A REGION OF SLBP DISTINCT FROM THE HISTONE MRNA BINDING AND
PROCESSING DOMAINS IS ESSENTIAL FOR DEPOSITION OF HISTONE MRNA IN THE
OOCYTE

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ABSTRACT

Jennifer M. Potter-Birriel:
A region of Drosophila SLBP distinct from the histone pre-mRNA binding and
processing domains is essential for deposition of histone mRNA in the oocyte
(Under the direction of Bill Marzluff)

Metazoan histone mRNAs are the only eukaryotic mRNAs that are not polyadenylated. Instead they end in a 3'end Stemloop (SL). Processing of the histones pre-mRNAs is accomplished by an endonucleolytic cleavage after the SL. The stem loop binding protein (SLBP) binds to the SL, and SLBP is a key factor in all steps of the life cycle of histone mRNAs. We are studying the role of SLBP in *Drosophila melanogaster* in vivo. In *Drosophila* each histone gene contains a cryptic polyA site after the histone processing site, and when histone pre-mRNA processing is defective histone mRNAs are polyadenylated. Using FLY-CRISPRCas9 we obtained a 11 deletion ($SLBP^{\Delta 11}$) null mutant and a 30 nucleotide deletion ($SLBP^{\Delta 30}$) in the N-terminal domain (NTD) of SLBP. The 30nt deletion removed 10aa from the N-terminal domain of SLBP in a region of unknown function distinct from the processing domain. The $SLBP^{\Delta 30}$ mutant was viable, but females were sterile. They laid eggs, but the eggs didn't hatch, because they didn't store histone mRNA in the egg. In Drosophila nurse cells produce large amounts of histone mRNA at the end of oogenesis which is translated and stored in the egg to allow the development of the embryo until zygotic histone gene transcription turns on. The stored histone mRNA is produced in absence of DNA replication. Despite having normal amount of SLBP, the SLBP30 mutant expresses polyadenylated histone mRNA at all stages.

In the ovary histone mRNA expression is normal in the rapidly replicating nurse cells throughout oocyte development but very little histone mRNA is expressed at the end of oogenesis after nurse cell replication is completed. Immunofluorescence data shows that the SLBP$^{\Delta 30}$ protein is mainly localized in the cytoplasm at this stage, suggesting the deleted region is important for nuclear import of SLBP at the end of oogenesis. The histone locus bodies present at the histone genes are also defective, and do not activate transcription of the histone genes. These results suggest that defective nuclear import of SLBP$^{\Delta 30}$ may lead to a defect in HLBs and histone gene transcription.

I want to dedicate my thesis work to my family specially my Mom (Carmen), my Dad (Osvaldo), my Grandma (Aida) and my Sister (Nashaly); I am truly grateful for all the support you all have given me throughout my doctoral career.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

AA- Amino Acid

AED- After egg deposition

Cdk1- Cyclin Dependent Kinase 1

Chk-2- Checkpoint kinase 2

CRISPR- Clustered regularly interspaced short palindromic repeats

CTD- C-Terminal Domain

DAPI- 4′,6-diamidino-2-phenylindole

dH3 – Drosophila Histone 3

dH1 – Drosophila Histone 1

dSLBP - Drosophila Stem Loop-Binding Protein

FLASH- Flice-associated huge protein

GFP- Green fluorescent protein

HCC- Histone cleavage complex

HDE-Histone downstream element

HLB- Histone Locus body

MPM2- Mouse monoclonal Mitotic proteins antibody

Mxc- Multi sex combs

NPAT- Nuclear Protein, Coactivator of Histone Transcription

NTD- N Terminal Domain

PAM- Protospacer adjacent motif

PCR- Polymerase Chain Reaction

RBD-RNA binding domain

RCME- Recombination-mediated cassette exchange

RPD- RNA processing domain

RPN2- Regulatory particle non-ATPase 2

RT-PCR- Real-Time Polymerase Chain Reaction

SL- Stem-loop

SLBP- Stem-loop binding protein

U7 snRNA- U7 small nuclear RNA

7SK (snRNA) - small nuclear RNA

CHAPTER 1: INTRODUCTION

OVERVIEW

Proper stoichiometry of histones is necessary for the maintenance of genome architecture and, most importantly, gene expression. Replication dependent histone mRNAs (H4, H3, H2A, H2B, and the linker H1) are needed in high amounts during DNA replication. During this time, histone proteins are synthesized to wrap up the newly synthesized DNA into nucleosomes and subsequently into chromatin. Histone proteins not only package the DNA but also plays essential roles in regulating gene expression through their post-translational modifications. For this reason, understanding how histones are produced coordinately with DNA replication is critical for understanding the fundamental mechanisms of cell proliferation.

Histone mRNA biogenesis.

Animal histone mRNAs are the only mRNAs in the cell that are not polyadenylated. They end instead in a 3' end stem-loop (SL), which is conserved in evolution and critical for its regulation. These genes also do not contain introns, but the addition of introns interferes with 3' end proceesing (Pandey et al., 1990). Processing of histone pre-mRNAs is accomplished by an endonucleolytic cleavage after the SL (fig.1.1) (Dominski et al., 2005). This orchestrated process is initiaded by bindgin of the stem-loop binding protein (SLBP) to the SL and binding of the U7 small nuclear RNA (U7 snRNA) to the histone downstream element (HDE) a purine rich sequence (fig.1.1) (Cotten et al., 1988; Dominski et al., 1999; Soldati and Schümperli, 1988).

The U7 snRNA is part of the U7 snRNP, which also contains a Sm ring, which is distinct from the Sm ring on the spliceosomal snRNAs. Binding of these proteins is needed for the recruitment of FLASH by Lsm11. FLASH, together with Lsm11, recruits the histone cleavage complex (HCC) composed of Symplekin, CPSF100, Cstf64, and the endonuclease CPSF-73, which cleaves 4 nt (invertebrates) or 5 nt (vertebrates) after the SL (fig.1.1) (Dominski et al., 2005; Sabath et al., 2013; Skrajna et al., 2017; Yang et al., 2013). Once the histone pre-mRNA is cleaved, the mature histone mRNA is transported to the cytoplasm where its translated (Whitfield et al., 2004) and then degraded at the end of S-phase (Zheng et al., 2003). SLBP stays bound throughout this process, and our lab has evidence that it is a key component in the entire life cycle of histone mRNAs (e.g. processing, nuclear export, translation, and degradation) (Krishnan et al., 2012; Pandey et al., 1994; Sanchez and Marzluff, 2002; Sullivan et al., 2009). SLBP was first detected about 29 years ago by Pandey et al., 1991 and cloned in 1996 by Wang et al. and Martin et al. Since then, its role in histone mRNA metabolism has been studied extensively by our group and others.

Figure 1.1: Obtained from Marzluff and Koreski, 2017
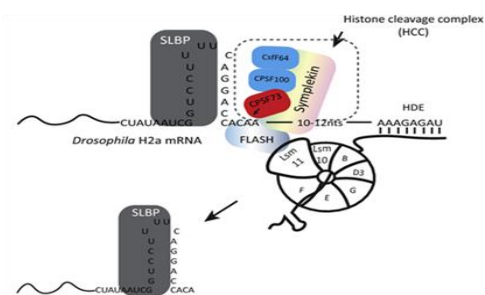
Figure 1.1: Histone mRNA biosynthesis. SLBP binds to the SL and recruits the U7 snRNA to the HDE sequence. The binding of both proteins leads to the recruitment of the HCC, which is composed of the polyadenylation factors Symplekin, CstF64, CPSF100, and the endonuclease CPSF73. SLBP stays bound to the mature histone (Marzluff and Koreski, 2017).

SLBP is a key component of histone mRNA regulation in mammalian cells.

Histone mRNAs are cell cycle-regulated, and in mammalian cells, this regulation is mainly post-transcriptionally regulated (e.g. 3′ end processing and the half-life) (fig.1.2) (Alterman et al., 1984; Harris et al., 1991; Lüscher and Schümperli, 1987; Schümperli, 1986). As histone mRNA levels increase before entering S-phase, SLBP is translationally regulated (fig.1.2) (Djakbarova et al., 2014; Whitfield et al., 2000). During S-phase, SLBP protein levels remain constant to process histone pre-mRNAs (fig.1.2). As cells complete DNA synthesis, histone mRNAs are degraded since there is no longer a demand for histone protein (Marzluff and Hanson, 1993). Degradation of SLBP is accomplished in an orchestrated cell-cycle regulated manner. The SFTTP motif located at the N-terminal domain of SLBP is the region required for its degradation (Zheng et al., 2003). In vitro analyses done by our group has shown that both threonines are necessary for the degradation of SLBP at the end of S-phase (Zheng et al., 2003). Degradation begins with the phosphorylation of the second threonine Thr61 by cyclin A/Cdk1, and then the first threonine Thr60 is subsequently phosphorylated by CK2 (Koseoglu et al., 2008). Mutation of either of these residues leads to SLBP stability at the end of S-phase (Zheng et al., 2003). Studies have shown that a constitutive or stable SLBP does not affect histone mRNA degradation at the end of S phase (Zheng et al., 2003).

The knockdown of SLBP in U2OS cells decreases DNA synthesis but increases the number of cells in S-phase (Sullivan et al., 2009). Cells enter S-phase but progress very slowly through S-phase due to the low production of histone mRNAs (Sullivan et al., 2009). Histone pre-mRNA is still processed, but the processed mRNA remains in the nucleus (Sullivan et al., 2009). Thus, SLBP is required in large amounts (1molecule histone mRNA) for proper S-phase progression.

Figure 1.2: Obtained from Marzluff et, al 2008.
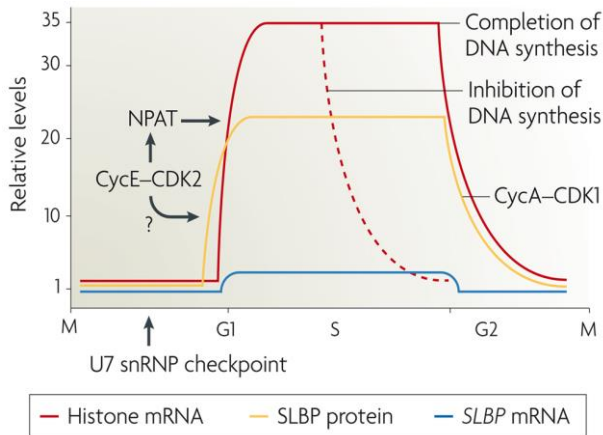
Figure 1.2: Histone mRNAs and SLBP are cell-cycle regulated. As cells enter S phase, Cyclin E-CdK2 levels increase and phosphorylate the co-activator Nuclear Protein, Coactivator of Histone Transcription (NPAT). Phosphorylation of NPAT stimulates histone gene expression. At the same time, SLBP is translationally regulated to processes the necessary histone pre-mRNAs which will subsequently be translated into histone proteins and used to assemble the synthesized DNA into chromatin (Marzluff et al., 2008).

Histone genes and histone mRNAs in Drosophila.

Histone genes in Drosophila melanogaster are clustered in a tandem array of about 100 copies of each histone gene (H4, H3, H2A, H2B, and the linker H1) at a single locus (fig.1.3 A). The histones genes are present in the Histone locus bodies (HLB). The HLB is a nuclear body that concentrates factors necessary for histone mRNA biosynthesis (fig.1.3 B) (Liu et al., 2006). As cells enter S-phase, histone gene transcription increases to meet the demand of synthesized histone proteins with DNA replication. The expression of histones genes in the HLB initiates with the phosphorylation by cyclin E/Cdk2 of the Multi sex combs (Mxc) in Drosophila (NPAT is the homolog in mammals) (White et al., 2007). Phosphorylation is known to help stimulate histone mRNA expression and to recruit factors including the HCC to the HLB for histone mRNA biosynthesis (White et al., 2011). Mxc and FLASH aggregates are observed in

Drosophila embryos at cycle 10, and the complete HLB is assembled in cycle 11, at the same time as the activation of zygotic histone gene transcription at cycle 11 (White et al., 2007a). During the first 90 minutes of embryogenesis, the embryo relies on the maternal supply of histone mRNA and protein that comes from the egg due to the absence of transcription (Lanzotti et al., 2002; Marzluff et al., 2008). Zygotic transcription only starts after cycle 11 of embryogenesis (Lanzotti et al., 2002). The RNAs and proteins, including histones and SLBP that are necessary for DNA replication and chromatin assembly during the initial rapid cell cycles, are all stored in the egg (Lanzotti et al., 2002; Marzluff et al., 2008). Once the embryos reach cycle 14, there are both G1 and G2 phases in the mitotic cell cycles. The maternal histone mRNA is degraded, and the normal cell-cycle regulation of histone mRNA is initiated.

The amount of dSLBP is a key component not only for the early embryo but for later stages of embryogenesis. Histone mRNAs are not normally polyadenylated. Drosophila histone genes, unlike histone genes in other species, have cryptic polyadenylation sites 3' of each processing signal, which is used if histone mRNA processing is defective (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001; Tatomer et al., 2016). Thus, cultured cells with SLBP knocked down continue to grow and produce primarily polyadenylated histone mRNAs (Wagner et al., 2005). Northern blot analysis or nuclease protection assays are used to detect polyadenylated histone mRNAs (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001; Tatomer et al., 2016). An allele of dSLBP that has a p-element insertion in the 5' UTR of SLBP is not viable but accumulates misprocessed histones beginning at the end of embryogenesis, and it continues through the larvae stages (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001). Eggs from females that contain a reduced amount of dSLBP exhibit defects during the syncytial nuclear divisions and embryos do not hatch due to a low amount of histone

mRNA/protein loaded into the egg (Lanzotti et al., 2002; Sullivan et al., 2001). These syncytial

embryos have chromosome condensation defects and fail to separate during anaphase and die

before initiation of zygotic transcription (Sullivan et al., 2001).

As expected, reduced amounts of histone protein lead to unsuccessful completion of

embryo development. A similar phenotype was observed in the C. elegans SLBP homolog CDL-

1. RNAi experiments done to reduce the amount of cdl-1 arrested in the mitotic cycles at the 2-4

cell stage as a result of chromosome condensation and morphological defects (Kodama et al.,

2002; Pettitt et al., 2002). Mammalian cells arrest when SLBP is knocked down (Sullivan et al.,

2009). In both cases, unlike in Drosophila, there are not sufficient polyadenylation histone

mRNAs produced to maintain cell growth.

Figure 1.3: Obtained from Marzluff and Koreski, 2017
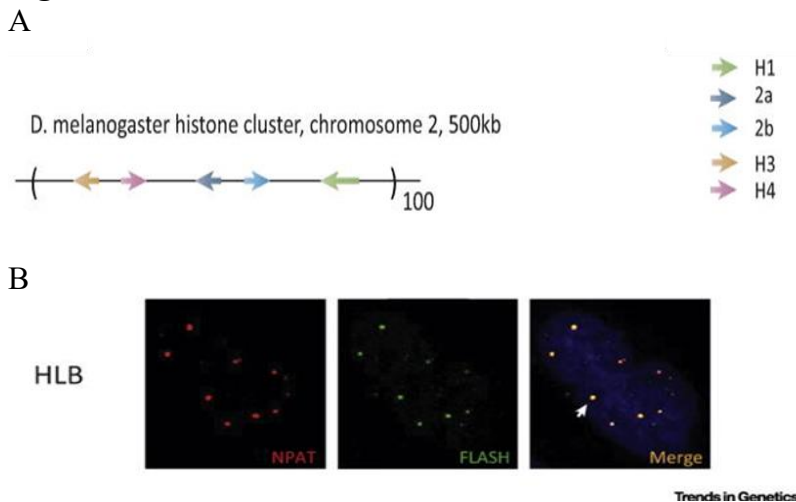
Figure 1.3: Drosophila histone cluster and HLB expression. **A** Drosophila histone genes are clustered at a single locus on chromosome 2, and it contains each histone gene H4, H3, H2A, H2B, and the linker H1. **B** Staining of NPAT (red) and FLASH (green) in HeLa cells. HLB expression can be visualized by identifying factors involve in HLB formation like FLASH (Marzluff and Koreski, 2017).

Histone storage is required for early development in Drosophila.

Organisms with rapid embryonic cycles have developed mechanisms to survive

development with the lack of zygotic transcription. Histone mRNAs and protein in Drosophila

are maternally supplied, and this regulation begins in oogenesis. Drosophila females contain two

ovaries, and each ovary contains a single layer of cells composed of a germarium, egg chambers, and an oocyte (fig.1.4) (Bastock and St Johnston, 2008; Wu et al., 2008; Yamashita, 2018) At the base of the germarium, stem cells divide and differentiate into 16 cells, where 15 cells are the nurse cells, and one cell becomes the oocyte (Bastock and St Johnston, 2008). The nurse cells continue to grow by endoreduplication, providing material for the growing oocyte (Bastock and St Johnston, 2008). Each egg chamber completes development, the nurse cells and follicle cells go through apoptosis and die, producing a single mature egg (fig.1.4) (Bastock and St Johnston, 2008; Wu et al., 2008).

In the late 1980s, two groups published papers addressing how histones were being regulated during oogenesis and why its regulation was relevant for the early embryo. Ambrosio and Schedl characterized histone gene expression by doing in situ hybridization experiments and quantitative analyses of histone mRNA levels. They divided histone gene expression into two phases. The first phase goes from stage 5 to stage 10A; during this phase, they observed a mosaic pattern of histone gene expression, meaning that not all cells expressed the same histone levels (Ambrosio and Schedl, 1985). During this time, the nurse cells are undergoing cycles of endoreduplication. This regulation was coupled to DNA replication, and histones were being degraded at the end of each S-phase (Ambrosio and Schedl, 1985). The second phase goes from stage 10B to stage 14. During this phase, histone mRNA levels were induced, and expression was observed in all the nurse cells (Ambrosio and Schedl, 1985). However, histone synthesis was not couple to DNA replication (Ambrosio and Schedl, 1985). After histone mRNA synthesis, nurse cell breakdown initiates by stage10B/11, and the synthesized histones mRNAs are then transported or dumped into the growing oocyte (fig.1.4) (Bastock and St Johnston, 2008).

In the second paper, Ruddell and Jacobs-Lorena, reached a similar conclusion using biochemical experiments on microdissected egg chambers, demonstrating the synthesis of the histone mRNA in the oocyte after the completion of nurse cells endoreduplication (Ruddell and Jacobs-Lorena, 1985). The histone mRNA produced for storage in the oocyte is translated, and large amounts of histone protein are also stored in the oocyte (Ruddell and Jacobs-Lorena, 1985).

Figure 1.4: Obtained from Bastock and St Johnston, 2008.
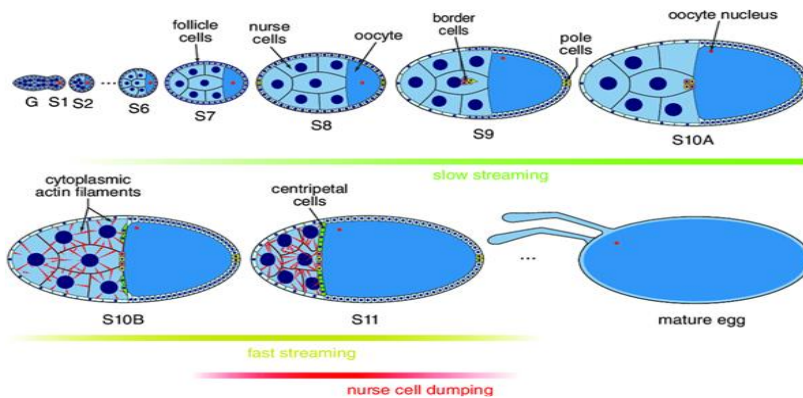
Figure 1.4: Drosophila oogenesis. The germarium is in the anterior region; stem cells divide and differentiate into 16 cells. The egg chamber contains 15 nurse cells and one oocyte and is surrounded by somatic or follicle cells. By stage 10B/11, nurse cell breakdown initiates, and it "dumps" the mRNAs and ribosomes among other particles into the oocyte (Bastock and St Johnston, 2008; Wu et al., 2008).

Other strategies for regulating histone mRNAs and proteins in oogenesis.

