E, F) H3 *in situ* hybridization of w^{1118} and Snp^{c00465} 3rd instar eye discs. The asterisks indicate the morphogenetic furrow, where all cells are arrested in G1. The arrows indicate the 2nd mitotic wave where all cells are undergoing a synchronous round of S-phase and express H3. (G) H3 northern blot with total RNA extracted from w^{1118} and Snp^{c00465} 3rd instar eye discs. rp49 serves as the loading control.

Snp does not regulate RNAi.

Mutations of *C. elegans* ERI-1 enhance the efficacy of RNAi through an unknown mechanism (Kennedy et al., 2004). To determine if Snp modulates the RNAi response to exogenous dsRNAs in *Drosophila*, we tested how Snp^{c00465} adult mutant flies responded to dsRNA producing transgenes. The first transgene, called GMR*w*IR, expresses a hairpin RNA of the third exon of the *white* gene under the control of an eye specific promoter (Lee & Carthew, 2003). One copy of GMR*w*IR silences *white* expression resulting in a faint orange eye color (cf. Figure 4.6A and 4.6B). Mutation of *Dicer-2*, which is required for the production of siRNAs from exogenous dsRNAs (Lee et al., 2004), suppresses the effect of GMR*w*IR and substantially restores eye pigmentation in GMR*w*IR flies (Figure 4.6C). If Snp acts as a negative regulator of RNAi, as does ERI-1, then *Snp* mutations should enhance the ability of GMR*w*IR to silence *white* expression, resulting in a white or pale yellow eye (Lee & Carthew, 2003). Homozygous *Snp* mutants containing GMR*w*IR had no further reduction in eye color when compared to siblings that are heterozygous for *Snp* (Figure 4.6D, 4.6E, 4.6F). This result suggests that Snp is not a modulator of RNAi.

To further test this, we employed a transgene (*y*IR) that expresses an inverted repeat of the *yellow* (*y*) gene under the control of a UAS element (Piccin et al., 2001). Expression of *y*IR with a *daughterless (da)*-Gal4 driver phenocopies the y^2 hypomorphic mutation, where the body of the fly is yellow and the bristles remain wild type in coloration (Fig. 4.6G-I). Other drivers (e.g. Actin-Gal4) of *y*IR result in a more widespread phenotype in which both the bristles and body turn yellow, as caused by a null y^1 mutation (Piccin et al., 2001). We expressed the *y*IR transgene under the control of *da*-Gal4 in a *Snp* mutant background to determine if the loss of *Snp* expression is able to enhance *y* silencing via the *y*IR construct

and produce yellow bristles or enhance the yellow body coloration. *Snp*^{c00465} mutant flies with *y*IR driven by *da*Gal4 showed no enhancement of bristle color or yellow body color when compared to heterozygous siblings (Figure 4.6 I, J). Therefore, the loss of *Snp* failed to enhance the silencing of two genes by RNAi, indicating that Snp unlikely plays a role in negatively regulating RNAi in *Drosophila*.