Interestingly, Drosophila only has one SLBP, but in other organisms like Xenopus and Zebrafish histone mRNAs are regulated by two SLBPs. Amphibians and egg-laying fish have developed a mechanism to regulate histone mRNAs in oogenesis and embryogenesis by using one SLBP, the mammalian orthologue for processing, and the other one for storing and regulating mRNAs for the early embryonic cycles (Marzluff et al., 2008). How this mechanism is regulated or how the two proteins can interchange during development is not well understood. Recently in He et al., they characterized both SLBPs in Zebrafish. RT-PCR and In-situ

hybridization experiments showed that SLBP2 is only expressed in the ovary during oogenesis, and SLBP2 protein persists during the early stages of embryogenesis (He et al., 2018). A knockout of SLBP2 causes defects in the nuclear cleavage cycles since there is not sufficient histone mRNA or protein stored in the egg (He et al., 2018). This phenotype is similar to what happens in Drosophila when SLBP is produced in low levels, resulting in the deposition of small amounts of histone protein and mRNA in the egg. The canonical SLBP, SLBP1, increases at the mid blastula stage when the zygotic genome is activated and functions in processing and translating zygotic histone mRNAs for subsequent cell proliferation (He et al., 2018). Since SLBP1 is the only SLBP active in pre-mRNA processing, SLBP1 must process the histone mRNA during oogenesis, and it is then stored bound to SLBP2 for early embryogenesis (He et al., 2018). How both SLBP's exchange during this process is not well understood. In Xenopus, there are also two SLBPs with similar functions as in zebrafish, although the details of what happens in development are slightly different. Wang et al., demonstrated that SLBP2 is involved in storing histone mRNAs, which are made early in oogenesis. These histone mRNAs end in short (5-10 nts) oligo(A) tails. At oocyte maturation, SLBP2 is degraded, and theoligo(A) tail is removed. SLBP1 can then bind to the histone, and it can activate the translation of histone mRNAs for the early stages of embryogenesis (Gorgoni et al., 2005; Sanchez and Marzluff, 2002; Wang et al., 1999). The stored histone mRNAs in Xenopus together with stored histone protein provide the histones required until the mid-blastula transition (Wang et al., 1999).

In Sea urchins, histone mRNAs are regulated quite differently; instead of one class of histone mRNAs its indeed regulated by two different classes of histone mRNAs (Angerer et al., 1985; Grunstein et al., 1981; Hieter et al., 1979; Jarvis and Marzluff, 1989; Marzluff et al., 2006; Maxson et al., 1983) In both classes, the histone mRNA ends in a 3'end SL and do not contain

any introns (Marzluff et al., 2006). The first class is the early or the alpha histone genes, which are clustered in a tandem array containing each histone gene, but it is only expressed in the unfertilized egg and in early embryogenesis (Cohn et al., 1976; Marzluff et al., 2006) . The expression of these histone mRNAs is silence during the embryonic cleavage's cycles. Once the embryo reaches the blastula stage, it expresses the second class of histone mRNAs (late histone genes), which interestingly are not clustered in a tandem repeat unit and are expressed late in embryogenesis and throughout development (Marzluff et al., 2006; Maxson et al., 1983)

In mouse, oocytes that lack SLBP do not develop past the 2-cell stage due to a reduced amount of histone levels, which results in genomic instability and infertility (Zhang et al., 2009). SLBP is synthesized in mouse oocytes arrested at G2 a time when SLBP in somatic cells would be degraded due to the absence of DNA replication (Allard et al., 2002). SLBP stays bound to the histone mRNP and translation of histone mRNA is activated (Whitfield, 1999; Whitfield et al., 2004). The data suggest that the expression of SLBP and translation of histone mRNA is necessary for the synthesis of histone proteins for the first S-phase.

Organisms have developed mechanisms to regulate the rapid cell cycles of embryogenesis in order to survive development. Histone genes are an essential component to properly package the DNA into chromosomes and to maintain the integrity of the genome. The complexity and uniqueness of SLBP and its role in histone mRNA biogenesis is impressive. SLBP is conserved, binds only histone mRNAs, and is the only known protein to bind to the 3'end SL (Dominski and Marzluff, 2001; Pandey et al., 1991). Having just one SLBP does not mean that Drosophila has not developed a different strategy to store mRNA and histone proteins than frogs and zebrafish.   In this thesis, I report a distinct region of SLBP that is essential for the deposition of histone mRNAs and protein into the oocyte. I believe that we are just starting to

uncover unknown functions of this protein and how important it is for Drosophila development.

SLBP structural domains.

dSLBP has an N-terminal domain and an RNA processing domain (RPD) composed of two parts, an RNA binding domain (RBD) of 72 amino acids and a C-terminal domain of 18 amino acids. (fig.1.5). Our group has studied the functionality of these domains in vitro and in vivo. In most of the organisms, SLBP has a similar domain structure, although the RPD is located closer to the middle of the protein, so there is also a C-terminal domain after the RPD. In the next three paragraphs, I will explain in more detail the importance of each domain for dSLBP function.

Figure 1.5 Schematic of dSLBP structure

Figure 1.5: Schematic of dSLBP structure. dSLBP contains an N-terminal Domain (NTD) and an RNA Processing Domain composed of an RNA Binding Domain (RBD) and a C-Terminal Domain (CTD).

N-Terminal Domain

The N-terminal domain of dSLBP has not been studied in detail. Initial analyses done by Dominski et al. demonstrated that this region was not necessary for histone pre-mRNA processing in vitro (Dominski et al., 2002). A conserved motif in this domain, the SFTTP, is highly conserved in metazoans (fig.1.6). It controls SLBP degradation at the end of S-phase in vertebrate SLBP. This motif is known to become phosphorylated in both Threonine's, and phosphorylation is necessary for SLBP degradation at the end of S-phase in mammalian cells (Koseoglu et al., 2008; Zheng et al., 2003). However, this motif in dSLBP is SFTPP, and in Lanzotti et al., a point mutant of the Threonine changed to Alanine did not affect the viability and had normal processing of histone pre-mRNAs (Lanzotti et al., 2004). Recent work by

Iampietro et al. showed that phosphorylation in the Serine by Chk-2 is necessary during

genotoxic stress to induce dSLBP degradation in the cytoplasm (Iampietro et al., 2014). This

event serves as a surveillance mechanism that leads to histone nuclear retention and nuclear

fallout to maintain the fidelity of the genome (Iampietro et al., 2014). However, it's still

unknown if this region has other functions in Drosophila development (e.g. embryogenesis or

oogenesis).

Figure 1.6 Sequence alignment of the N-Terminal Domain of SLBP

```
HUMAN       56    RPESFTTPEGPKPRSRCSDWASAVEE
CHICKEN     56    RADSFTTPEGPGPLSRCLDWAAAVEE
XENOPUS     54    RPDSFTTPESHKPVPRCKDWGSAVEE
ZEBRAFISH   87    RSESFTTPESEGPVSRIKNWGDEVEE

DROSOPHILA 115    RRHSFTPPSNENSRSNSPNSSNSSAN
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Figure 1.6: Sequence alignment of the N-Terminal Domain of SLBP. The SFTTP
or SFTPP motif is conserved in Human, Chicken, Xenopus, Zebrafish, and Drosophila.

RNA-Binding Domain

The RBD contains helices αA, αB, and αC and is 55% conserved between Humans and

Drosophila. One of the most conserved and studied residues is the TPNK motif. The TPNK

motif is highly conserved in Humans, Mammalian, Drosophila, Xenopus, Zebrafish, and C.

elegans (fig.1.7). The Threonine is phosphorylated, and crystallography analyses showed that

phosphorylation is critical to stabilize the RBD when is in complex with the SL (Zhang et al.,

2014; Zhang et al., 2012). Furthermore, the same analyses demonstrated that it is an important

region to bring the three α-helices (αA–αC) together (Zhang et al., 2014). In Lanzotti et al., they

made a point mutant (APNK), and according to their in vitro assays, this mutant did not affect

either binding or processing of histone pre-mRNAs (Lanzotti et al., 2004). However, genetic

analyses using an HA-tagged dSLBP in a null background of dSLBP showed that this APNK

mutant did not rescue viability (Lanzotti et al., 2004). Embryo analyses showed that this point

mutant (APNK) is mislocalized and mainly in the cytoplasm (Lanzotti et al., 2004). The evidence demonstrates that this residue is essential for the proper binding of SLBP to the SL in vivo. Another conserved motif is the YDRY located in the helix B of SLBP, in vitro studies have shown that point mutations of the tyrosine abolishes binding of SLBP to the SL (Dominski et al., 2001). However, the two residues in the middle; the DR are required for processing (Dominski et al., 2001). As I mentioned earlier, in Xenopus and Zebrafish, histone mRNAs are regulated by two SLBP's. Interestingly, SLBP 1 in both organisms contain the DR residues between the two tyrosines, which are known to be required for histone mRNA processing in vitro; however, SLBP 2, which has a different function in both organisms, does not contain this conserved amino acids (Ingledue et al., 2000; Wang et al., 1999). In SLBP 2 the DR is replace with QC/QN, and this SLBP has be been described to have a role in the deposition of maternal histone mRNAs during development (He et al., 2018; Wang et al., 1999). However, its not well known how histone deposition is regulated in Drosophila, since this organism only has one SLBP.

Figure 1.7 Sequence alignment of the RNA Binding Domain of SLBP

```
Human          129   ETDESVLMRRQKQINYGKNTIAYDRYIKEVPRHLR
Mouse          129   ETDESVLMRRQKQINYGKNTIAYDRYIKEVPRHLR
X. laevis 1    128   ETDPAVLTRRQKQISYGKNTIAYDRYIKAVPRHLR
Danio rerio 1  134   ETDETTLIRRQKQINYGKNTLAYDRYIKEVPKHLR
X. laevis 2    102   ETDEATLHRRQKQIDYGKNTVGYQCYLQQVPKTER
Danio rerio 2  129   E-INEAVLKRRKQIQYGKNTCGYQNYVQQVPKRLR
Drosophila     189   EADPAILSRRQKQIDYGKNTAAYERYVEMVPKDER
C. elegans     189   CTDEAVLKRRSREIDRAKEKAVYQRYTSEVPLRDR

Human          164   QPGIHPKTPNKFKKYSRRS
Mouse          164   QPGIHPRTPNKFKKYSRRS
X. laevis 1    162   EPNVHPRTPNKFKKYSRRS
Danio rerio 1  169   QPGVHPKTPNKFKKYSRRS
X. laevis 2    137   KSGVHPRTPNKSKKYSRRS
Danio rerio 2  160   QPGVHPKTPNKFKKYSRRS
Drosophila     224   -TRDHPRTPNKYGKYSRRA
C. elegans     241   IKGQHPRTPNKLINFSRRS
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Figure 1.7: Sequence alignment of the RNA Binding Domain of SLBP. The YDRY in humans or YERY in Drosophila is a highly conserved motif located in helix B of the RBD. The tyrosine's (black and bold) are known to be required for SLBP binding in vitro. The DR/ER has a role in histone mRNA processing in vitro (purple and bold). The TPNK motif (red) is highly conserved in Human, Mouse, Xenopus (X. laevis), Drosophila, C. elegans and Zebrafish (Danio rerio). The TPNK helps to bring all three helices together and help to position SLBP to the SL.

C- Terminal Domain

The C-terminal domain of Drosophila is unique, shorter in length, and is essential for processing, although it does not directly interact with the RNA when is in complex with the SL (Dominski et al., 2002; Zhang et al., 2014). The C-terminus of dSLBP ends with the SNSDSDSD, and these serines are extensively phosphorylated in baculovirus SLBP and in vivo (fig.1.8) (Zhang et al., 2014). NMR and Crystallography studies have shown that the highly phosphorylated C-terminus of dSLBP interacts with basic residues in SLBP, promoting a conformation that can bind RNA (Zhang et al., 2014). This change in entropy helps the molecule to become more stable by increasing its affinity to the RNA SL (Thapar et al., 2004). Phosphorylation of these four Serine's located at the end of the dSLBP C-terminus is required for the processing of histone pre-mRNAs in vitro (Skrajna et al., 2017). In Skrajna et al., they did in vitro analyses with point mutants with the purpose to abolish phosphorylation (4S-4A). First, the in vitro binding assays showed that phosphorylation is not required for dSLBP to bind to the SL (Skrajna et al., 2017). However, in nuclear extracts where dSLBP is depleted in S2 cells, the 4S-4A partially processed histone pre-mRNAs and decreased the recruitment of the HCC, including the endonuclease CPSF-73 (Skrajna et al., 2017). This phenotype was rescued with a wild-type dSLBP and the 4S-4E (mimics phosphorylation) (Skrajna et al., 2017). Nevertheless, it is unclear what it is the precise role of the phosphorylation site in vivo, or whether phosphorylation is regulated in vivo.

Figure 1.8 Sequence alignment of the C-Terminal Domain of SLBP in Human and Drosophila.

Figure 1.8: Sequence alignment of the C-Terminal Domain of SLBP in Human and Drosophila. The red letters indicate the acidic residues, and the stars indicate the amino acids that are phosphorylated.

Dissertation Goals

My overall goal was to understand the role of different regions of SLBP in histone RNA metabolism in vivo. A lot is known about the role of specific regions of SLBP in processing in vitro. Studying the conserved residues within the structure will continue to help understand SLBP function and its role in histone mRNA metabolism. As part of my work, I conducted genetic analyses using the multicellular organism Drosophila melanogaster to address the role of SLBP in an in vivo system. With the advance of science and the development of new technology, we can now address questions in a more sophisticated and precise manner compared to 20 years ago.

For my thesis work, my first aim was to create a null allele of dSLBP. Previously, we did not have a null allele of dSLBP in the lab. For this purpose, I used the Fly CRISPR-Cas9 system, and this system allowed me to create mutations in the dSLBP gene. This approach with the use of molecular and biochemical analyses gave us insight about dSLBP role in histone mRNA biosynthesis in Drosophila embryogenesis and oogenesis.

My second aim was to study in more detail residues analyzed previously in vitro but not in vivo. Point mutants were created and incorporated in embryos by Recombination Cassette Mediated Exchange assay (RCME) to integrate by homologous recombination each transgene into the same target site. With this approach, I was able to determine if these conserved residues have any role(s) in vivo, and these studies gave us a better understanding of their importance in dSLBP function in the cell.

A second mutant obtained in the CRISPR experiment was a deletion of 10 amino acids in a region of SLBP, which had not previously been investigated. Surprisingly this mutant had a

novel phenotype, and characterization of this mutant resulted in the elucidation of previously unknown features of the deposition of histone mRNAs and proteins into the Drosophila oocyte. The proper stoichiometry of histones proteins is critical for the packaging of the newly synthesized DNA into chromosomes to maintain the fidelity of the genome. The study of histone mRNAs will bring more insight into how this mechanism is being regulated. My studies provide new insight into how the supply of histones during early development, before activation of zygotic transcription, is achieved to allow development to proceed through the initial syncytial cell cycles.

My thesis work advanced the knowledge of the structural function of dSLBP and its biological role in a multicellular organism, and elucidated details of the pathway for providing the histone mRNAs to the developing oocyte and the role of SLBP in that process.

CHAPTER 2: PHENOTYPE OF SLBP NULL ALLELE

INTRODUCTION

Histone mRNAs end in a 3'end SL that is critical for its regulation (Liu et al., 1989; Pandey and Marzluff, 1987). SLBP binds to the SL of histone pre-mRNAs and does not bind to any other mRNAs (Brooks et al., 2015; Dominski and Marzluff, 2001; Dominski et al., 1995; Marzluff et al., 2008). My first goal was to create a null allele of dSLBP and to ask the followed questions: Is SLBP necessary for the early stages of embryogenesis, and histone pre-mRNA processing? Is SLBP maternally supplied?

In early embryogenesis histone mRNAs are not cell-cycle regulated in Drosophila; instead large amounts of histone mRNAs are instead synthesized at the end of oogenesis and stored in the egg (Ambrosio and Schedl, 1985; Lanzotti et al., 2002; Ruddell and Jacobs-Lorena, 1985; Sullivan et al., 2001), where they are stable for the syncytial cell cycles.

Previous studies had been carried out with an SLBP mutant, SLBP15, which we now know is likely a hypomorph, due to a cryptic promoter in the p element inserted into the 5' UTR (Lafave and Sekelsky, 2011; Sullivan et al., 2001). This mutant survived until 3rd instar larva, but no flies were obtained. On the other hand, a less severe hypomorph (dSLBP10) had a smaller fragment of the p-element in the 5'UTR of the SLBP gene, was a maternal effect lethal, due to reduced amount of histone mRNAs being store into the egg (fig.2.4 B) (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001). Females were viable but sterile and males were fertile.

When I came to the Marzluff lab, they did not have a null mutant of dSLBP. I used Fly- CRISPR Ca9 to create a null allele of dSLBP. I characterized the alleles with genetic and biochemical analyses to study histone mRNA biogenesis and SLBP levels during fly development. We previously did not have a good antibody of dSLBP, that was appropriate for Western blotting and immunofluorescence.

In this chapter, I describe a knockout of dSLBP generated by CRISPR, and the molecular phenotype in histone mRNA expression, and characterized the levels of SLBP protein during early development

MATERIALS AND METHODS

CRISPR Design

        The protocol was obtained from CRISPR Fly design. The designed oligos were

synthesized by Integrated DNA Technologies, Inc. (IDT). Each oligo was phosphorylated at the

5' end. The antisense and sense oligo were annealed in boiling water (95°C for 5 minutes) and

oligos were allowed to cool down at Room Temperature. Annealed oligos were cloned into the

pu6-BbsI-gRNA plasmid. The plasmid was digested with the BbsI enzyme and dephosphorylated

with Calf intestinal Alkaline Phosphatase (NEB). The annealed oligos were ligated with the

digested plasmid overnight at 16°C. Each reaction (ligation) was transformed into competent

bacteria. The insertion was confirmed by sequencing with both T7 and T3 primers. The DNA

was sent to BestGene Inc., the DNA was injected into embryos (stock: BDSC#54591) expressing

the Cas9 protein.

SLBP CRISPR GUIDE Sense
/5Phos/CTTCGAACGAGTGTCGGCGCTTGT
SLBP CRISPR GUIDE Antisense
/5Phos/AAACACAAGCGCCGACACTCGTTC

Screening of flies

        The Guide RNAs were inject into the BDSC#54591 fly line. The company injected 300

embryos and about 110 larvae survived. The company sent 4 vials, from which I obtained 69

flies; 35 were females and 34 were males. The first cross (G0) is to cross out the Cas9 source in

the first generation. Single males were selected and crossed to 3-5 virgin females with the

respective genotype w; TM3/ TM6B. Only 2/34 males were fertile. Since, most of the males

were infertile, single males were used for the next cross G1. To confirm that the gene of interest

was edited, DNA was prepared from the single progeny of these flies to conduct a PCR (Primers

used: 18# GenomSLBP F and GenomSLBP Rev 1) annealed at 57°C for 45 seconds; with an

extension at 72°C for 1 minute). Each PCR product was sent for sequencing (DmSLBPSeqFor1). Note that these flies all have a wild-type SLBP gene from the mother. The PCR products that contained two peaks near the PAM site (flies still have one wild type allele of dSLBP) were subcloned into the pbluescript plasmid to separate both alleles and were submitted for sequencing. I obtained three flies with mutated SLBP genes. One with a 1nt deletion, one with a 11nt deletion and one with a 30nt deletion.