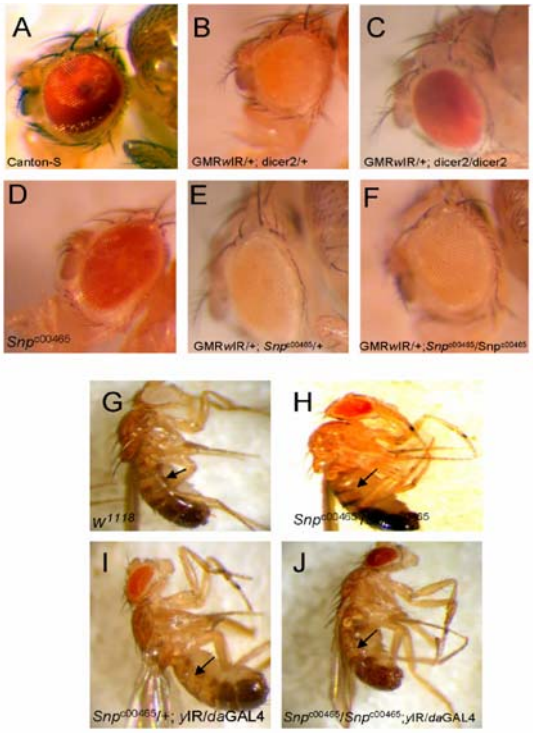


Figure 4.6. *Snp* mutation does not affect transgene-mediated RNAi silencing. Panels A-E contain a photomicrographs of an adult eye. A) Wild type Canton-S fly. B) A fly carrying one copy of the GMR-*w*IR construct, which silences the *white* gene and reduces pigment deposition, and heterozygous for a mutation of *Dicer-2*. C) A GMR-*w*IR fly homozygous mutant for Dicer-2. Note the restoration of a eye color. D) A *Snp*^{c00465} homozygous fly. The eye color results from the *mini-white* in the pBac transposable element. E) A fly carrying one copy of GMR-*w*IR and heterozygous for *Snp*^{c00465}. F) A fly carrying one copy of GMR-*w*IR and homozygous for *Snp*^{c00465}. Note that silencing of the *mini-white* gene by GMR-*w*IR is not affected by loss of *Snp* expression. G) A *w*^{1118} male control fly with wild type body color. H) A *Snp*^{c00465} homozygous male fly with wild type body color. I) A *yIR/da-GAL4* +/*Snp*^{c00465} male fly. Note that the thorax (arrow) is lighter than the controls (G,H) resulting from silencing of the *yellow* gene by activation of the yIR transgene with *da*-Gal4. J) A *yIR/da-GAL4 Snp*^{c00465}/*Snp*^{c00465} male fly. Note that there is no obvious change in coloration when compared to the fly in panel I.

Snp does not play a role in apoptosis.

CRN-4 is a *C. elegans* member of the ERI-1 subfamily of exonucleases that when mutated causes a persistence of TUNEL labeled DNA in apoptotic cells, suggesting that it plays a role in the degradation of apoptotic DNA (Parrish & Xue, 2003). To determine if Snp might play a role in digesting apoptotic DNA in *Drosophila*, TUNEL staining was performed on *Snp* mutant embryos and the amount and intensity of TUNEL staining in the *Snp* mutant embryos was compared to heterozygous siblings. We detected no obvious difference in the amount of TUNEL positive cells in *Snp* embryos compared to heterozygous siblings (Figure 4.7A and 4.7B). As a control, DNaseII mutant embryos were also stained with TUNEL. DNaseII is required for degradation of apoptotic DNA in *Drosophila*, and apoptotic DNA persists in embryos and ovaries (Mukae et al., 2002). We detected an elevated level of TUNEL positive apoptotic DNA in 32% (n=274) of the population collected from heterozygous parents (Figure 4.7C). These embryos likely represent DNaseII mutant embryos, while the embryos with normal levels of TUNEL positive nuclei are heterozygous siblings. This result indicates that we can score for differences in TUNEL labeling, and we therefore conclude that Snp does not play a major role in the clearance of apoptotic DNA in *Drosophila*.