First cross G0

w; TM3   X   nosCas9; [SLBP]
   TM6B   +

Second cross G1

w; TM3   X   +;   [SLBP]
   TM6B   TM3 or TM6B

          SLBP
          TM3
          Stock

PAM Site

```
Wild type w^1118  GAGCGCCTGGTCAAGGAGGAGAAGCTAAAAACGC[CCT]ACAAGCGCCGACACTCGTTCACGCCGCCCAGCAACGAG
           1nt   GAGCGCCTGGTCAAGGAGGAGAAGCTAAAAACGCCCTACA- GCGCCGACACTCGTTCACGCCGCCCAGCAACGAG
          11nt   GAGCGCCTGGTCAAGGAGGAGAAGCTAAAAACGCC ---- -- -- ---GACACTCGTTCACGCCGCCCAGCAACGAG
          30nt   GAGCGCCTGGTCAAGGAGGAGAAGCTAAAAACGCCC --- -- -- ------ ----- ----- ---- - --- AGCAACGAG
```

Amino Acid   E   R   L   V   K   E   E   K   L   K   T   P   S   N   E
           101   ★   ★   125
                                      Y   K   R   R   H   S   F   T   P   P
                                      113   122

CRISPR Sequencing Results (note each sequence has two alleles)

PAM Site
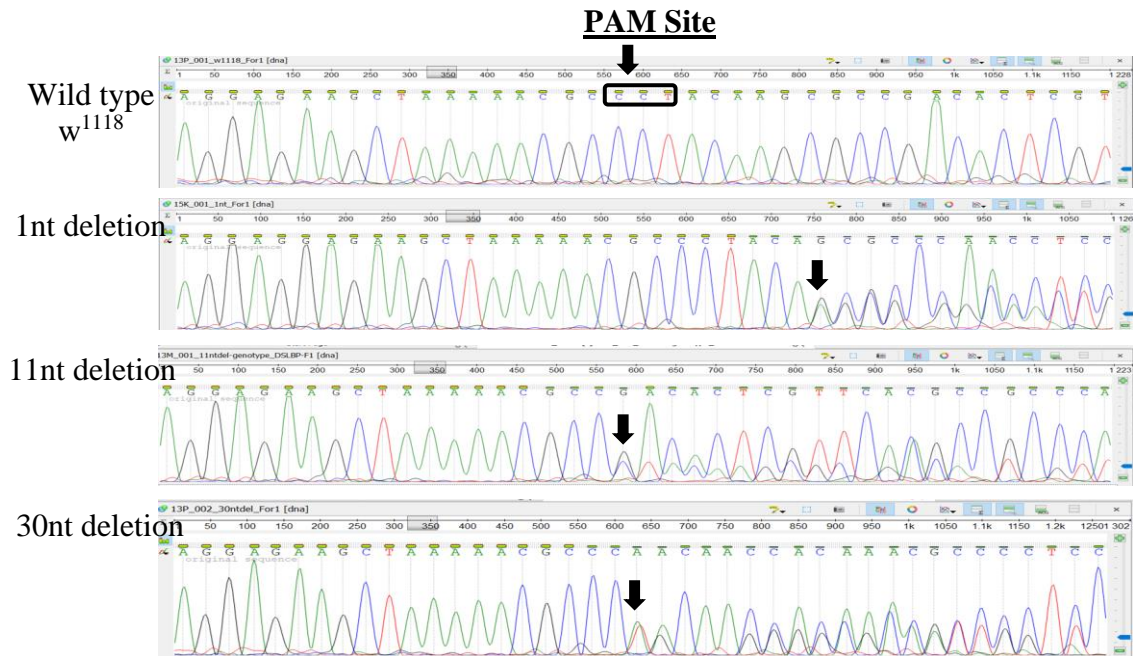
Primer Name	Primer Sequence (5' to 3')
GenomSLBPF	ACCATGCCATCTAGATTGTTTTCGGGGAA
GenomSLBP Rev 1	TGAGGAGTTCGTGAAATGGTTT
(Sequencing Primer) DmSLBPSeqFor1	GAACACACCGCAGAAAGGCT

Northern Blotting

Embryos, larvae or ovaries were analyzed. The samples (20 embryos in 20 ul water, 10 larvae in 100 ul water and 10 females (20 ovaries) in 50 ul water) were homogenized in 1ml of TRIzol Reagent (RNA Isolation Protocol) in a frosted glass homogenizer. The RNA was recovered by precipitation with isopropanol. An RNA loading dye was used to resuspend the samples. The quality of the RNA was confirmed using an agarose gel and staining with ethidium bromide. The loading dye contains xylene cyanol and bromophenol blue, which served as a marker to follow the migration of the samples (it allows to approximate the size). For Northern

blotting, the samples (3ug/ul embryos, 6ug/ul larvae, and 8ug/ul ovaries) were loaded onto a 6% 7M Urea-Acrylamide gel and run at 500V. After both dyes ran off the gel (~1:10hr), the gel was ran for another 30 minutes. The transfer was performed at 30V for 0.8hrs in a Bio-Rad Electro transfer apparatus, and the RNA was transferred onto a Positively Charged Nylon Transfer Membrane from GE Healthcare. The membrane was allowed to dry and UV crosslinked 3X at (12000 Joules x 100). Membrane was pre-hybridized with 5 mL of the Amersham Rapid-hyb buffer at 60°C for 1 hour. Probes for dH3, dH1 and 7SK were generated from PCR products containing the coding region of each RNA and labeled with the Prime-It II Random Primer Labeling kit from Agilent Technologies, and 5 uC/ul of dCTP. Each probe was added to 100ul of Salmon Sperm DNA, boiled for 5 minutes and mixed with 1 mL of pre- hybridization solution. The blot was hybridized with probe overnight at 60°C. It was then washed at 55°C, 3X with 10ml of wash solution (0.8% SSC, 0.08% SDS) for 15 minutes each time. The membrane was dried and the hybridized DNA visualized either on film or in the Amersham Typhoon Biomolecular Imager.

Western Blotting

Embryos were incubated with 50% bleach to remove the vitelline membrane for 5 minutes, then the dechorionated embryos were rinse with dH2O 2X. The samples were collected and homogenized in SDS-PAGE Protein Loading Buffer with either a pestle (embryos) or a 25G needle (ovaries). Samples were loaded into an 8% polyacrylamide gel and run at 100V until the Bromophenol blue reached the bottom of the gel. The samples were transfer into a Nitrocellulose membrane 0.45um from BIO-RAD at 100 V for 1 hour. The membrane was incubated for 1 hour with 5% milk in 0.1 % PBST (Phosphate Buffered Saline and Tween 20). The primary antibody dSLBP (1:1000) was incubated overnight. The secondary antibody ECL Anti-Rabbit IgG,

Horseradish (1:500) from GE Healthcare UK Limited was incubate it for 1 hour. After

visualizing the SLBP the membrane was rinsed and then treated with the loading control

antibody to actin and the secondary antibody ECL Anti-Mouse IgG, Horseradish Peroxidase GE

Healthcare were incubated each for 1 hour. After each incubation the membrane was rinsed with

1% of PBST 3x for 5 min.

RESULTS AND DISCUSSION

      Drosophila SLBP has 276 amino acids, and the SLBP gene contains two exons and one

intron (fig.2.1). As it is shown in figure 2.2, lane 1 and fig. 2.3 A, embryos collected at 1-hr after

egg deposition (AED) contain histone mRNAs and SLBP protein, in a time where transcription

is absent. After activation of the zygotic genome the levels of both (histone mRNA and SLBP

protein) are high from 4 to 12 hrs AED in a period when cells are replicating at a faster pace

(fig.2.2 lane 3 to 4, and fig.2.3 B lane 2 to 3). Later in development, once DNA replication slows

down from 16 to 20 hrs AED histone mRNA and SLBP protein levels decrease (fig. 2.2, lane 6

and fig. 2.3 B, lane 5).

Figure 2.1
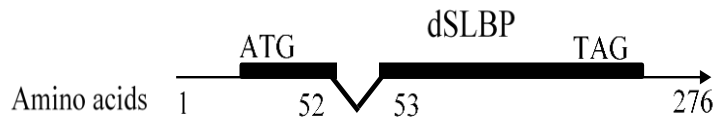
Figure 2.1: Gene structure of Drosophila SLBP. dSLBP contains two exons and one intron.

Figure 2.2
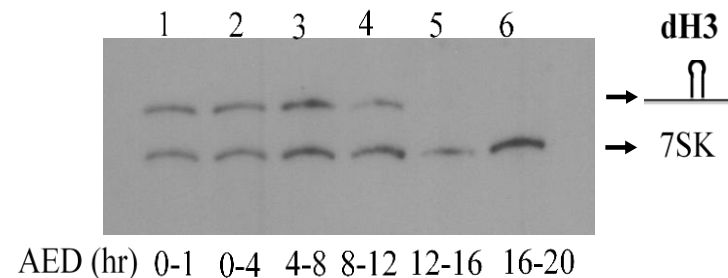
Figure 2.2: Histone mRNA expression in the Drosophila embryo. Wild type embryos were
collected at 1hr and every for 4 hours. Lane 1 represents the histones mRNAs that were loaded
into the egg (e.g. maternally supplied). Histone mRNA levels are high during periods of active
DNA replication and decrease after 12 hrs. as the number of cells replicating DNA decreases.
7SK RNA was used as a loading control.

Figure 2.3

A

AED (hr)　　　0-1

B

AED (hr)　0-4　4-8　8-12　12-16　16-20
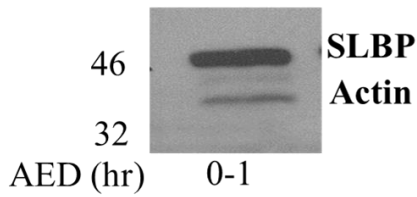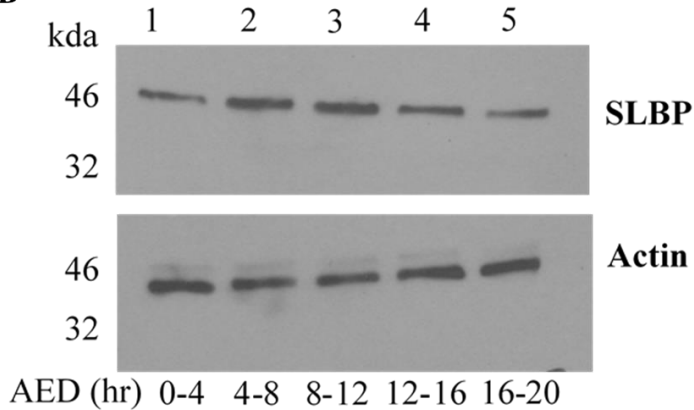
Figure 2.3: SLBP protein expression during embryogenesis. Wild type embryos were collected at 1hr and every 4 hours AED. A It represents the amount of dSLBP protein that was loaded into the egg. B In normal embryos, SLBP levels increases from 4-12hrs AED, and then decreases slowly as development proceeds.　Actin was used as a loading control.

Figure 2.4

A
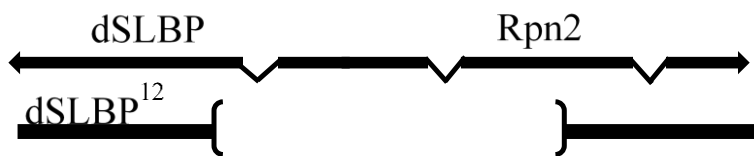
B
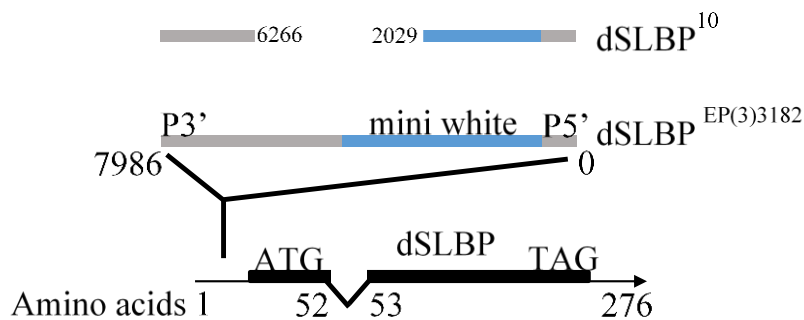
Figure 2.4: dSLBP excision alleles. SLBP locus is located on the right arm of chromosome 3. A dLSBP12, the promoter and portions of the coding region of both dSLBP and RPN2 are deleted. B dSLBP10 contains fragments of a p-element (EP (3)3182) in the 5'UTR of SLBP gene (Sullivan et al., 2001).

To create a null allele of dSLBP, I used the Fly CRISPR-CAS9 system. I designed a guide RNA at the beginning of the second exon of SLBP (fig. 2.5). Once I obtained the CRISPR mutant alleles, I did a genetic cross to first remove the CAS9 (enzyme), and I did a second cross to obtain single CRISPR events (materials and methods). DNA was isolated from flies to conduct PCR and sequencing analyses, to search for mutations (e.g. deletion, insertion) in the SLBP gene (materials and methods). In our CRISPR experiment, I obtained two frameshifts a 1 (SLBPΔ1) and an 11nt (SLBPΔ11) deletion located in the NTD of dSLBP (Materials and Methods).Genetic analyses were done to ask whether these CRISPR alleles were viable over a deficiency of dSLBP (dSLBP12). Each CRISPR allele (SLBPΔ1 and SLBPΔ11), was over a balancer that contained a GFP-tagged chromosome, which allowed me to distinguish homozygous embryos (GFP negative). The genetic cross is illustrated in figure 2.6. Both alleles were not viable, and they died at the first larvae stage. Since, both deletions were not viable, I determined the levels of SLBP in SLBPΔ11 embryos to determine when the maternal supply of SLBP ran out. Embryos were collected from 7 to 10 hrs and 16 to 19 hrs AED, and blot analyses show that the maternal SLBP was gone by 16 to 19 hrs AED (fig. 2.7, lane 4). Interestingly, northern blot analyses demonstrate that these embryos begin to accumulate polyadenylated histone mRNAs (dH3 and dH1) from 4 to 8 hrs AED (fig.2.8 and 2.9, lane 3), and it continues through the first larvae stage (fig.2.10, lane 2), suggesting that the reduced levels of SLBP at these stages led to some misprocessing of the histone mRNA. Effects in histone mRNA processing were seen early in development after activation of the zygotic genome in other SLBP mutants (Sullivan et al., 2001).

To validate that the reduced protein levels of dSLBP were the cause of this phenotype. Genetic analyses were done to rescue the null phenotype with a wild type transgene of SLBP (TG/+; SLBPΔ11/ dSLBP12). The wild type transgene was inserted on the 2nd chromosome and it rescued the phenotype; flies were viable over this background, and females were fertile. Northern blot analyses done from the ovaries of these females (TG/+; SLBPΔ11/ dSLBP12), did not express any polyadenylated histone mRNAs (data not shown). These results confirm that this deletion affects SLBP synthesis or its stability during embryogenesis. Embryos 7 to 10hrs AED (Fig. 2.7, lane 1 (wild type) and 3 (mutant) still had significant amounts of SLBP. It is evident that the organism needs a specific amount of dSLBP (e.g. mRNAs and protein) for histone pre-mRNA processing. We don't know exactly why the organism can't survive with polyadenylated histone mRNAS. In the literature it has been shown that maternal storage of H2A, H2B and H2AV protein is accomplished by binding to lipid droplets which are sequestered by the Jabba protein (Cermelli et al., 2006; Welte 2007; Li et al., 2012 and Li et al., 2014). Sequestering histone proteins into lipid droplets serves as a supply for the early rapid cycles of Drosophila embryogenesis (Li et al., 2012 and Liu et al., 2014). An embryo that expresses a knockout of Jabba doesn't have histone proteins for H2A and H2B, presumably these proteins are degraded (Li et al., 2012). However, this embryo survives with the presence of SLBP which contributes for the synthesis of maternal histone proteins. In a double mutant of Jabba and an SLBP hypomorph, these embryos don't survive, due a lack of histone protein deposited onto the chromatin (Li et al., 2012). Imbalance deposition of histones onto chromatin leads to chromosome defects and compromise organismal survival. This effect might happen from an excess of histones bound to the chromatin or free histones in the cytoplasm. Embryos expressing SLBPΔ11 probably encountered problems to properly package the DNA with histones into

chromatin, which will result in genome instability and cell death (Salzler et al., 2009; Sullivan et al., 2001).

An interesting question is why the organism is not able to survive and continue development with these polyadenylated transcripts. In other mutants, like dSLBP10, which begins to accumulate polyadenylated histone mRNAs late in embryogenesis (Godfrey et al., 2006; Lanzotti et al., 2002), the flies are viable. However, they still contain a substantial amount of properly processed histone mRNAs, which is enough to complete development. It will be interesting to investigate how much SLBP per cell is required to properly process histone mRNAs? or why the cell can only tolerate a reduce amount of polyadenylated histone mRNAs during Drosophila development? Addressing these questions will help us understand the biology and molecular dynamics of this process.

This is the first null allele of dSLBP created in the Marzluff lab, and the phenotype of this mutant is more severe than other dSLBP mutants characterized in the lab. It validates that SLBP is a key component for histone mRNA biogenesis and that it is necessary for 3' end processing in vivo.

Figure 2.5
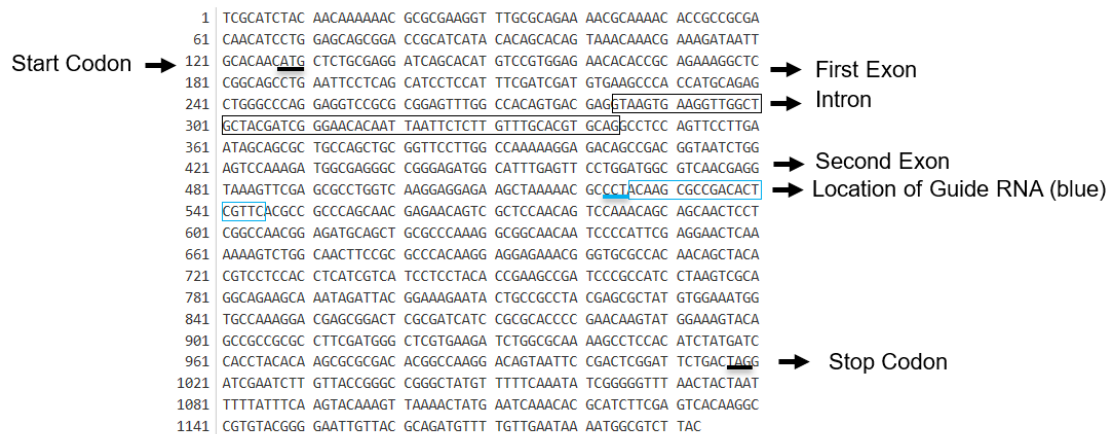
Figure 2.5: Genomic sequence of dSLBP to indicate location of the Guide RNA. dSLBP contains two exons and one intron. In blue is indicated the location of the designed Guide RNA.

28

Figure 2.6

Virgin Females   Males

$$\frac{SLBP^{\Delta 11}}{TM3\ Ser\ P[act\text{-}GFP]} \quad X \quad \frac{dSLBP^{12}}{TM3\ Ser\ P[act\text{-}GFP]} \quad \longrightarrow \quad \frac{SLBP^{\Delta 11}}{dSLBP^{12}}$$

33% Homozygous embryos
(not green)
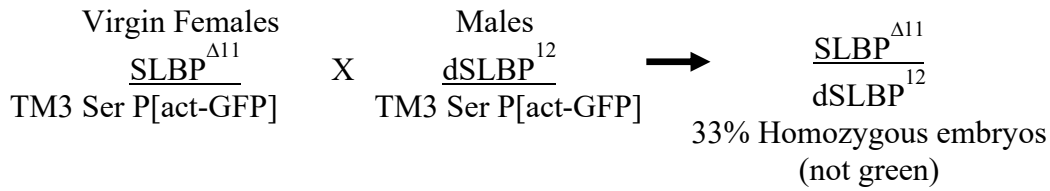
Figure 2.6: Genetic analyses of CRISPR mutants over dSLBP12 (null). Each mutant was over a balancer chromosome that contained a GFP-tagged (heterozygous). Homozygous embryos were identified by the absence of GFP.

Figure 2.7
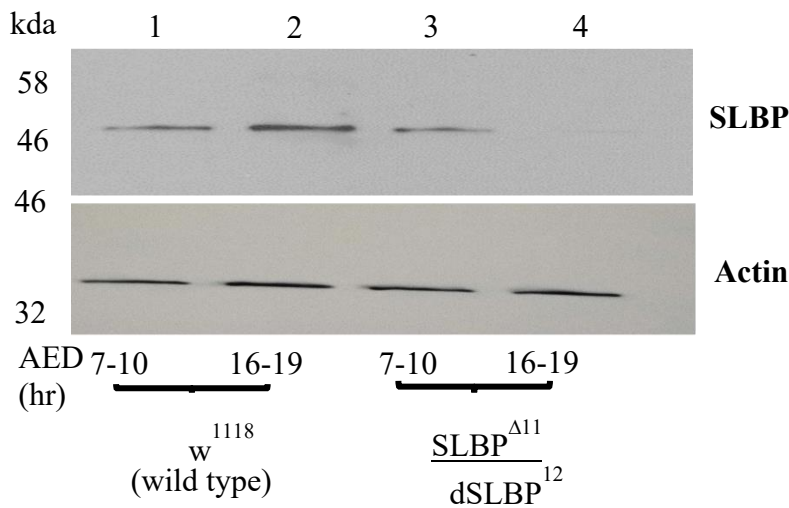
Figure 2.7: SLBPΔ11 runs off maternal SLBP at the end of embryogenesis.
Wild type and homozygous embryos were collected from 7-10 and 16-19 hrs AED. SLBP protein levels declined in SLBPΔ11 by 16-19 hrs AED. Actin was used as a loading control.

Figure 2.8

29
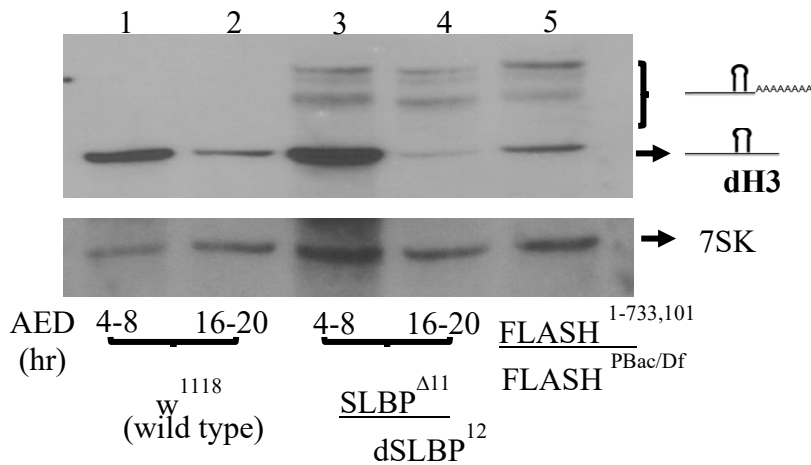
Figure 2.8: SLBPΔ11 begins to accumulate poly A+ after activation of the zygotic genome. Wild type and homozygous embryos were collected from 4-8 and 16-20 hrs AED. Polyadenylated dH3 accumulates at 4-8hrs, and it persists later in development. 7SK RNA was used as a loading control and FLASH 1-733,101/ FLASH PBac/Df as a positive control.