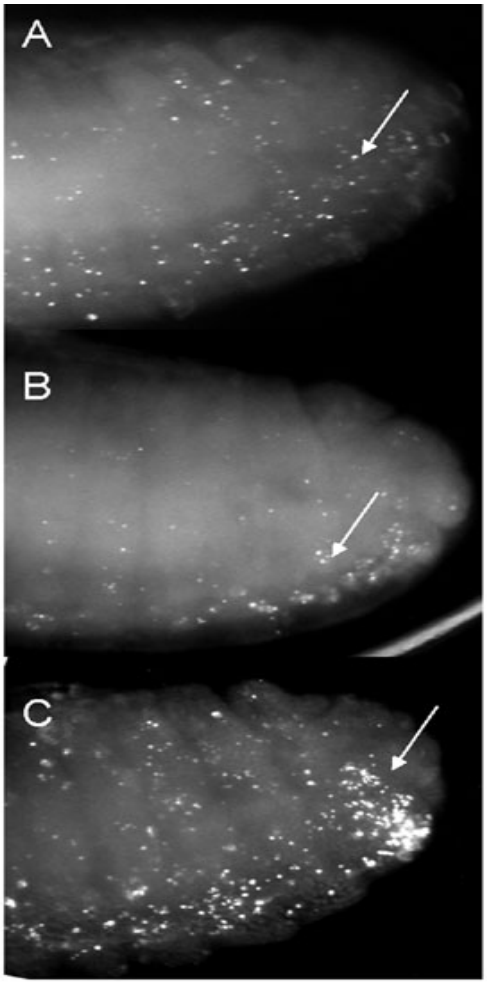


Figure 4.7. Effect of *Snp* mutation on digestion of apoptotic DNA in embryos. Each panel shows TUNEL positive nuclei in the posterior portion of a stage 13 embryo (arrows). A) Snp^{c00465} /*CyO*. B) Snp^{c00465}/ Snp^{c00465}. Note the intensity and number of TUNEL positive nuclei is similar to the heterozygous sibling shown in panel A. C) DNaseII ^{lo} homozygous mutant embryo. Note the increase in the amount of TUNEL positive nuclei, particularly in the posterior CNS (arrow).

DISCUSSION

We have presented a genetic characterization of a novel member of the DEDDh family of nucleases that we call Snipper (Snp). Our studies show that Snp is a 3'-5' exonuclease, which is capable of binding to a synthetic histone 3' end *in vitro*. Similar to *in vivo* studies on 3'hExo (Dominski et al.), we have not been able to obtain evidence that Snp

plays a significant role in cell cycle regulated histone mRNA degradation *in vivo*. It is possible that 3'hExo and Snp act redundantly with other exonucleases, perhaps that act at the 5' end, to destroy histone mRNA at the completion of S phase. 3' hExo and Snp may also play orthologous roles in histone mRNA metabolism other than coupling histone mRNA degradation with the cell cycle. Alternatively, Snp and 3'hExo may not be functional orthologues, and Snp may participate in cellular functions other than histone mRNA metabolism. Our data demonstrate that any such putative functions for Snp are not required for *Drosophila* development.

Recent work has revealed that ERI-1 is a member of a complex containing Dicer-1, the RNA dependent polymerase RRF-3, and the proteins ERI-3 and ERI-5 that functions to produce and process a class of siRNAs required for silencing of endogenous genes. The exact role of the exonuclease ERI-1 in producing endogenous siRNAs is unknown, but it is hypothesized that ERI-1 might recognize short stem loop structures in mRNAs that are targets of endogenous siRNAs, and remove any unpaired 3' nucleotides producing a stem loop structure suitable for the RNA dependent polymerase, RRF-3, to prime the formation of a dsRNA molecule that can be processed by Dicer-1 (Duchaine et al., 2006). In addition, ERI-1 does not bind and degrade the 3' overhangs on siRNAs *in vivo* as previously thought from the ability of ERI-1 mutations to enhance the efficacy of gene silencing by exogenous dsRNA (Duchaine et al.). Recent studies suggest that this phenomenon is caused by the competition for Dicer between the exogenous RNAi complex and the endogenous RNAi complex, which both require Dicer. When ERI-1 is lost, more Dicer is available for the complex that processes exogenous dsRNAs, thus causing increased efficiency in silencing by dsRNAs (Duchaine et al., 2006). No orthologues of RRF-3 have been found in *Drosophila*,

and this pathway of transitivity of RNAi does not exist in *Drosophila.* This suggests that the specific contribution of ERI-1 to RNAi may not exist in *Drosophila*, consistent with our failure to observe a modulation of RNAi in *Snp* mutants. Alternatively, Snp may not be functionally orthologous to ERI-1.

TUNEL labeling studies also indicate that Snp does not play a major role in the clearance of apoptotic DNA. In *C. elegans,* Parrish and Xue have shown that several exonucleases and endonucleases, many of which have other known functions, and many of which also are not required for viability, are required for the clearance of apoptotic DNA, and they hypothesize the presence of an apoptotic degradosome. The lack of an increase in TUNEL staining in *Snp* mutant embryos does not exclude the possibility that Snp plays a role in the clearance of apoptotic DNA, since Parrish and Xue's work indicate that the clearance of apoptotic DNA involves several nucleases. Thus, it's possible that redundancy with several other nucleases masks Snp function in apoptosis.

In the absence of genetic insights, the functional role/s of this promiscuous exonuclease remains a mystery. Our biochemical data suggest a bias towards double stranded DNA or RNA substrates with a 3' flank, although it is also very active on single-stranded DNAs or RNAs, including polyA. The biochemical properties of Snp are remarkably similar to those of the *E.coli* DEDDh exonuclease RNase T (Deutscher & Li, 2001), which like Snipper, is also a broad specificity enzyme and has been implicated in regulating 3' maturation of a number of stable RNAs that include tRNAs (Deutscher et al., 1985) and 23S ribosomal RNAs (Li et al., 1999) and in the turnover of tRNAs. Substrates of RNase T (like those of Snp) generally consist of a double stranded stem followed by four unpaired 3' nucleotides. RNase T is also a DNase and binds ssDNA more tightly as

compared to RNA degrading these single-stranded substrates in a non-processive manner (Zuo & Deutscher, 2002). RNase T can suppress UV repair effects in *E.coli* mutants that lack DNA repair specific exonucleases (Zuo & Deutscher, 1999). A unique feature of both RNase T and Snp is their ability to trim the 3' ends of structured RNAs very close to the double-stranded stem (Ref). Most nucleases are unable to act near the base of a stem-loop or DNA duplex. Snp is predicted to be structurally distinct from RNase T. RNase T is a dimer and the conserved regions identified to play a role in substrate recognition in RNase T are absent in Snp (Zuo & Deutscher, 2002). Given the similarities in their substrate specificities, it is possible that Snp may play analogous roles as RNase T does in *E.coli* (Deutscher, 2006). Snp may function in DNA damage repair or recombination where this structure appears during the intermediate steps of repair. Snp may also be involved in the turn over of tRNA or in the 3' maturation of stable RNAs. The 3' end of tRNA has a very similar hairpin structure to that of histone mRNAs, and the final 4 nucleotides of the 3'end of tRNA and all replication dependent histone mRNAs are similar, each ending in CCA. Therefore tRNAs appear to be an ideal substrate for Snp and future studies will test these hypotheses.

In conclusion, our data indicates that Snp is most similar to the uncharacterized human protein AAH10503, and suggest that these proteins along with 3'hExo and ERI-1 define a new subclass of exonucleases that show 3'-5' exonucleolytic activity towards adverse array of substrates and may participate in cellular processes other than histone mRNA metabolism and RNAi.

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Chapter V

Discussion

SLBP is required for histone pre-mRNA processing throughout development.

In this body of work we extend the analysis of *SLBP* function for the embryo to later stages of development. Previous work has shown that when *SLBP* is mutated in the embryo longer polyadenylated forms of each of the replication dependent histone are observed and the polyadenylated histone mRNAs appear to have a longer half-life than normal histone transcripts and perdure outside of S-phase in the embryonic gut (Sullivan et al., 2001; Lanzotti et al., 2002). An identical mis-processing phenotype was seen in *SLBP* mutant 3rd instar imaginal eye discs with longer polyadenylated transcripts being produced. However unlike the embryonic gut, which is endocycling, a perdurance of mis-processed H3 was not seen in the proliferating cycles of the eye discs. Clearance of mis-processed H3 appeared to be normal when cells were arrested in G1 in the morphogenetic furrow; clearance of the mis-processed H3 also appeared to happen normally when cells exited S-phase for the final time. This result suggests a potential interesting difference in how different cycling cell populations might be able to handle these mis-processed histone transcripts. To try and further support this idea, the endocycling cells of the larval salivary gland should be examined to determine if they are able to properly degrade the mis-processed histone transcripts as the cells exit S-phase.

SLBP is required for histone mRNA processing in the female germline.