Figure 2.9
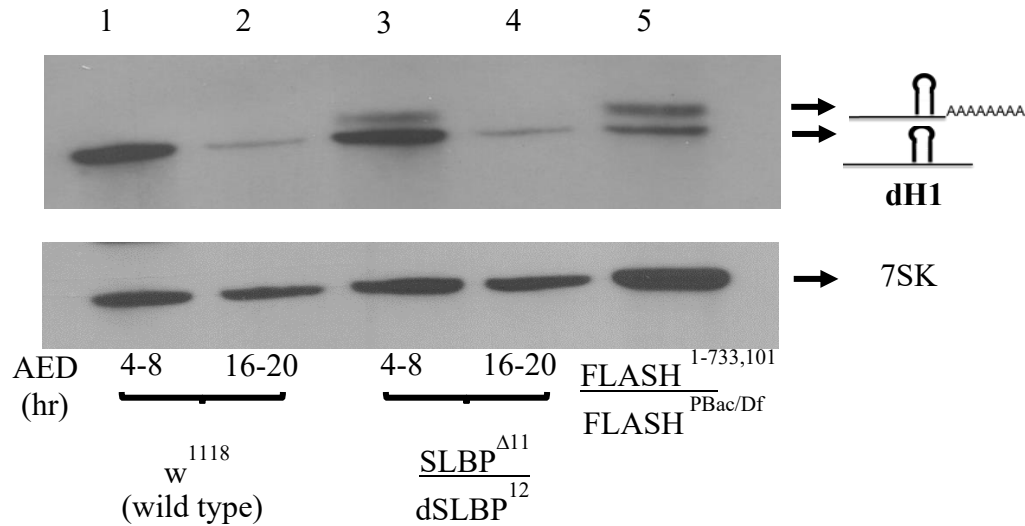
Figure 2.9: SLBPΔ11 accumulates polyadenylated transcrips after the activation of the zygotic genome. Wild-type and homozygous embryos were collected from 4-8 and 16-20 hrs AED. Polyadenylated dH1 accumulates at 4-8hrs, and its stability declines by 16-20hrs. 7SK RNA was used as a loading control and FLASH 1-733,101/ FLASH PBac/Df as a positive control.

Figure 2.10
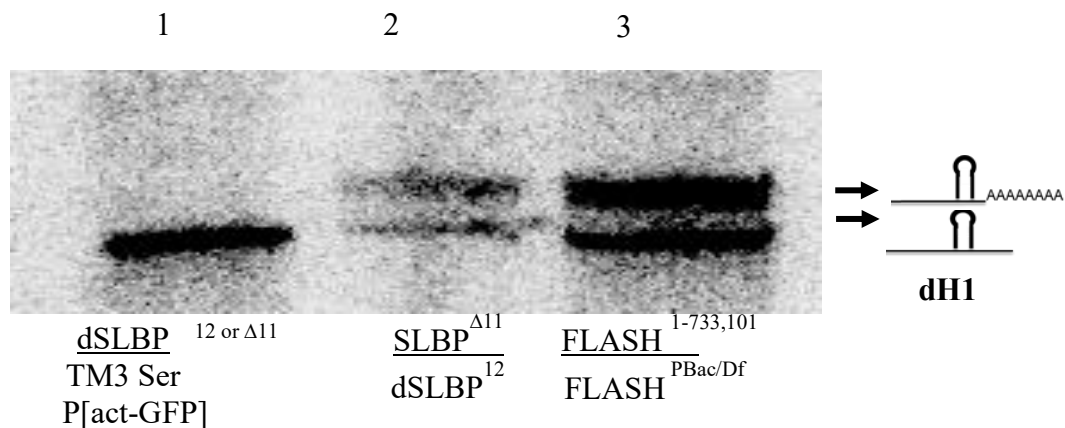
Figure 2.10: Polyadenylated transcripts persists in the larval stages. Homozygous SLBPΔ11/ dSLBP12 and heterozygous dSLBP12/TM3 Ser P [act-GFP] larvae were collected at the first larval stage. SLBPΔ11 expresses polyadenylated dH1 at the first larvae stage. The FLASH 1-733,101/ FLASH PBac/Df was used as a positive control.

CHAPTER 3: ROLE OF PHOSPHORYLATION AND SLBP IN AN IN VIVO SYSTEM

INTRODUCTION

The Marzluff lab has primarily investigated the proteins required for histone mRNA biosynthesis, including SLBP, in vitro. Based on these biochemical analyses, the lab has wanted to ask these same questions in a vivo system. In the previous chapter, I described that SLBP is necessary for histone mRNA biogenesis in animals and, most importantly, for histone mRNA processing. In this chapter, I describe regions of SLBP that has been tested for activity in processing in vitro, but not in vivo. I focused on amino acids that are known to be phosphorylated in vivo and in vitro. The region in SLBP required for RNA processing is the 73 aa RNA binding domain followed by 15-20 amino acids, which are not required for RNA binding but is required for processing. The RNA binding domain is very conserved in animals, while the amino acids C-terminal to the RBD are more variable. In mammals, the RNA binding and processing domain is located in the center of the protein, but in Drosophila it consists of the last 90 amino acids of the protein.

The first phosphorylated region located in the RBD, is the TPNK motif, which is present in SLBP in all organisms (fig.3.1). The threonine is phosphorylated, and according to mass spectrometry analyses, all the SLBP molecules are phosphorylated, both in proteins expressed in baculovirus and in Drosophila S2 cells. Structural analyses revealed that this phosphorylation stabilizes SLBP structure and is necessary for high affinity binding of SLBP to the stem-loop (Zhang et al., 2012). The other phosphorylated region is the C-terminal domain of SLBP, which

contains a hyperphosphorylated region (e.g. SNSDSDSD sequence) (fig.3.4). As determined by

mass spectrometry this region is also quantitatively phosphorylated in baculovirus expressed

SLBP (Borchers et al., 2006) and in vivo. The C-terminal region of SLBP is required for histone

mRNA processing in vitro (Dominski et al., 2002; Skrajna et al., 2017; Zhang et al., 2014), and

maximal activity depends on phosphorylation of the C-terminus. Point mutants in SLBP that

prevent phosphorylation in this region shows that histone mRNA processing is reduced, and it

decreases the recruitment of the U7 snRNP to the histone pre-mRNA (Skrajna et al., 2017).

However, it is unclear what is the precise role of the phosphorylation in vivo, or whether

phosphorylation is regulated in vivo. Now that I have created a null allele of SLBP (SLBPΔ11), I

proceeded to conduct genetic analyses with these point mutants in vivo, to determine if they had

biological function in vivo in the presence of the null mutation.

MATERIALS AND METHODS

Genotyping of rescued flies

Transgenes that would express the mutant SLBP from the SLBP promoter were constructed in the pATTB vector and injected into embryos by BESTGENE. The transgenes were inserted at the 25C site in the 2nd chromosome. To confirm that the analyzed transgenes were correct; DNA was isolated from rescued males that contained the respective genotype on the 2nd and third chromosomes: TG/+; SLBPΔ11/dSLBP12, where a single copy of the transgene is inserted (Orange eye and Non-tubby flies). PCR and sequencing analyses of the respective genotypes were analyzed (4S-4A and 4S-4E), and NPNK males with the respective genotype: TG/+; +/TM3. Note that the C-terminus of SLBP is present in the dSLBP12 mutant, and there are two wildtype SLBP gene in the males carrying the SLBP transgenes.

NPNK (genotype: TG/+; +/TM3)

4S-4A

4S-4E

34
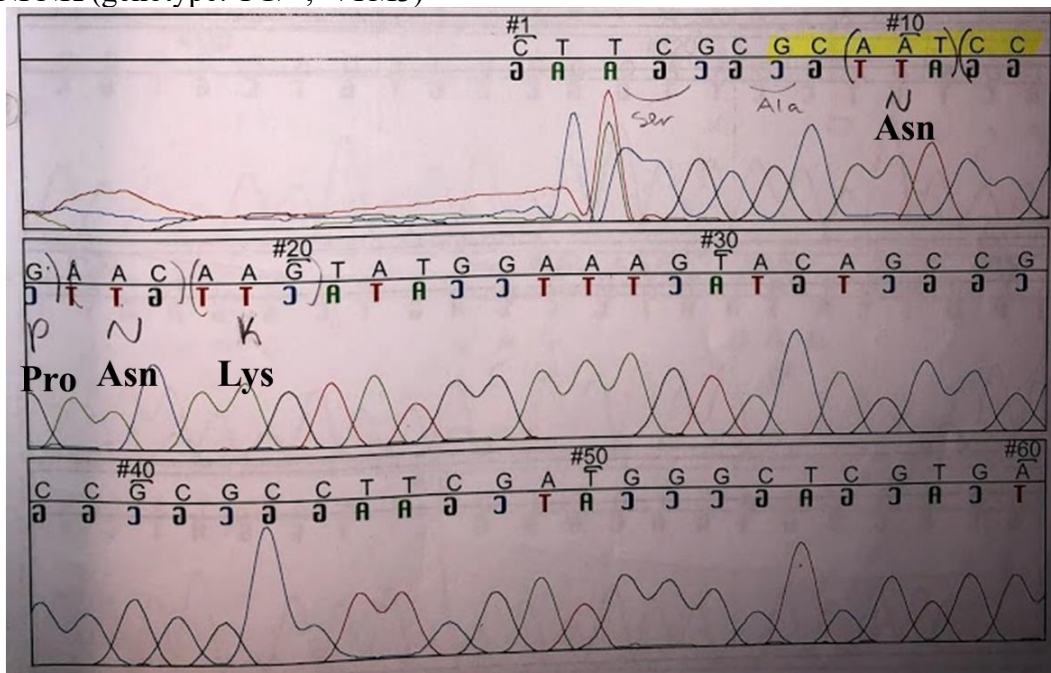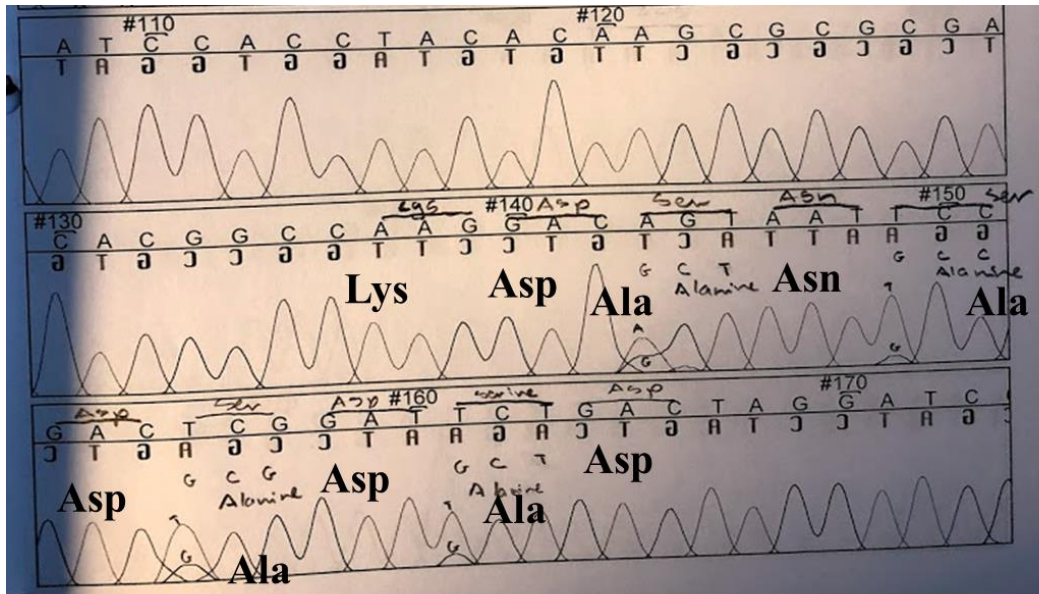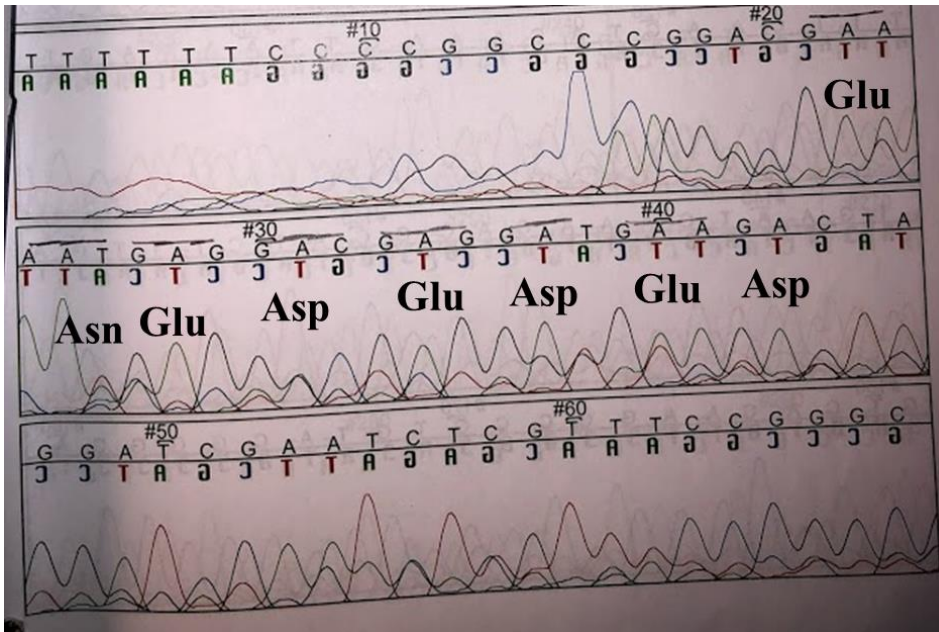
RESULTS AND DISCUSSION

We introduced four different transgenes, a wild-type gene, and three mutant genes, T231N, and two mutations changing the 4 serines at the C-terminus to either 4 alanines or 4 glutamic acids. Phosphorylation of T231 stabilizes the structure of SLBP bound to the SL. TPNK is in the center of a loop and the phosphate on T231 that bridges the three α -helices make up the SLBP RBD, stabilizing the structure. Substituting an asparagine for threonine potentially restores some of the bonds between the phosphothreonine and the three α -helices (Fig. 3.2). Previously Lanzotti et al. showed that a point mutant, T231A, that abolishes phosphorylation (APNK), results in weaker binding of SLBP to the SL but was not necessary for SLBP to bind to the SL and for histone mRNA processing in vitro (Lanzotti et al., 2004). However, genetic analyses expressing the point mutant APNK, was not viable and accumulated polyadenylated histone mRNAS in the Drosophila embryo (Lanzotti et al., 2004). This data demonstrates that the phosphorylated residue is critical for SLBP to bind to the SL in vivo.Point mutants were created by PCR and cloned into the pattB vector. The transgenes were integrated by RCME in the 2nd chromosome at the 25C site (Groth et al., 2004). Since we have a null allele SLBPΔ11, I conducted genetic crosses to rescue with a transgene the lethality of this null allele (fig.3.3). The first region I analyzed was the TPNK motif located in the RBD. I asked if we could rescue the APNK phenotype obtained in Lanzotti et al., by mutating the Threonine to Asparagine. Interestingly, the NPNK transgene did not rescue the phenotype, and embryos died at the first larvae stage, presumably due to failure of efficiently misprocess histone mRNAs. The phosphate on the hydroxyl group that the threonine contains plays a role in bringing all three α-helices (αA–αC) together (fig.3.2) (Zhang et., 2014). The lysine located in helix αA, its an important amino acid for this connection (fig.3.2) (Zhang et., 2014). A point mutant of this lysine abolishes the

the binding of SLBP to the SL in vitro (Dominski et al., 2001). In Zhang et al., 2014; they also

showed with crystallography analyses that this side chain (e.g. lysine) might be important to

interact with the phosphate group in the threonine (fig.3.2). Based on this data it explains why

this threonine is highly conserved throughout evolution (fig.3.1) and the importance of the -

phosphothreonine OH to stabilize SLBP to the SL. Note that if the T is changed to E, the

structure of the loop is partially restored, but most of the loop is still disordered (fig.3.2, Zhang et

al., 2014).

Figure 3.1

```
Human           164   QPGIHPKTPNKFKKYSRRS
Mouse           164   QPGIHPRTPNKFKKYSRRS
X. laevis 1     162   EPNVHPRTPNKFKKYSRRS
Danio rerio 1   169   QPGVHPKTPNKFKKYSRRS
X. laevis 2     137   KSGVHPRTPNKSKKYSRRS
Danio rerio 2   160   QPGVHPKTPNKFKKYSRRS
Drosophila      224   -TRDHPRTPNKYGKYSRRA
C. elegans      241   IKGQHPRTPNKLINFSRRS
```

Figure 3.1: Sequence alignment of a portion of the RNA Binding Domain of SLBP. The TPNK motif is highly conserved in Human, Mouse, C. elegans, Xenopus, Zebrafish and Drosophila.

Figure 3.2: Obtained from Zhang et al., 2014.
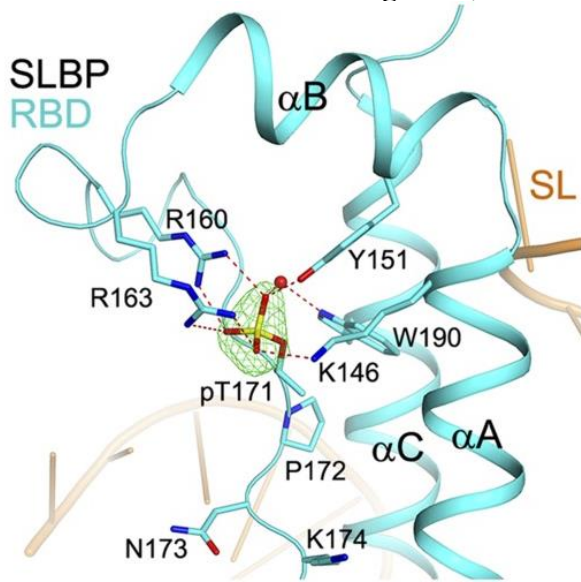
Figure 3.2: Crystallography of the Human SLBP bound to the SL. Illustrated is pT171, the Phosphorylated Threonine at this position helps to forms a bridge with the three helices side chains (Zhang et al., 2014).

Figure 3.3

Virgin Females   Males

$$\frac{\underline{+}}{+} \; ; \; \frac{SLBP^{12}}{TM6B} \quad X \quad \frac{Transgene}{+} \; ; \; \frac{SLBP^{\Delta 11}}{TM6B}$$

Rescues

2nd $\frac{Transgene}{+}$; 3rd $\frac{SLBP^{\Delta 11}}{SLBP^{12}}$

Orange eye and Non-tubby flies
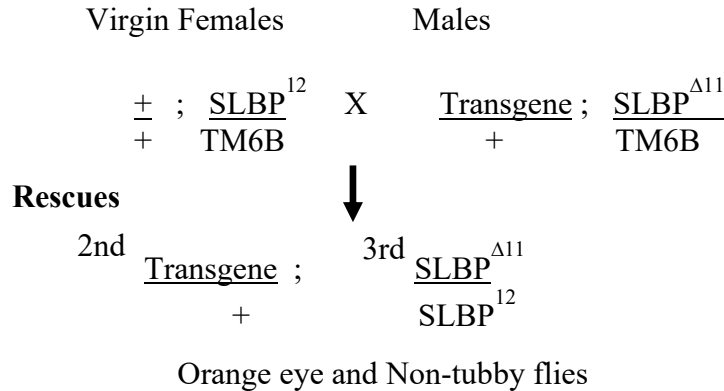
Figure 3.3: Genetic analyses of point mutants in the null background. Each mutant was over a balancer chromosome. Homozygous flies were identified by the absence of TM6B (tubby phenotype). The presence of the transgene was identified by the orange eye color.