Previous studies have indicated that females homozygous for a hypomorphic mutation of *SLBP* show a dramatic depletion of histone mRNAs loaded into the eggs. However the RNA that is loaded into the egg is properly processed, potentially indicating that SLBP might not be required for the processing of histone pre-mRNAs in the female germline, or that the amount of SLBP present in the germline of the hypomorph is sufficient to carry out pre-mRNA processing. Since null mutations of *SLBP* are lethal we created mosaic germlines to assess *SLBP*'s role in processing in a null situation. In these null egg chambers almost all the RNA produced is mis-processed. However there is much less RNA than in wildtype egg chambers, indicating that SLBP is indeed required for histone pre-mRNA processing in the germline. Interestingly the nurse cells in these *SLBP* null egg chambers become very abnormal in appearance and appear to degenerate prematurely in late stage egg chambers, thus these females lay a very sparse amount of eggs. One potential explanation for why the nurse cells degenerate is that as mentioned less histone mRNA is produced in these mutant egg chambers so it could be possible that there is not enough histones around to support the endocycles of the nurse cells and the nurse cells arrest in the cycle and then undergo apoptosis. Recent characterization of U7 mutants has revealed a very similar phenotype to that of the SLBP null egg chambers, poly(A) histone message is produced but there is much less histone message compared to wild type egg chambers and the nurse cells also degenerate prematurely in the U7 mutant egg chambers (Godfrey et al., 2006). In somatic tissues no such dramatic cellular phenotypes have been described so far in *SLBP* mutants, potentially indicating that the germline is much less tolerant to the loss of *SLBP* than somatic cells.

A role for Drosophla SLBP in translation of histone mRNAs?

Based on the growing evidence from vertebrate systems that *SLBP* is also required for the efficient translation of histone messages, it is intriguing to think that essential 53 amino acid region of the *Drosophila* N-terminus might also be playing a role in regulation of translation. However as mentioned previously there is very little conservation between vertebrate SLBPs and *Drosophila SLBP*, and a direct match of the conserved putative translation activation domain **DW**X_3**E**E found in the N-terminus of vertebrate SLBPs could not be found in this 53 amino acid region of *Drosophila* SLBP. However a stretch of amino acids within this region does have similar amino acid identities: KFX_3VKEE. Furthermore *Drosophila* does contain a putative homologue of SLIP-1(CG13124) so it is intriguing to think that this region might be acting in a similar manner to the translation activation domain of vertebrate SLBP.

To test this hypothesis that this KFX_3VKEE sequence motif might be the essential region of this 53 amino acid region of the N-terminus, a point mutant was created mutating the motif to AAX_3AKAA. Initial studies indicate that mutation of this motif fails to restore viability in an *SLBP* mutant background and future studies will be done to characterize if pre-mRNA processing is normal in this point mutant. Also *in vitro* pull down assays will be done to determine if *SLBP* is capable of interacting with the putative *Drosophila* SLIP-1 homologue CG13124. If full length SLBP can interact with CG13124, then the deletion constructs will be tested and point mutant will be tested to determine where CG13124 interacts with *SLBP*. It will be very interesting to determine if SLBP's role in stimulating translation of histone transcripts is conserved in *Drosophila*.

Why do SLBP flies die?

One tantalizing question is why the loss of SLBP is lethal to the organism. *SLBP* null flies die as pharate adults or 3rd instar larvae, however this analysis has shown that replication dependent histone mRNA is present during all stages of development in amounts that appear to be equal to or slightly greater than that found in wild type organism except in the germline. This analysis has found that the majority of this mis-processed RNA is also regulated normally in regards to the cell cycle. During embryogenesis the only mis-regulation of the mis-processed RNAs were seen in the gut, all other cell types in the embryo appeared to regulate the poly (A) histone mRNAs normally with respect to the cell cycle. While during larval development in the proliferating cells of the 3rd instar eye disc, it appeared that the poly (A) histone mRNAs were regulated normally. One possibility this data suggests is that the production of mis-processed poly (A) histone mRNAs is not lethal to the organism, but *SLBP* may play an essential role later in histone mRNA metabolism. Studies from vertebrate systems has shown that SLBP might be required for the efficient translation of histone transcripts (Sanchez & Marzluff, 2002). Furthermore recent studies have shown that mutation of the U7 snRNA also results in the production of poly (A) histone mRNAs. However in contrast to the loss of *SLBP*, the loss of U7 is not lethal to the organism. *U7* mutant flies are viable but female and male sterile, despite having the presences of mis-processed histone mRNAs. This result could potentially suggest that the mis-processing of histone mRNAs is not lethal in itself and that *SLBP* may play an essential role in the production of histones other than its role in processing such as playing a role in the regulation of translation of the mature histone transcript (Godfrey et al., 2006).

Its been previously suggested that one possibility why *SLBP* mutant flies die and *U7* mutant flies live is due to the fact that the phenotype of mis-processing of histone mRNAs occurs later in development in *U7* mutant flies because of a large maternal supply of the U7 snRNA and the production of poly (A) histone mRNAs is delayed until the 2nd instar larval stages. Whereas in *SLBP* mutants the production of poly (A) histones is coincident with the activation of the zygotic genome due to the lack of a detectable maternal supply of *SLBP*. Therefore it has been suggested that in the *SLBP* mutants the reason for the lethality is the long term presence of these poly (A) histone messages slowly poisons the animal and leads to a build up defects that eventually kills the organism, while in the U7 mutants the poly (A) messages are not present long enough for enough of these defects to occur to kill the animal before adulthood.

A second potential explanation is that the differences in phenotypes are due to different role for *SLBP* and *U7* snRNA in histone metabolism. It is known that both U7 and *SLBP* share a required role in histone pre-mRNA processing(Sullivan et al., 2001; Lanzotti et al., 2002; Godfrey et al., 2006), however this report shows that *SLBP* has an essential function for viability of the organism that is independent of its role in histone mRNA processing. If this second function for *SLBP* is its requirement for efficient translation of the histone transcripts this could be the reason for the lethality in *SLBP* mutant flies. SLBP might exert some control in how much histone protein might be produced to ensure a 1:1 ratio of all the core histones is produced. While in an *SLBP* mutant it is likely that a translation complex not meant for translating histone messages is used and potentially some control on histone protein production might be lost and for example this might cause the core histone not to be produced in a 1:1 ratio, which could potentially be detrimental to a cell.

While in a U7 mutant it is likely that *SLBP* is still present in these cells and potentially could still bind to these poly (A) histone transcripts and ensure that proper translation occurs, this could be one potential reason the U7 mutants are still viable. It will be interesting to determine the defects that are killing the *SLBP* mutant flies.

What's Snipper's real role in a cell?

In this report we examined the role of an 3-5' exonuclease, Snipper, in the regulated destruction of histone mRNAs at end of S-phase based on its homology to 3'hEXO a human exonuclease that has been implicated in the degradation of histone mRNAs (Dominski et al., 2003). However we could not find any role for Snp in the regulated destruction of histone mRNAs, nor could we find Snp playing a significant role in any of the published functions of the members in this 3'hEXO/ERI-1 subfamily of exonucleases. This begs the question as to what Snp's role actually is? To try and gain more knowledge about Snps function yeast two hybrid assays have been performed with the potential human orthologue of Snp, ExoD1 (AAH10503). At least one interaction found from this is with a BRCA2 associated factor. BRCA-2 is involved in the repair of structured DNA such as collapsed replication forks, (Gudmundsdottir & Ashworth, 2006). *In vitro* data suggests that Snp is a promiscuous exonuclease that can degrade both DNA and RNA, and that Snp's highest activity in on DNA or RNA that is structured such as a piece of DNA that has a 3' flap (Kupsco et al., 2006). While no BRCA-2 homologues have been found in *Drosophila*, it is intriguing to think that Snp may possibly play a role in DNA repair. Future studies will investigate Snp's role in DNA repair by testing Snp mutant's sensitivity to various DNA damaging agents.

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