The other region that I evaluated was the CTD of SLBP. Point mutants were created and cloned in the pattB vector. Transgenes were integrated by RCME in the 2nd chromosome at the 25C site (Groth et al; 2004). Two four amino acid mutants were created to test if phosphorylation at the CTD was required for histone mRNA processing in vivo. The purpose of the first mutant

was to abolish phosphorylation at the CTD; for this reason, the serines were changed to alanines (4S-4A). The second mutant was to mimic phosphorylation, and the serines were mutated to glutamic acid (4S-4E). I conducted a genetic cross for each transgene in the background of a null allele of dSLBP illustrated in figure 3.3, for 4S-4A, 4S-4E, and a wildtype SLBP (fig.3.5). The wild type transgene rescued the null phenotype, and restored processing. Surprisingly, both mutants also rescued the null phenotype, and they were viable and fertile. Western blot analyses were done from dissected ovaries, and SLBP protein levels are normal compared to wild type (fig.3.6). I further conducted a northern blot analyses, and these ovaries did not express any polyadenylated histone mRNAs (fig.3.7). These results demonstrate that phosphorylation at the CTD is not an essential factor for SLBP function in vivo. We believed based on the in vitro and crystallography analyses that phosphorylation at the CTD was required for SLBP stability when is bound to the SL; however, the requirement for phosphorylation of the CTD for folding SLBP is not essential in vivo, suggesting that there must be other factors present in vivo to assure SLBP folding correctly.

The phosphorylation of TPNK behaves the opposite; it is much more important in vivo than in vitro. This phosphorylation does not have as large an effect as phosphorylation of the C–terminal in vitro, but it is essential in vivo. Its possible that failure to phosphorylate the TPNK promotes aggregation of the SLBP with other cellular proteins.

Figure 3.4


```
               ★ ★ ★ ★
DROME 260 TQARDTAKDSNSDSDSD
HUMAN 201 AEEGCDLQEIHPVDLESAESSSEPQTSSQDDFDV
```

Figure 3.4: Sequence alignment of the CTD of the Human and Drosophila SLBP. The CTD of SLBP, contains a hyperphosphorylated region (e.g. SNSDSDSD sequence), that is required for histone mRNA processing in vitro.

Figure 3.5

Transgene	Position	Sequence
Wild type	C-term	TQARDTAKD**S**N**S**D**S**D**S**D
4S-4A	C-term	TQARDTAKD<u>A</u>N<u>A</u>D<u>A</u>D<u>A</u>D
4S-4E	C-term	TQARDTAKD<u>E</u>N<u>E</u>D<u>E</u>D<u>E</u>D

Figure 3.5: Point mutants created in the CTD of Drosophila SLBP. Illustrated are the transgenes that were created and cloned in the the pATTB vector. Point mutants were created to either abolish 4S-4A or mimic 4S-4E phosphorylation of SLBP. A wild-type transgene of dSLBP was used as a control.

Figure 3.6
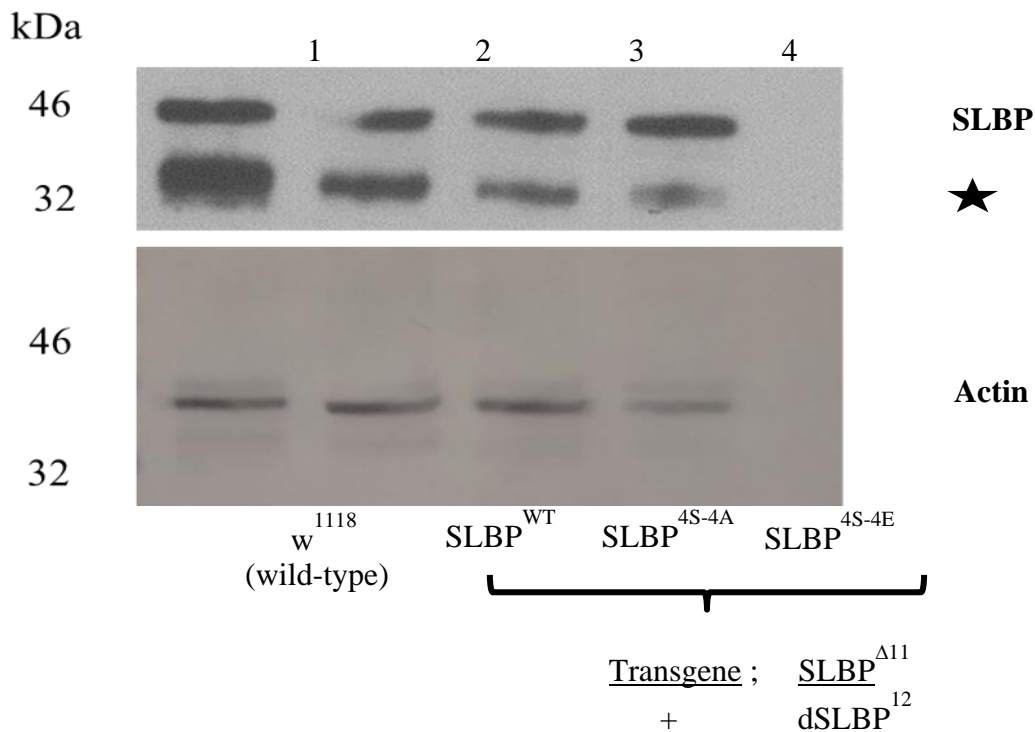
Figure 3.6: SLBP protein expression is normal in point mutants. Ovaries were dissected from wild type (w1118) (no transgene) and from the transgenes (WT SLBP) (4S-4A) (4S-4E). SLBP protein levels in points mutants (lane 3 and 4) are normal compared to wild type (lane 2). Actin was used as a loading control. The two bands for SLBP are due to partial degradation of SLBP in the N-terminal domain.

Figure 3.7
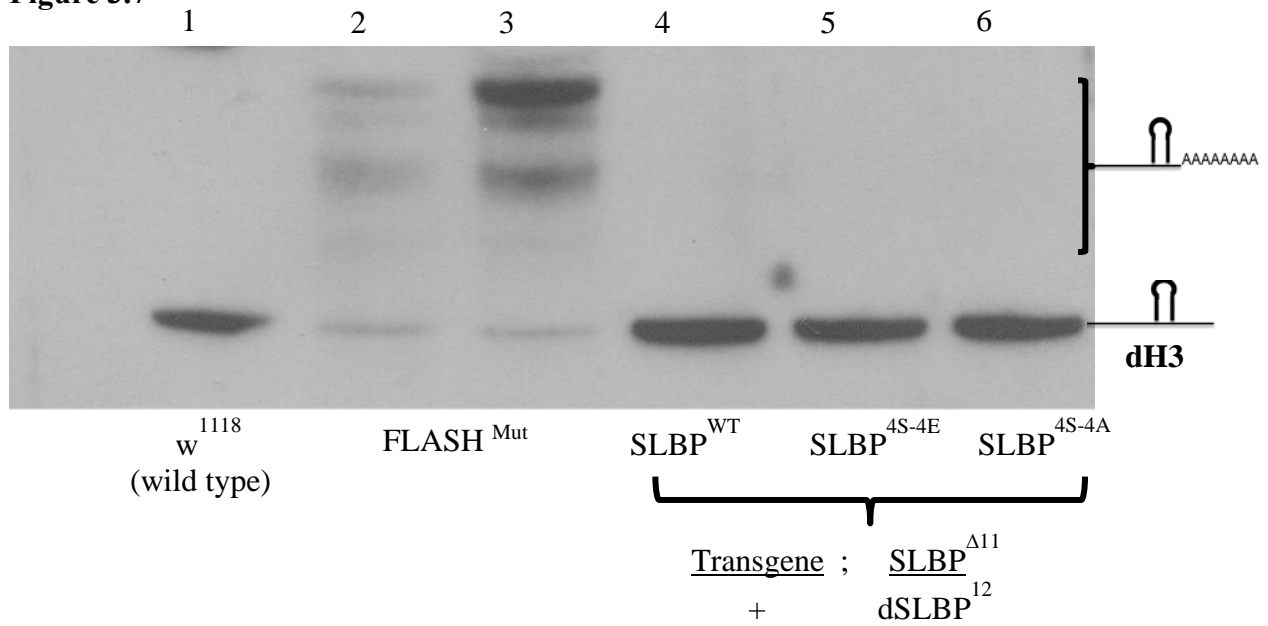
Figure 3.7: Point mutants don't express polyadenylated histone mRNAs. Dissected ovaries were collected from the respective genotypes w1118, SLBP WT, SLBP 4S-4E, and SLBP 4S-4A. Point mutants don't have polyadenylated transcripts dH3 being express in the ovary. FLASHMut was used as a positive control.

CHAPTER 4: OVARIES EXPRESSING THE SLBP Δ30 FAILED TO DEPOSIT HISTONES MRNAS INTO THE EGG

INTRODUCTION

In previous chapters, I demonstrated that a null allele of SLBP is 1st instar larvae lethal, and it accumulates polyadenylated histone mRNAs early in embryogenesis. I also conducted studies in conserved residues in the structure of dSLBP, and some of these residues are not essential in vivo. In this chapter, I will describe another mutation made within the same CRISPR experiment in a region that has not been previously studied in detail.

In this chapter, I will answer unknown questions that can be addressed with the current technology. How histone mRNA synthesis is regulated during oogenesis to deposit large amounts of histone mRNA into the egg? Does SLBP have a specific role during this regulation? SLBP is not cell cycle regulated in the embryo. Is it cell regulated during oogenesis? Drosophila females contain two ovaries. Each ovary is composed of a string of cells that contains a germarium located in the anterior region. In this region stem cells divide and differentiate into a daughter cell (Bastock and St Johnston, 2008). The daughter cell goes throughout 4 mitotic divisions which develop as a cyst (egg chamber) of 16 cells, one of which will differentiate into an oocyte (Bastock and St Johnston, 2008). The egg chamber contains 15 nurse cells and the oocyte and is surrounded by a layer of follicle (somatic) cells (Bastock and St Johnston, 2008). Egg chambers will continue to develop with multiple endoreplication in the nurse cells, until a mature egg is produced at stage 14 . (Bastock and St Johnston, 2008). In this chapter I report that the expression of histone mRNAs in steg 10B of oogenesis that will be deposited into the oocyte,

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differs from the expression of histone mRNAs during S-phase. A mutant of SLBP which has a small deletion in the N-terminal region of the protien, that I isolated in a CRISPR experiment does not support histone mRNA expression in stage 10B. The defect is a failure to transcribe the histone genes in stage 10B. Flies expressing this mutant are viable and males are fertile. The mutant flies produce small amounts of polyadenylated histone mRNA throughout development, indicating there is also a defect in the efficiency of processing histone mRNA.

MATERIALS AND METHODS

Genotyping of rescued flies

Males that contained the same genotype as females were analyzed by PCR to confirm the presence of the transgene. The flies that rescued have the transgene in the 2nd chromosome and the 3rd chromosome contains the SLBPΔ11 and dSLBP12 alleles. Since dSLBP12 has an internal deletion that deleted the first exon, we designed primers in the first exon and a second primer after the PAM site. This PCR allowed us to detect the transgene and SLBPΔ11.
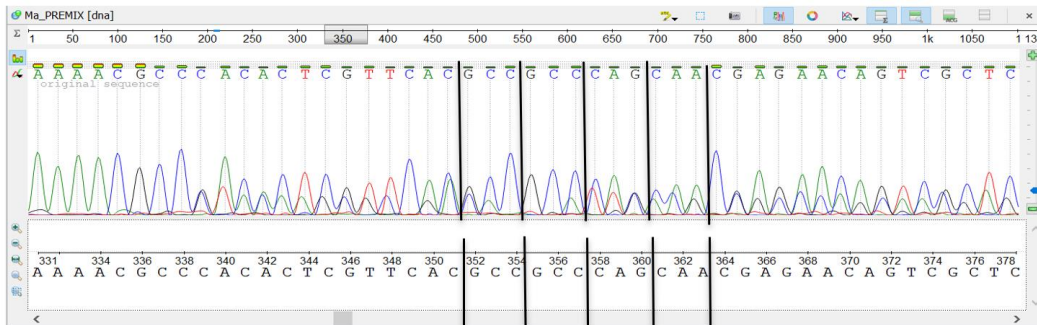
SLBP^{Δ11} CAC GCC GCC CAG CAA C
SLBP$^{S \text{ to } A}$ CGA CAC **GCA** TTC ACG C

.
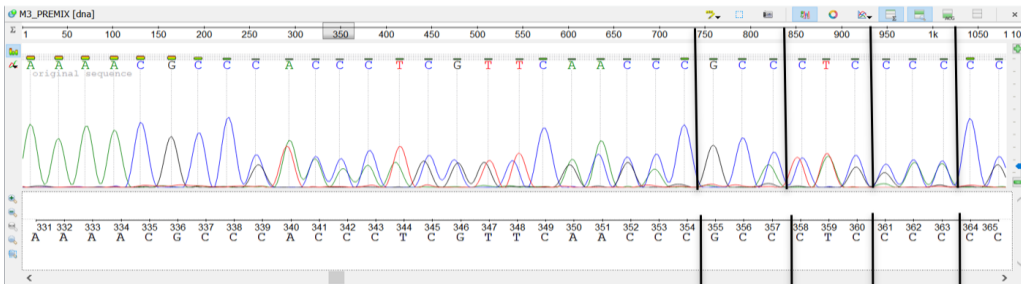
SLBP^{Δ11} G C C G C C C A G C A A C
SLBP$^{S/T \text{ to } AA}$ C A C **G C A** T T C **G C C** C

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w[1118]   AAAACGCCCTACAAGCGCCGACAC**TCG**TTC**ACG**CCG

SLBP[Δ11] AAAACGCC - - - - - - - - - - - GACACTCGTTCACGCCG

       K   T   P   Y   K   R   R   H   **S**   F   **T**   P

SLBP[S to A]   AAAACGCCCTACAAGCGCCGACAC**GCA**TTCACGCCG

       K   T   P   Y   K   R   R   H   **A**   F   T   P

SLBP[S/T to A] AAAACGCCCTACAAGCGCCGACAC**GCA**TTC**GCC**CCG

       K   T   P   Y   K   R   R   H   **A**   F   **A**   P

RESULTS AND DISCUSSION

Histone mRNA expression in the ovary was divided into two phases by two groups, Marcello Jacobs-Lorena and Paul Schedl. The first phase goes from stage 5 to stage 10A. During this time, the nurse cells undergo endoreduplication, with S and gap phases and histone mRNA expression is periodic and is not observed in all cells at the same time (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985). We used single-molecule fluorescent in situ hybridization (smFISH) analyses were done to detect histone mRNA expression. A histone coding probe (H3) to visualize histone mRNAs shows that expression is mainly observed in the cytoplasm (Fig. 4.1). During these stages' DNA replication correlates with histone mRNA synthesis and cells in S-phase can be visualized by staining of the histone locus bodies (HLBs) with a mitosis-specific monoclonal antibody MPM-2 and the HLB antibody FLASH (fig.4.2). Mpm-2 antibody detects phosphorylated Mxc, which is present only in S-phase cells as a result of its phosphorylation by Cyclin E/Cdk2 (Calvi et al., 1998; White et al., 2007b). HLBs are present in all the nurse cells, but anti-Mpm-2 only stains the HLBs in S-phase cells. After the completion of endoreduplication in the nurse cells at stage 10A all the histone mRNAs used during endoreduplicaition have been degraded (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985).

The second phase of histone mRNA synthesis goes from stage 10B to stage 14. Stage 10B is the stage at which histone gene expression starts. At this stage histone mRNA is induced, and histone mRNA synthesis occurs in all the nurse cells (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985). This synthesis is not coupled to DNA replication. We visualized this dynamic regulation using a histone coding probe (H3) in figure 4.3 in stage 10B oocytes. H3 foci

is observed in all the nurse cells (fig.4.3). These transcription foci in the nuclei are at HLBs and stain FLASH. Surprisingly they do not stain with Mpm-2 (Fig. 4.3), indicating that Mxc is not phosphorylated. Thus the regulation of histone gene transcription in the stage 10B nurse cells differs from the regulation during earlier stages of oogenesis. After histone mRNA synthesis, nurse cell breakdown initiates, and the synthesized histones mRNAs and proteins are transported into the growing oocyte (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985) SLBP is required to properly process histone pre-mRNAs in all cells, including during oogenesis (Godfrey et al., 2006), and and for deposition of histone mRNAs into the egg (Lanzotti et al., 2002; Sullivan et al., 2001). However, litlle is known about SLBP expression or localization during oogenesis. Previously, it has been shown that SLBP doesn't have any nuclear export signal, and it depends on binding to the histone mRNA SL to be transported to the cytoplasm (Erkmann et al., 2005a). Previously, in Erkman et al, they mapped and characterized two potential nuclear import localization signals. One signal located at the NTD, the RKRR and the KVRH located at the CTD (Erkmann et al., 2005b). GST-GFP-SLBP contructs of each point mutant were transfected into HeLa cells, and each mutation reduces significantly SLBP nuclear localization (Erkmann et al., 2005b). SLBP in mammalian cells is cell cycle regulated and SLBP is nuclear during G1 and G2 but located in the cytoplasm during S-phase (Erkmann et al., 2005b). SLBP in Drosophila is not cell cycle regulated and little is known if it contains any nuclear localization signals (e.g. export or import).

Figure 4.1
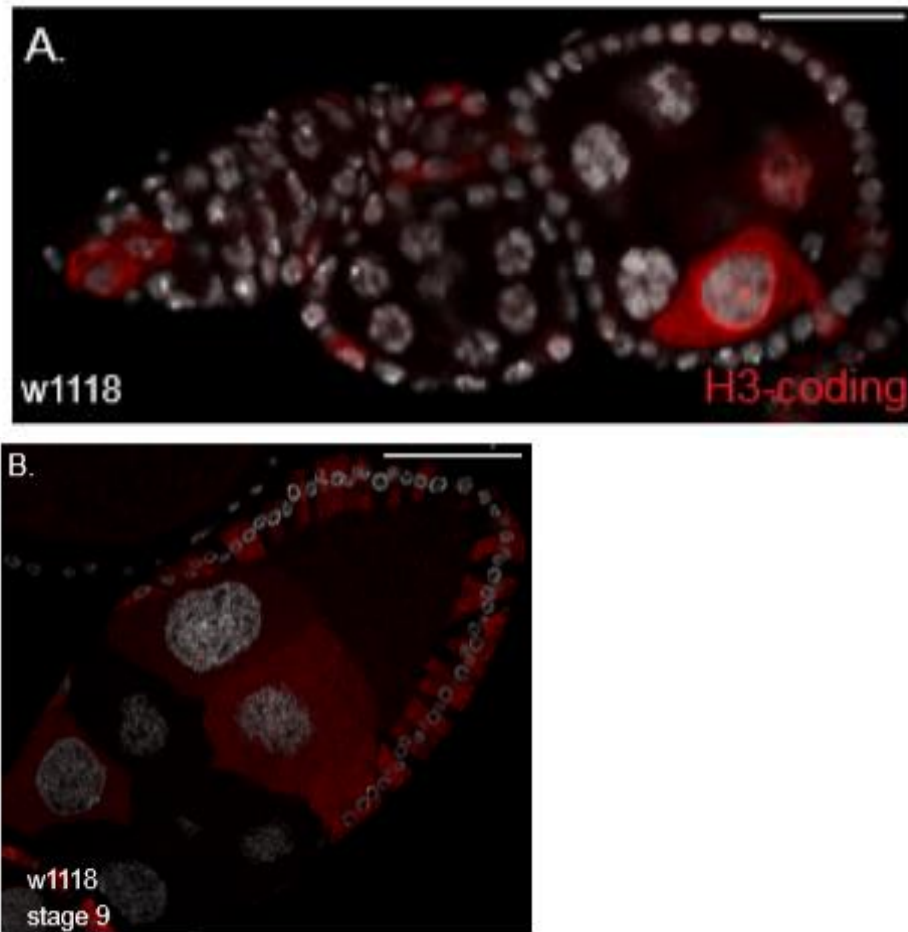
Figure 4.1: Histone mRNA expression at egg chambers during the first phase of oogenesis. A H3-coding probe was used to visualized histone mRNA expression in the ovaries of wild type females. A Representation of egg chambers from early stages of oogenesis. B H3 expression in follicle cells and the cytoplasm at stage 9.

Figure 4.2
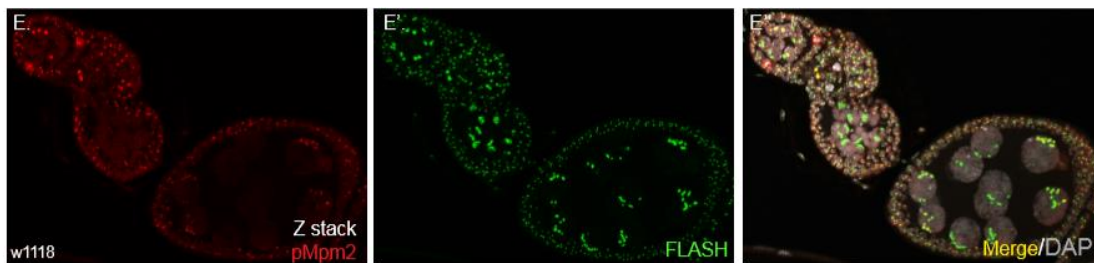
Figure 4.2: HLB expression during oogenesis. Mpm2 (red) recognizes mitotic cells. FLASH was used to visualize HLB formation

Figure 4.3
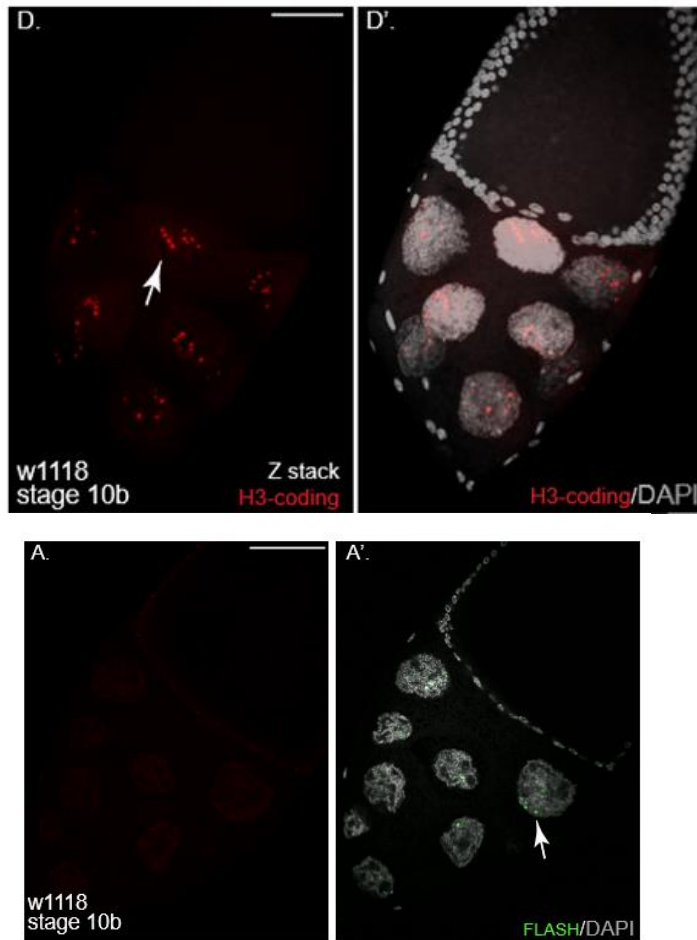
Figure 4.3: Histone mRNA expression in the second phase of oogenesis. Wild type ovaries were probe at stage10B. A Histone mRNA is expressed in all nurse cells (H3-coding probe) B FLASH correlates with HLB assembly and DAPI was used to visualize the DNA.

SLBPΔ30 is viable but female sterile.

In the same CRISPR experiment described in chapter II, I obtained a 30nt deletion (SLBPΔ30) that deletes 10 amino acids at the NTD of dSLBP (fig.4.4) (Chapter II- materials and methods). I conducted genetic analyses to ask whether SLBPΔ30 was viable over a deficiency dSLBP12. The flies were viable, but the females were sterile. Previously, the Duronio and Marzluff labs described dSLBP10, which is viable in a null background of SLBP, but females were sterile due to a reduced amount of histone mRNA and protein loaded into the egg (Lanzotti

et al., 2002; Sullivan et al., 2001). As a control, I used the same flies to compare with my CRISPR mutant, which had the same phenotype (e.g. female sterility). The cause of dSLBP10 phenotype was thought to reduced amounts of SLBP protein, although reagents to determine the protein levels were not available. To determine whether SLBP levels were affected in SLBPΔ30, I proceed to ask whether SLBPΔ30 and SLBP10 had the same molecular phenotype. I dissected ovaries from these flies and did western blot analyses. The ovaries of both alleles were similar in size and developed normally compared to wild type, and they did not have any morphological defects (fig.4.5). Ovaries expressing dSLBP10 had reduced protein levels of dSLBP, but the SLBPΔ30 expressed normal levels compared to wild type (fig.4.6). This experiment was the first time we were able to visualize the protein levels of dSLBP. I continued to ask whether these mutants had any defects in histone pre-mRNA processing in the ovary. Previously, Godfrey showed that dSLBP10 did not have any detectable polyadenylated histone mRNAs in the ovary, but there were reduced levels of processed histone mRNAs in the ovary (e.g. 3'end histone mRNA) (Godfrey et al., 2006). I extracted and isolated RNA from ovaries expressing each allele for northern blot analyses. The dSLBP10 (fig.4.7 lane 2) had lower levels of histone mRNA compared to wild type (fig.4.7 lane 1) as shown previously. However, SLBPΔ30 (fig.4.7 lane 3) not only had a reduced amount of histone mRNAs, but it also expressed polyadenylated transcripts. This result was a surprise but suggested that histone mRNA levels were relevant to why these females were sterile.

Figure 4.4
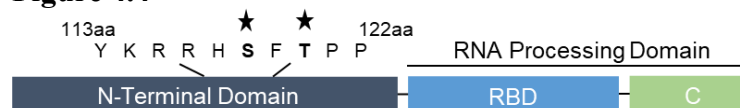
Figure 4.4: dSLBP structural domains. Illustrated are the 10 amino acids that are deleted at the NTD. Notice that this deletion removes two residues that are known to become phosphorylated.

Figure 4.5

w^{1118} dSLBP^{10} SLBP$^{\Delta30}$

Figure 4.5: Ovaries from females expressing each mutant. Ovaries of females expressing each genotype were dissected about 4 to 6 days after they eclosed. The ovaries of each mutant developed normally compared to wild type.

Figure 4.6
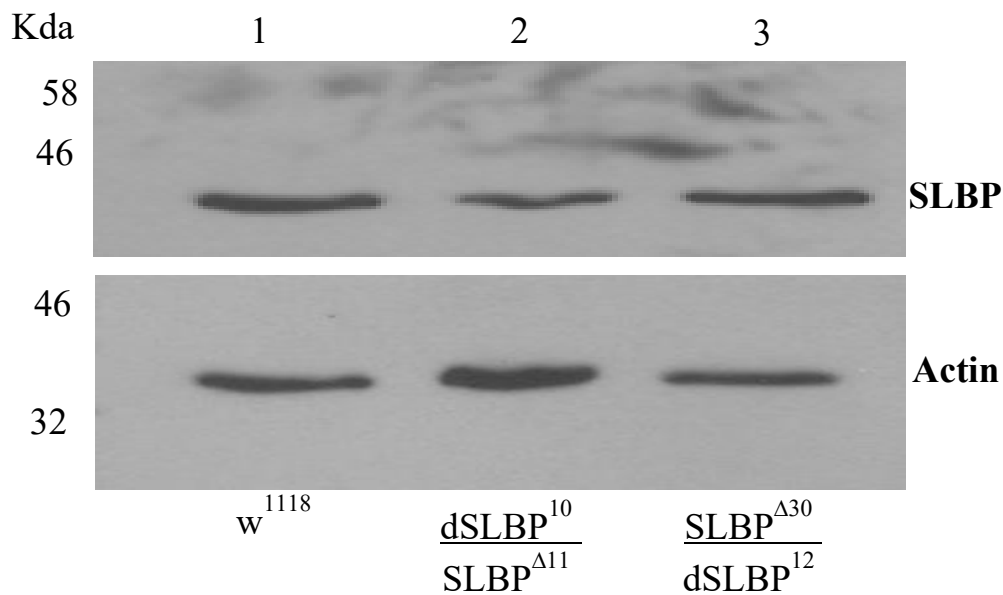
Figure 4.6: dSLBP protein expression in both hypomorphs. Ovaries from each genotype were dissected and homogenized with 2X SDS loading buffer. dSLBP10 (lane 2) expresses lower levels of dSLBP compared to w1118 (lane 1) and the SLBPΔ30 (lane 3). Actin was used as a loading control.

Figure 4.7
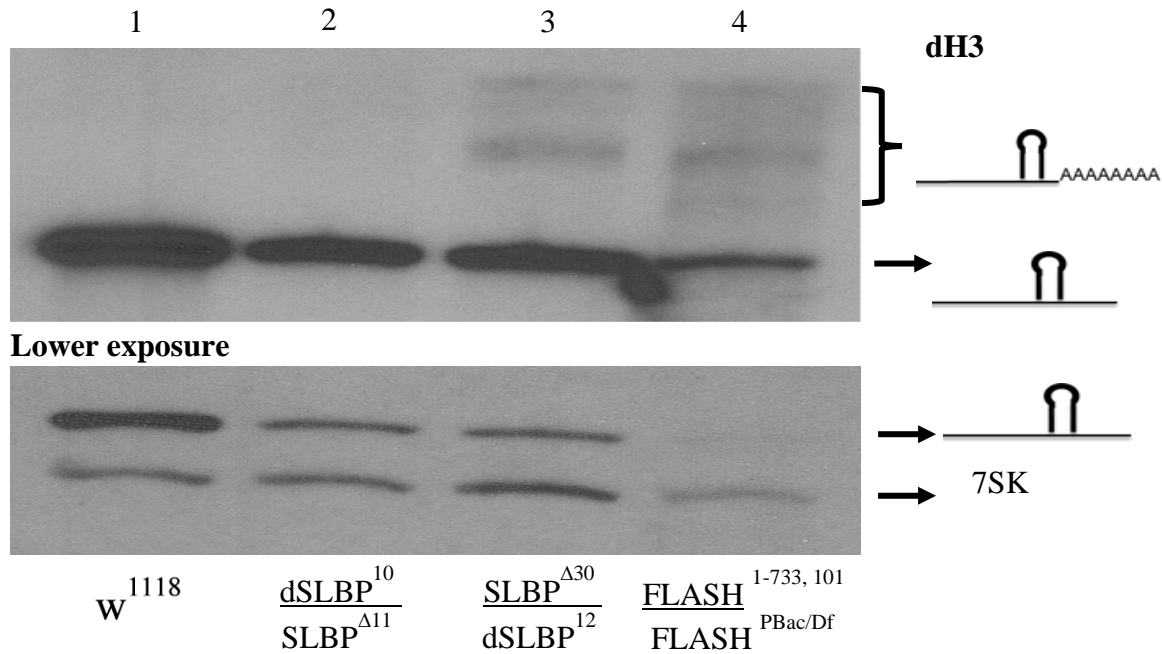
Figure 4.7: The SLBPΔ30 expresses polyadenylated histone mRNAs in the ovary. Ovaries from wild type and both hypomorphs dSLBP10 and SLBPΔ30 were dissected. RNA was isolated using the Trizol Reagent. FLASH MUT was used as a positive control and 7SK as a loading control. Histone mRNA levels are reduced in both hypomorphs (lane 2) and (lane 3) compared to w1118 (lane 1). However, the SLBPΔ30 (lane 3) expresses both properly process and polyadenylated histone mRNAs in the ovary. Two exposures of the gel are shown to clearly show the polyadenylated histone mRNA.

Both hypomorphs have a reduced amount of histone mRNA loaded into the egg.

Females expressing the dSLBP10 had a reduced amount of histone mRNA loaded into the egg (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001). In order to see if the same was the case for SLBP30, I collected embryos at ~45minutes AED before activation of the zygotic genome. At this time, all the RNAs, including Histone mRNAs come from the mother, since there has been no activation of transcription. Northern Blot analyses on embryos at this stage had a low amount or almost no histone mRNA (dH3 or dH1) loaded into the egg (fig.4.8). dSLBP10 embryos (fig.4.8 A lane 2) have more histone mRNA (dH3) deposited when we compared to SLBPΔ30 and normalized to 7SK RNA (fig.4.8 C lane 2). The data shows that these

embryos did not hatch because they do not contain enough maternal histone mRNA to support the early cleavage cycles of the Drosophila embryo.

It was indeed surprising that embryos expressing SLBPΔ30 did not process histone mRNA or any misprocess transcripts (fig.4.8 D lane 2) despite having polyadenylated histones mRNAs in the ovary. In the hypomorph dSLBP10, insufficient maternal supply of histones mRNAs and proteins causes chromosome defects, and embryos arrest before completing the syncytial cycles. To determine if the same happens in SLBPΔ30, I collected embryos at 2-hours AED and stained with DAPI to visualize the DNA. These two hypomorphs have chromosomal defects at the early cleavage stage of embryogenesis (fig.4.9 B and C), consistent with too little histone protein being deposited in the oocyte. SLBPΔ30 embryos die sooner than SLBP10 and contains less nuclei DNA during this stage. Replication dependent histone genes are not polyadenylated and are indispensable for histone synthesis and chromatin assembly. Since there is no expression of new mRNAs early in Drosophila embryogenesis, histone mRNAs and proteins must be stored in the egg to support chromatin assembly. It is undebatable that SLBP plays a main role in the production of histone proteins, and it was surprising that this mutant of SLBP was not able to carry out this function during stage 10B of oogenesis.

Figure 4.8
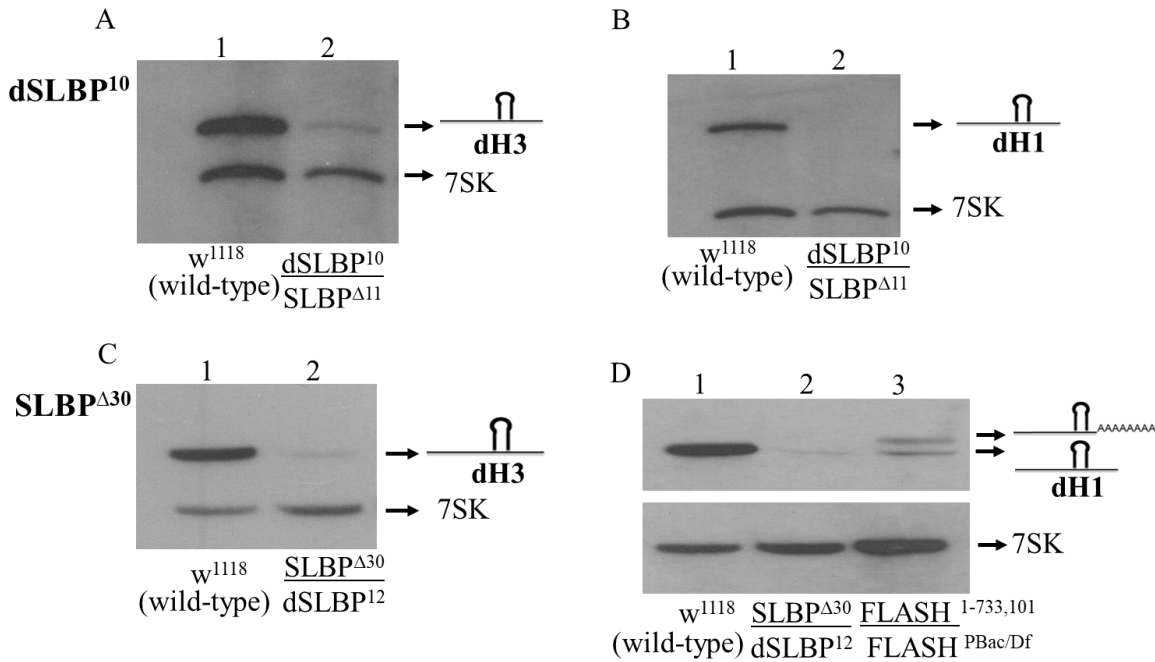
Figure 4.8: The SLBPΔ30 do not deposit enough histone mRNA into egg. Embryos were collected at ~45 minutes AED, to determine the amount of histone mRNAs that are maternally supplied. Northern blot analyses were done for both hypomorphs and each blot was probe for dH3 (A and C) and dH1 (B and D). If we compare A (dSLBP10) and C (SLBPΔ30), the dSLBP10 has more histone mRNA in the egg than SLBPΔ30.

Figure 4.9
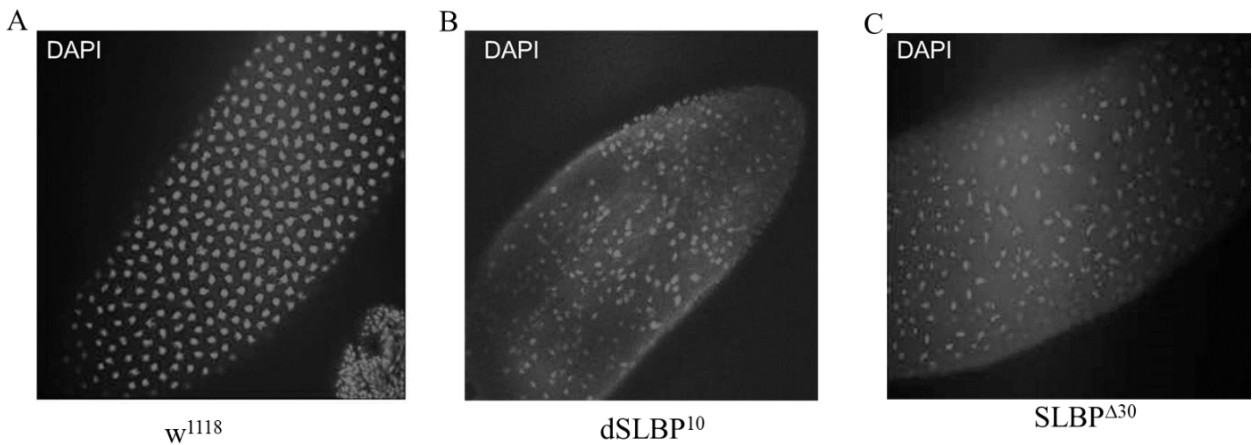
Figure 4.9: Maternal effect causes disruption of the early nuclear cleavage cycles. I stained 0-2hr embryos with DAPI. Females expressing wild type (A) and only the mutant dSLBP10 (B), SLBPΔ30 (C), protein lay normal amounts of eggs, which arrest before completing the syncytial cell divisions. Chromosomal defects are seen in both hypomorphs (B and C).

SLBPΔ30 expresses polyadenylated histones throughout development.

It has been shown that dSLBP10 larvae express polyadenylated histone mRNAs (Godfrey et al., 2006). In order to ask whether polyadenylated histones were being produced early in development in SLBPΔ30, I collected embryos expressing SLBPΔ30 from 5-8 AED and 16-20 AED. Northern blot analyses show that these embryos begin to express polyadenylated histone mRNAs late in embryogenesis (fig.4.10 lane 4), and this phenotype continues through the 3rd larvae stage (fig.4.11 A and B, lane 3). However, despite producing some polyadenylated histone mRNAs, the SLBPΔ30 continues to produce enough properly processed histone mRNAs to complete development.

Figure 4.10
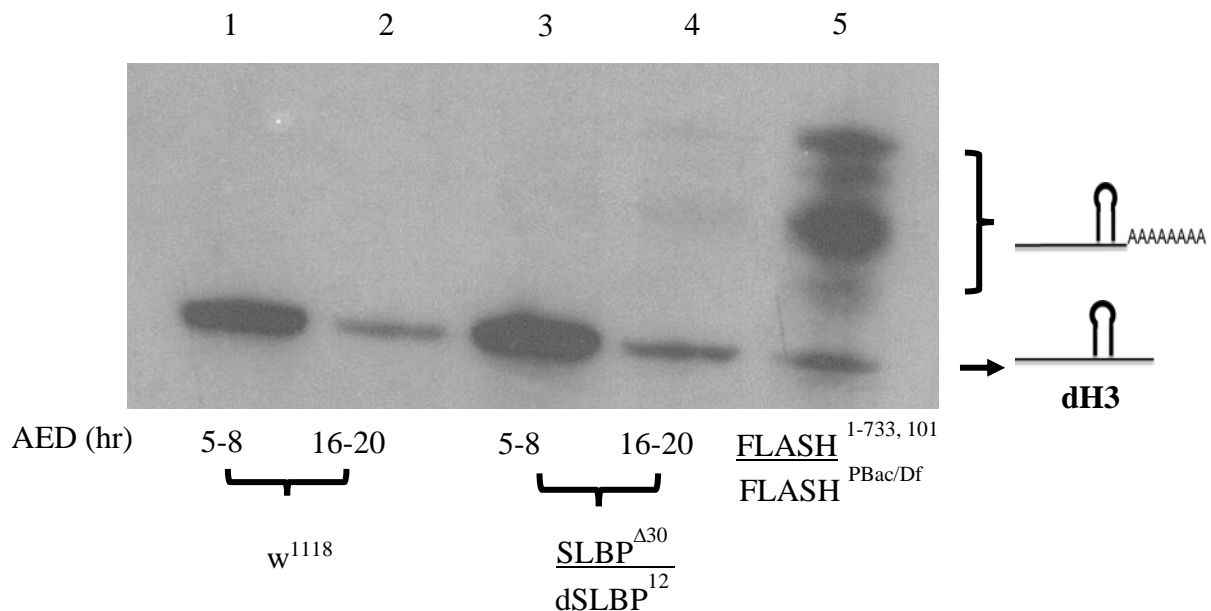
Figure 4.10: The SLBPΔ30 begins to express polyadenylated histone mRNAs late in embryogenesis. Embryos were collected for w1118 and SLBPΔ30 from 5 to 8hrs and 16 to 20hrs AED. By 5 to 8hrs both w1118 (lane 1) and SLBPΔ30 (lane 3), have properly process dH3. However, by 16 to 20hrs (lane 4) the SLBPΔ30 begins to accumulate polyadenylated histone mRNAs. The FLASHMUT was used as a positive control.

Figure 4.11
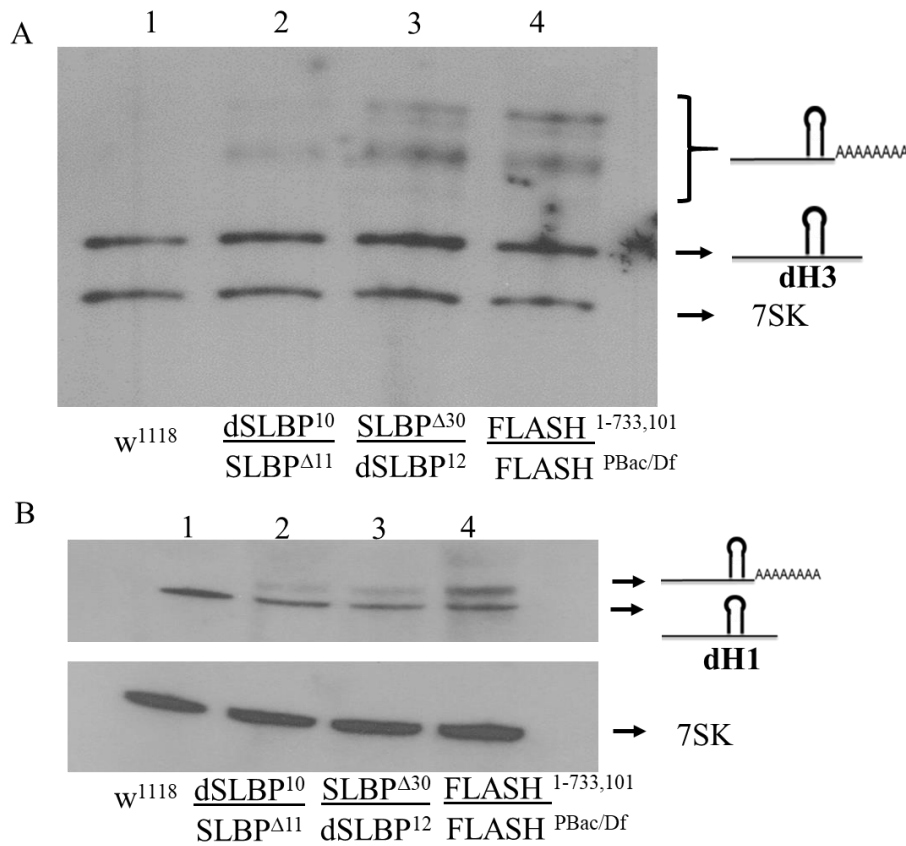
Figure 4.11: The SLBPΔ30 continues to express polyadenylated histone mRNAs in the 3rd larvae stage. 3rd larvae instar was collected from w1118, dSLBP10 and SLBPΔ30. RNA was extracted from each genotype, and each membrane was probe with dH3 (A) and dH1 (B). Histone mRNAs are polyadenylated in both hypomorphs in dH3 and dH1.

Histone mRNA expression in the Drosophila ovary and HLB expression.

The ten amino acid deletion has a more severe phenotype compared to dSLBP10. However, it was still unclear at what time of oogenesis is histone mRNA biosynthesis being disrupted. Since early stages (e.g. first phase) of histone mRNA metabolism appeared normal in both mutants compared to wild type; we continued to focus on stage10B of oogenesis. Single-molecule fluorescent in situ hybridization (smFISH) analyses were done to study histone mRNA expression. During stage 10B, histone mRNA expression is induced in all nurse cells, as is observed in wild type egg chambers using a histone coding probe (H3-coding) (fig.4.12 D).

However, in SLBP10 (fig.4.12 E), the histone nuclear foci (H3-coding) were not present in all the nurse cells and were not as bright as wild-type. The SLBPΔ30 stage 10B ovaries has no detectable signal (fig.4.12 F). Without a doubt, the defect in SLBPΔ30 protein is affecting histone mRNA transcription. Histone genes are present in the HLB, which concentrate factors required for histone mRNA biosynthesis (Tatomer et al., 2016; White et al., 2011). We next determined whether HLB expression was disrupted in this mutant. FLASH, which is a component of HLB formation, was used as a probe. FLASH nuclei foci are readily detected in both wild type and dSLBP10 (fig.4.12 G and H) ovaries. However, dSLBP10 had fewer nuclei foci compared to wild type. In SLBPΔ30 FLASH nuclei foci are smaller and disrupted (fig.4.12 I). SLBPΔ30 definitely affected HLB morphology but HLBs were present, but the major defect in both dSLBP10and dSLBP30 was at the level of transcription. We also conducted smFISH with a histone coding probe to visualize histone mRNA expression at stage 10B and later stages. In wild type egg chambers, we can observe that histone mRNAs are being deposited into the oocyte (fig.4.13 B and C); however, SLBP10 has lower amounts compared to wild type (fig.4.13 E and F). On the other hand, histone mRNAs in SLBPΔ30 are not detectable in the oocyte (fig.4.13 H and I). This result correlates with our genetic and biochemistry analyses. The ten amino acids deleted in the dSLBP30 mutant are contributing to this phenotype, and we must carefully analyze these residues to unrevealed what role they play in the ability of SLBP to promote transcription.

Figure 4.12
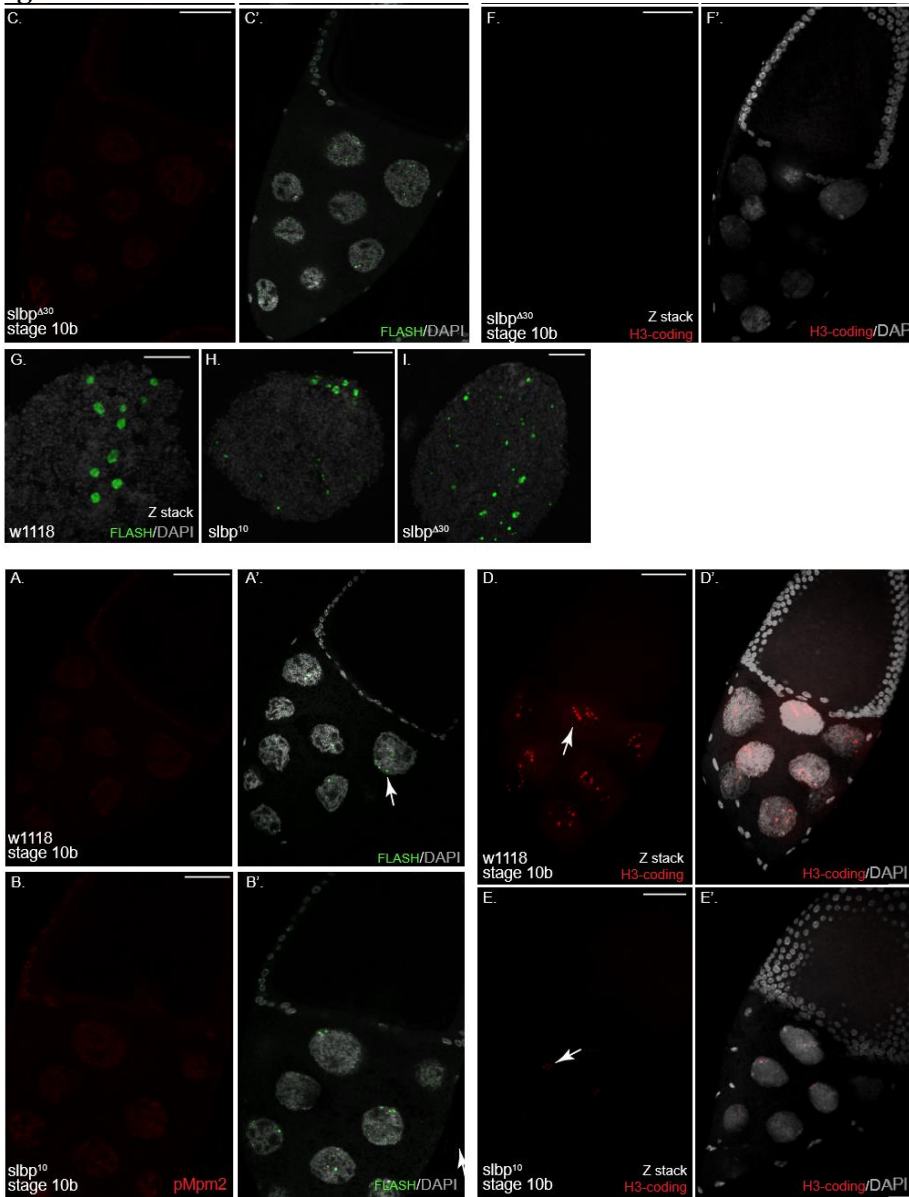
Figure 4.12: Histone mRNAs are not expressed in the SLBPΔ30 at stage 10B of oogenesis. The stage 10B of oogenesis was evaluated with MPM2, H3-coding (red), and FLASH (green) MPM2 expression is not observed at this stage due to the lack of DNA replication (A, B and C). Histone mRNA expression is expressed in all nurse cells (wild type, D), but expression is deficient in (dSLBP10, E) and (SLBPΔ30, F). FLASH nuclei are bigger in both wild type (G) and dSLBP10 (H), but smaller and disrupted in SLBPΔ30 (I). DAPI was used to visualize the DNA.

SLBPΔ30 does produce polyadenylated transcripts in nurse cells in early stages of

oogenesis and in follicle cells. Furthermore, to ask whether misprocessing is occurring in this

mutant since we observed polyadenylated histones mRNAs in the ovaries of SLBPΔ30, we used

a probe that hybridizes the downstream region of H3 (H3-downstream). Wild type (fig.4.13 A and A') or SLBP10 (fig.4.13 D and D') did not have any signal for the downstream probe, but SLBPΔ30 (fig.4.13 G and G') had detectable nuclei foci mostly in follicle cells, and some were detected in the nurse cells. The high abundance of detectable nuclei in follicle cells are likely cells going through or stuck in S phase. The same has been observed in embryos that have low levels of SLBP. In situ hybridization analyses in embryos expressing a null allele of SLBP have histone mRNAs in cells that have exited S phase (Lanzotti et al., 2002). In Godfrey et al., 2006; they conducted in situ hybridization analyses in eye imaginal discs which are a sack of ephitelial tissue and a great system to study cell cycle regulation in vivo. In this study they found that eye imaginal discs expressing SLBP10 contained missprocessed transcripts in synchronous replicating cells (e.g. S- phase), or the second mitotic wave (Godfrey et al., 2006). Eye imaginal discs from wild type only expressed histone mRNAs that were properly process during this time (Godfrey et al., 2006). Without a doubt polyadenylated histone mRNAs are more stable during S-phase and are encountering problems to progress through the cell cycle.

Clearly, histone pre-mRNA processing and histone synthesis are affected by the presence of SLBPΔ30. This protein not only failed to process histones pre-mRNAs properly but also to synthesize histones at the second phase of oogenesis.

Figure 4.13
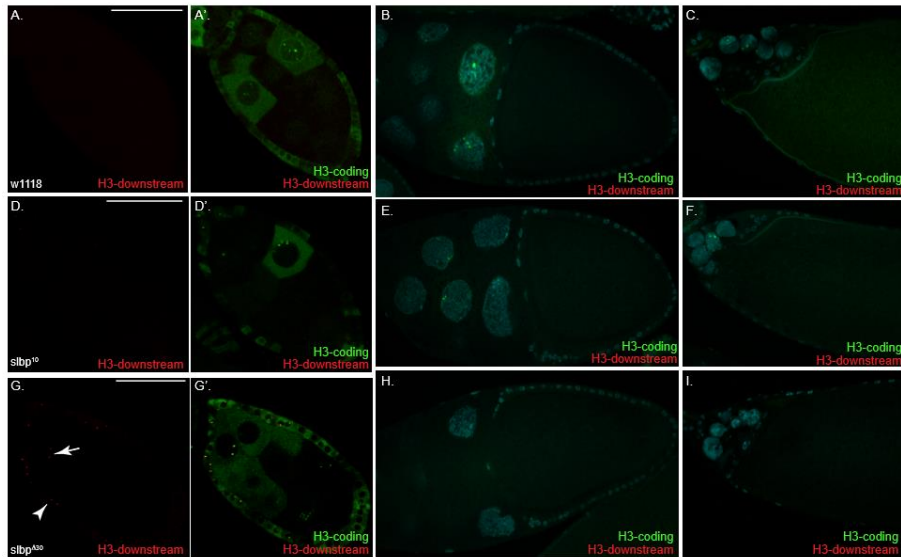
Figure 4.13: SLBPΔ30 expresses polyadenylated histone mRNAs in the ovary. A H3-coding (green) and H3-downstream (red) probe was used to visualize histone mRNA processing. A, D, G are mid oogenesis when nurse cells make histone mRNA. C, F, I are stage 14 when nurse cells dumping has largely occurred. In wild type egg chambers, histone mRNAs are deposited into the oocyte (B and C); however, SLBP10 has lower amounts compared to wild type (E and F). Histone mRNA expression in the SLBPΔ30 is not detectable in the oocyte (H and I). However, polyadenylated histones mRNAs (H3-downstream) in the ovaries of SLBPΔ30, are present in both follicle cells, and in the nurse cells (G and G') but not in w1118 (A and A') or SLBP10 (D and D').

Defect in SLBPΔ30 protein leads to failure to concentrate in the nucleus in stage 10B.

SLBPΔ30 has a more severe phenotype compared to dSLBP10. Both hypomorphs failed to deposit histone mRNAs into the oocyte at stage 10B during oogenesis. dSLBP10 has lower protein levels, which must cause its phenotype; however, still unclear what is happening in SLBPΔ30. To visualize SLBP expression during oogenesis, we conducted immunofluorescence (IF) analyses. To validate the SLBP antibody for IF, Graydon Gonsalvez carried out an RNAi experiment to knockdown dSLBP during oogenesis. SLBP in a wild type background is expressed and localized in both the nurse cell nuclei and the cytoplasm, with higher concentration in the nucleus. The knockdown (RNAi) of dSLBP decreases its expression in the nurse cells, validating that we are looking at dSLBP expression (fig.4.14). We used the SLBP

antibody to evaluate SLBP expression during oogenesis. Protein expression of SLBP in dSLBP10 is reduced, starting at the germarium through later stages of oogenesis in both the nurse and the germarium (fig.4.15 and 4.16), compared to the wild-type ovaries.

This result explains why at the end of oogenesis, a reduced amount of histone mRNAs is deposited into the oocyte. Previously, it has been observed that a knockdown of dSLBP in mammalian cells causes histone mRNA retention because cells do not have enough SLBP that can go to the nucleus and subsequently process and transport these histones mRNAs to the cytoplasm. The same phenotype has been observed in the Drosophila ovary; lower amounts of dSLBP are being produced in the ovary, which is not enough for the demand of histone pre-mRNAs that are needed to be produced. dSLBP expression in the SLBPΔ30 was unexpected, normal levels of dSLBP were observed in the western blot analyses. However, immunofluorescence using an antibody against SLBP, demonstrate that this expression did not correlate with its localization. SLBPΔ30 is present at equal concentrations in the nucleus and the cytoplasm, while in wild type flies there is a higher concentration of SLBP in the nucleus. SLBPΔ30 is mainly localized in the cytoplasm, starting from the germarium towards later stage of oogenesis (fig. 4.15 and 4.16).The histone mRNAs that are synthesized at stage 10B are loaded into the oocyte and required for the early cleavage cycles of embryogenesis. Further analysis of SLBP at this stage demonstrates that dSLBP10 enrichment in the nurse cells is reduced, but SLBP expression in SLBPΔ30 is disrupted, and mislocalized. Quantitative analyses of SLBP nuclear enrichment is illustrated in table 4.1. Based on these results, it can be implied that the nurse cells do not have enough SLBP in the nucleus in either the SLBPΔ30 or SLBP10 mutants, which is needed to transcribe the histone mRNAs, that are required for the syncytial stages prior to activation of the zygotic genome.

Figure 4.14
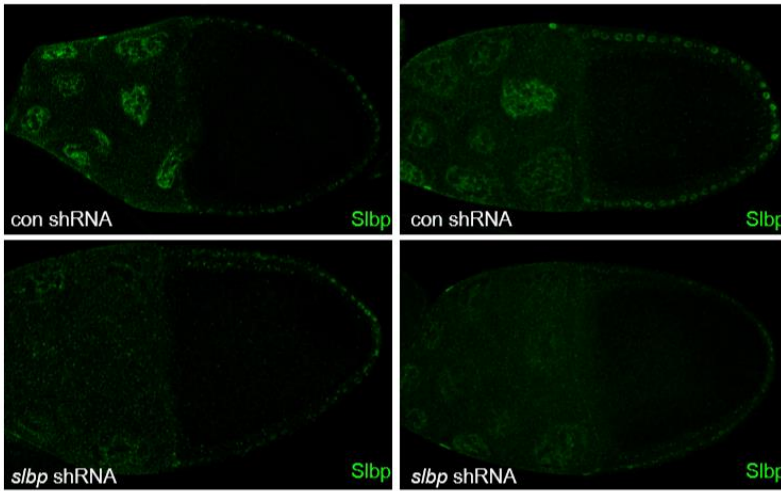
Figure 4.14: dSLBP knockdown decreases its expression and enrichment in nurse cells. Ovaries were processed for immunofluorescence using an antibody against Slbp (green). SLBP in a wild type background (con shRNA) no treatment, and (slbp shRNA) with treatment. The con shRNA expresses in both the nurse cells and the cytoplasm. The knockdown (slbp shRNA) decreases SLBP expression and enrichment in the nurse cells.

Figure 4.15
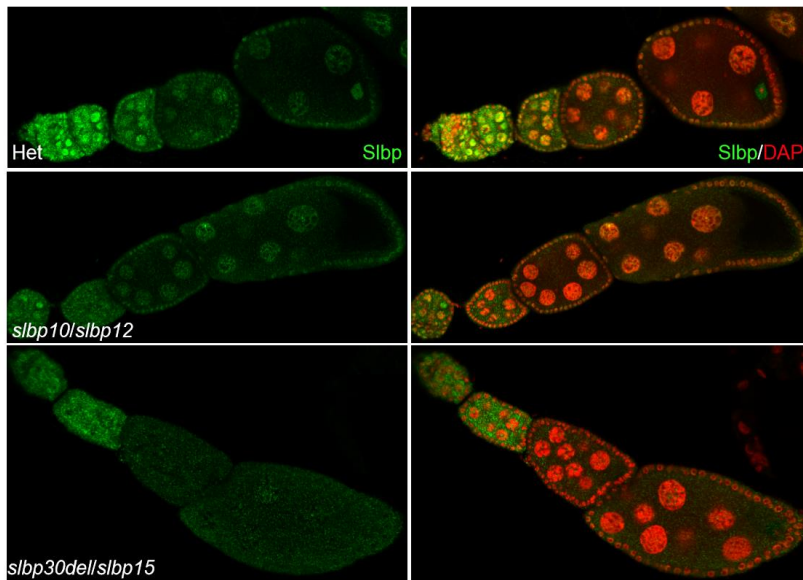
Figure 4.15: The SLBPΔ30 is localized in the cytoplasm during oogenesis. Ovaries were processed for immunofluorescence using an antibody against Slbp (green). The germarium and developed egg chambers were probe with a SLBP antibody (green) and DAPI (red). SLBP is highly enriched in the nurse cells but reduced in dSLBP10. SLBPΔ30 nuclei expression is not only reduced but its expression is mostly in the cytoplasm.

Figure 4.16
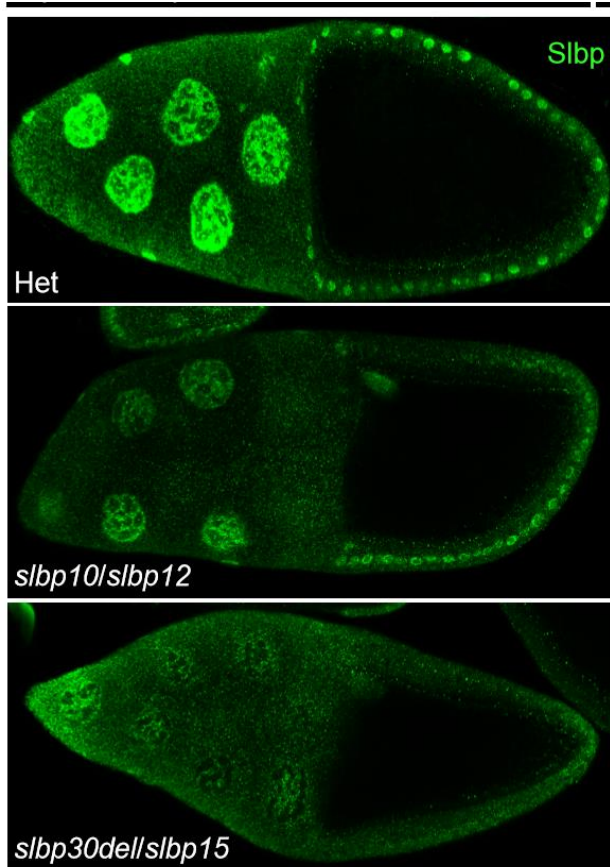
Figure 4.16: The SLBPΔ30 is mainly localized in the cytoplasm. Ovaries were processed for immunofluorescence using an antibody against Slbp (green). Stage 10B egg chambers from heterozygous, and both hypomorphs dSLBP10 and SLBPΔ30 was probe with a SLBP antibody. Hypomorphs have a reduced amount of SLBP protein levels in the nuclei and the cytoplasm compared to heterozygous. However, SLBPΔ30 is mainly enriched in the cytoplasm.

Table 4.1
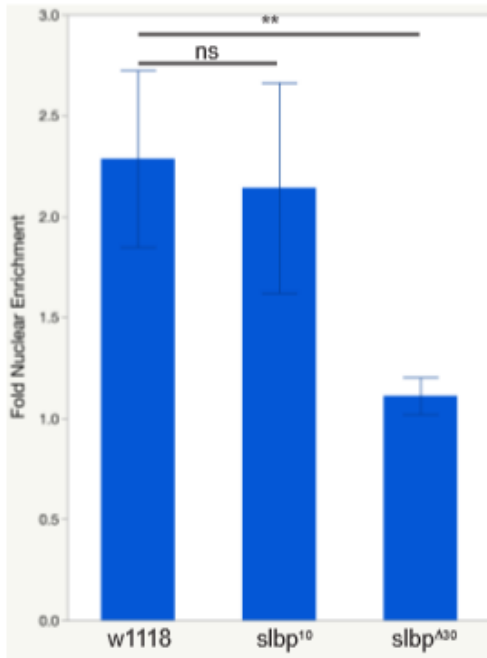
Table 4.1. Quantitative analyses of SLBP nuclear enrichment. SLBPΔ30 mutants have a lower enrichment of SLBP in the nuclei of either the nurse cells or follicle cells compared to SLBP10. ns represents not significant. ** p <0.05, unpaired t test.

The SFTPP motif is not essential for dSLBP nuclear localization during oogenesis.

The 10 aa deletion contains an SFTPP sequence which is known to be phosphorylated. We have characterized previously the serine and the threonine in a similar SFTTP motif in the N-terminal region of vertebrate SLBP, whose phosphorylation is required for hSLBP degradation at the end of S-phase in mammalian cells (Koseoglu et al., 2008; Zheng et al., 2003). The threonine in Drosophila SLBP, is phosphorylated in SLBP expressed in baculovirus and in cultured cells. Previously Dave Lanzotti made a point mutant in the SFTPP motif; the threonine was changed to alanine in flies, and it did not affect viability or histone pre-mRNA processing (Lanzotti et al., 2004). Recently, phosphorylation the serine by Chk2 was identified as necessary for dSLBP degradation in the cytoplasm during genotoxic stress, which leads to histone RNA nuclear retention (Iampietro et al., 2014). Based on these data, I created two point mutants S to A and S/T to AA, to determine if the absence of these residues in SLBPΔ30 was caused of the

phenotype. The same genetic analyses were done to rescue the SLBPΔ11 null phenotype (fig.3.3). As expected, both mutants were viable over this background; however, males and females were fertile. Both genotypes laid a normal amount of eggs that hatched and developed normally. Northern blot analyses with the ovaries expressing each point mutant did not have any polyadenylated histone mRNAs dH3 (fig.4.17 A and B, lane 2). These results suggest that phosphorylation on these residues is not important for deposition of histone mRNA into the oocyte. It is interesting that phosphorylation in the serine is required for histone nuclear retention during DNA damage, but dSLBP during normal conditions is not degraded after completion of S phase.Since nuclear import of SLBP seemed to be affected by the 10 amino acid deletion, we analyzed the SLBP amino acid sequence and we identified a potential nuclear import sequence. The only canonical nuclear import sequence identified contained a string of basic amino acids, LKTPYKRRHSFT, on which, is located at the NTD of Drosophila SLBP. Interestingly, this sequence is deleted in SLBPΔ30. Previously, the Marzluff lab characterized at least two regions in the mammalian SLBP which are required for nuclear import, the RKRR at the NTD and the KVRH at the CTD. To test the role of this sequence in SLBP localization during oogenesis. I created two additional mutations in the basic residues in 10 aa deletion, the ARAH and the AAAH. Transgenes bearing these mutations are currently being injected in Drosophila and flies expressing the transgenes being selected. If females are sterile, I will conduct further analysis for each allele to determine if it is identical in properties to the 10 aa deletion.

Figure 4.17

Transgene	Position	Sequence
AFTPP	N-Terminal Domain	YKRRH**A**F**T**PP
AFAPP	N-Terminal Domain	YKRRH**A**F**A**PP
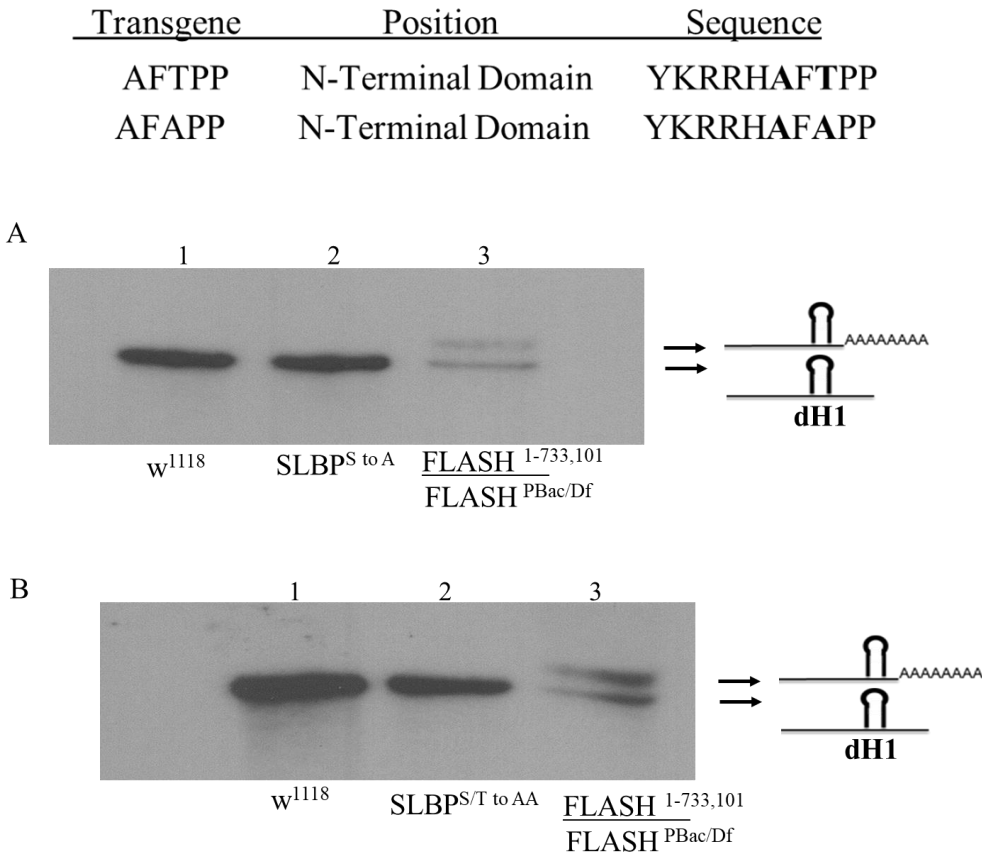
Figure 4.17: The SFTPP motif is not necessary for nuclear import of SLBP. Point mutants were made in both phosphorylated residues S to A and S/T to AA. Ovaries from wild type and both mutants SLBPS to A (A) and SLBPS/T to AA (B) were dissected. RNA was isolated using the Trizol Reagent. FLASH mut was used as a positive control. Histone mRNA levels are normal in both point mutants (A, lane 2) and (B, lane 2) compared to w1118 (A and B, lane 1). Neither of the mutants expressed any polyadenylated histone mRNAs

CONCLUSIONS

Maternal histone mRNAs are needed for the early cleavage cycles of embryogenesis to support development. Females expressing SLBPΔ30 are viable but sterile due to low amounts of histones mRNAs loaded into the egg. These histone mRNAs are synthesized at stage 10B in the absence of DNA replication. Immunoflerescense data shows that SLBP in mainly localized in the cytoplasm during oogenesis. Egg chambers expressing SLBPΔ30 did not express histone genes at stage 10B when in wild type egg chamber histone genes are express in all the nurse cells. This result suggest that reduce amounts of SLBP in the nurse cell nuclei at stage 10B affects the initiation of histone gene transcription. The histone locus bodies present at the histone genes are also defective, and do not activate transcription of the histone genes. Analysis of SLBP amino acid sequence demonstrate that the 10 amino acid deleted in SLBPΔ30 is part of a nuclear import signal. These results suggest that a defective nuclear import of SLBPΔ30 may lead to a defect in HLBs and histone gene transcription.

CHAPTER 5: DISCUSSION

I started my project with the goal of using the FLY-CRISPRCAS9 system to create a null allele of SLBP in Drosophila melanogaster. I obtained two frame shift mutations (1nt and 11nt deletion). Genetic analyses of these alleles were not viable and died at the first larvae stage. Collected embryos at 16-20hr AED demonstrates that the maternal SLBP mRNA did not last after this time. However, Northern blot analysis shows that these embryos encountered problems early in development, since polyadenylated histone mRNAs began to accumulate at 4-8hrs AED. The time when transcription of the zygotic genome is activated. Within the same experiment, I obtained a 30nt (10 aa) deletion that was characterized and resulted in a novel maternal lethal effect. Viable females expressed normal amount of SLBP protein but had greatly reduced amounts of histone RNAs being dumped into the oocyte. Immunofluorescence data shows that the SLBPΔ30 protein is mainly localized in the cytoplasm at this stage, suggesting the deleted region is important for nuclear import of SLBP at the end of oogenesis.

Role of phosphorylation of SLBP in processing differs in vivo and in vitro

The study of the structural domains of SLBP help us understand its role during histone mRNA biosynthesis and dynamics in the cell. The RBD and the CTD are essential for histone mRNA processing in vitro (Dominski et al., 2002). Conserved residues in the RBD were analyzed to investigate its function in vivo. The Threonine in the TPNK motif was mutated to Asparagine, genetic analyses with this allele shows that was not viable and died at the first larvae stage. Previous crystallography analyses show that this Threonine is critical for bringing all three helices together and stabilize to the SL (Zhang et al, 2014). The phosphohtronine OH in the

Threonine is essential for this interaction.

The hyperphosphorylated residues at the CTD are unique in Drosophila. Point mutants to abolish phosphorylation disrupts processing in vitro (Skrajna et al., 2017). We tested the same hypothesis in vivo, flies expressing each mutant allele were viable and did not affect histone mRNA processing. The CTD don't interact with the RNA SL, and we currently don't have any evidence that the free tail interacts with an unknown protein.

Maternal effect lethal mutants which have defects in histone pre-mRNA processing

I described two different alleles with a similar phenotype. SLBP10, which was described years ago, contains fragments of a p-element located upstream of the 5' UTR of SLBP, and SLBPΔ30, which I isolated has a 10 amino acid deletion in the NTD of SLBP. A single copy of either allele is viable in a null background of SLBP, but females are sterile due a reduced amount of histone mRNA and protein being loaded into the egg. The ovaries of both alleles developed normally and laid a normal amount of eggs. SLBPΔ30 has a more severe phenotype, and expresses polyadenylated histone mRNAs throughout development. SLBP has not been the only protein of the histone complex that resulted in female sterility when is mutated. Female sterility has also been shown before with other mutants required for histone mRNA processing like FLASH and U7 snRNA which are components of the HLB. However, in contrast to these SLBP mutants, a more severe mutation of SLBP, or U7 snRNA causes morphological defects in the ovaries and failure to complete oogenesis, with no eggs produced.The inability to produce enough SLBP, FLASH or U7 causes a maternal lethal effect which disrupts histone mRNA processing and histone mRNA synthesis (Godfrey et al., 2006; Lanzotti et al., 2002; Sullivan et al., 2001; Tatomer et al., 2016). In the two hypomorphs I studied there was a different effect, specific failure to synthesize histone mRNA in stage 10B of oogenesis, although normal

appearing eggs were produced.   It demonstrates how critical these proteins are to ensure the synthesis of histone proteins for the early cleavage cycles, to package the newly synthesized DNA into chromatin.

How are maternal stores to histone mRNA produced independent to DNA replication?

Together with Dr. Graydon B. Gonsalvez at the Medical College of Georgia, I investigated how histone mRNA is produced for deposition into the egg during oogenesis. According to previous data histones gene expression is periodic and correlate with DNA synthesis from stage 5 to stage 10A of oogenesis (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985). Stage 10 B is the key stage of oogenesis that provides the necessary components (e.g. histone mRNAs and proteins) for the early cleavage cycles of embryogenesis (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985). During this time histone genes are expressed in all the nurse cells, in the absence of DNA synthesis (Ambrosio and Schedl, 1985; Ruddell and Jacobs-Lorena, 1985). How histone mRNA synthesis occurs in the absence of DNA synthesis was not well understood. I discovered that histone mRNA synthesis in stage 10B differs from histone mRNA synthesis in other Drosophila tissues. HLB formation is independent of histone mRNA expression and occurs in G1. For example, HLBs form in G1 arrested cells in the epidermal cell nuclei, but histone mRNA is not expressed. Normally histone mRNA synthesis is activated by phosphorylation of Mxc (NPAT) in the HLB by Cyclin E/Cdk2 as cells enter S-phase. However in stage 10B of oogenesis, cyclin E/cdk2 is not activated and there is no phosphorylation of Mxc in the HLB, as determined by failure to detect Mpm2 staining in these HLBs. However histone gene transcription is activated in all the nurse cells nuclei and the histone mRNAs are properly processed. The SLBPdelta30 mutant allele fails to support histone gene transcription at stage 10B of oogenesis, but supports histone mRNA expression in other

stages of oogenesis, and well as in other tissues. In other tissues if SLBP levels are low, histone gene transcription is nomal histone pre-mRNA processing is not efficient, and polyadenylated histone mRNA is produced, and in cultured Drosophila S2 cells severe depletion of SLBP results in large amounts of polyadenylated histone mRNAs. There has never been an indication that SLBP is required for histone gene transcription. Thus there are different defects in the SLBPΔ30 mutant in different tissues.

The other surprise is there are near normal levels of SLBP in stage 10B of the SLBPΔ30 mutant, and the major defect is that SLBP is not concentrated in the nucleus. A similar phenotype is seen in SLBP10, although in this allele full-length SLBP is produced, although lower amounts of SLBP are produced, and as a result nuclear levels of SLBP are also lower in this mutant. This mutant also fails to transcribe histone mRNA in stage 10B of oogenesis. Thus what is required for histone gene transcription in stage 10B is high levels of SLBP in the nucleus, which must substitute for phosphorylation of Mxc.How the tissue can sense that SLBP is disrupted or that low levels of SLBP are being produced? Is SLBP necessary for histone transcription? Those are some questions that are needed to be address in order to have better understanding of the biology of this process.

Is SLBP necessary to concentrate the active U7 snRNP in the HLB in stage 10B?

One second difference between the HLB in G1 and S-phase in normal tissues is that there are not high levels of symplekin in the HLB except in S-phase, suggesting that U7 snRNP is converted to an active form as cells enter S phase (Tatomer et al, 2014). We don't have evidence yet that the HLBs in stage 10B contain U7 snRNP, and whether that U7 snRNP is active. It will be important to see whether SLBP is required to concentrate any factors (e.g. RNA pol II or U7 snRNP or symplekin) in the HLB in stage 10. During this analysis I showed that there the HLBs

are also disrupted in the SLBP mutants in stage 10B, resulting in many smaller HLBs rather than the approximately 10 large HLBs. Since the nurse cells are about 1000C, there must normally be large numbers of histone clusters in each HLB. Deirdre Tatomer characterized FLASH 1-733, a mutant that lacks the C-terminal domain, which is required for its localization to the HLB and recruitment of the U7 snRNP. Interestingly, this mutant produces the same protein levels, shows small amounts of misprocessing but does not concentrate FLASH or U7 snRNP in the HLB. Since this mutant is fertile, it will be interesting to determine whether the HLBs in stage 10B egg chambers are similar to wild-type, and if introducing SLBPΔ30 makes the phenotype of this mutant more severe. One possibility is that there are normally small amounts of SLBP in the HLBs, which would be difficult to detect by IF, and that the SLBPΔ30 may not be able to concentrate in the HLB.

This would explain the small amounts of misprocessing throughout development. The removal of the 10 aa may have impacted an HLB localization signal. What if SLBP is indeed necessary in the HLB to initiate this process? More analysis must be done to determine whether there is some SLBP present in the HLB.

Did we discover a nuclear import signal?

Molecular analyses of these ovaries show evidence that SLBP is mislocalized from early stages of oogenesis. We have not yet examined other tissues. In both hypomorphs the amount of SLBP in the nucleus is reduced but SLBPΔ30 is not concentrated in the nucleus while SLBP10 has lower total SLBP although the SLBP is still concentrated in the nucleus. A downstream histone probe was used to determine the severity that this allele had on histone mRNA processing. SLBPΔ30 expresses the downstream histone probe in follicle and in the nurse cells. Based, on this analysis, the nurse cells don't have enough SLBP near the site of transcription to

rapidly process all the histone mRNA. Analysis of the SLBP amino acid sequence identified only one possible canonical nuclear import sequence, the identified nuclear import sequence is a strength of basic amino acids, the KRRH which is located at the NTD of Drosophila SLBP, and which overlaps with the region deleted in SLBPΔ30. My results suggest that this nuclear localization signal may be particularly important in Drosophila egg chambers. When it is deleted it results in reduced levels of SLBP in the nuclei resulting in a large reduction in transcription at stage 10B of oogenesis. Thus, in addition to its other roles in histone mRNA metabolism, I have shown that SLBP has an additional function in histone gene transcription in stage 10B of oogenesis. In mammalian cells, SLBP has at least two nuclear import localization signals (Erkmann et al., 2005b). There is no SLBP in G1 cells, unlike the situation in Drosophila, where SLBP is present throughout the cell cycle. SLB is present in both the nucleus and the cytoplasm in S-phase cells. When DNA replication is inhibited in S-phase cells, histone mRNA is rapidly degraded and SLBP increases in the nucleus. , nuclear and cytoplasmic at S phase and completely nuclear at G2. Mammalian SLBP is likely only present in the cytoplasm when it is bound to histone mRNA.

What's next?

I am currently conducting genetic analyses by changing the amino acids KRKH to alanine, to see if this reproduces the phenotype of the 10 aa deletion, and also affect nuclear localization. I would also like to add a nuclear localization signal to the SLBPΔ30 mutant, and see if a transgene expression would restore fertility to the SLBPΔ30 mutant. If a canonical NLS rescues the phenotype of SLBPΔ30 then the failure to transcribe the histone genes would be due to not providing enough SLBP in the nucleus. If it restores the nuclear localization of SLBP, but does not rescue the fertility defect then the specific sequence that was deleted ma have an

additional function, directly stimulating transcription of the histone genes.

Regardless of whether simply restoring SLBP to the nucleus rescues the phenotype, there is the still the question of how histone gene transcription is activated in the absence of phosphorylation of Mxc. Answering this question will require analysis of the HLB in stage 10B, and comparing it with the HLBs in nurse cells undergoing endoreplication. Whether all the known components are present can also be determined by immunofluorescence. In addition there are a number of mutants in HLB components, such as the localization mutants of FLASH, which could be used to probe changes in structure and function of the HLBs in stage 10B.

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