MECHANISMS MEDIATING CYCLIN E/CDK2-REGULATED REPLICATION-DEPENDENT HISTONE mRNA BIOSYNTHESIS

Anne Eileen White

A dissertation submitted in the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology.

Chapel Hill
2010

Approved by:

Dr. Robert J. Duronio

Dr. William F. Marzluff

Dr. Mark Peifer

Dr. Kerry S. Bloom

Dr. Stephen Rogers

ABSTRACT

Anne E. White: Mechanisms mediating Cyclin E/Cdk2-regulated replication-dependent histone mRNA biosynthesis
(Under the direction of Dr. Robert J. Duronio)

Nuclear organization is a dynamic modulator of gene expression. Subcellular compartments called nuclear bodies concentrate factors regulating gene expression and change in size, composition, and activities in response to cellular inputs. Thus, understanding the mechanisms that assemble and organize nuclear bodies is important for appreciating how they contribute to genome function. We explored the relationship between histone locus bodies (HLBs) and replication-dependent histone mRNA biosynthesis as a model for understanding nuclear body function. HLBs are localized near the histone genes and are enriched with factors required for histone mRNA biosynthesis. Cyclin E/Cdk2 is necessary for cell cycle-dependent histone mRNA biosynthesis. However, the molecular mechanisms of this regulation are not fully known. Using the MPM-2 monoclonal antibody as a tool for exploring cell cycle-mediated control of HLB function, we show that in *Drosophila* cells MPM-2 detects Cyclin E/Cdk2-dependent nuclear foci that co-localize with nascent histone transcripts and are coincident with HLBs. To identify MPM-2 reactive HLB proteins, we performed a genome-wide RNAi screen and used mass spectroscopy to identify novel HLB proteins. We show that one of these factors, Mxc, is MPM-2 reactive. We propose that Cyclin E/Cdk2 regulates histone mRNA biosynthesis by modulating the activity of Mxc.

Interestingly, Mxc is required for HLB assembly and efficient histone pre-mRNA processing. These results suggest that Cyclin E/Cdk2 employs multiple levels of control to ensure that histone gene expression and DNA replication are properly coordinated during cell division.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor Dr. Robert Duronio, and committee members Dr. William Marzluff, Dr. Mark Peifer, Dr. Steve Rogers, and Dr. Kerry Bloom, for their support and advice during the course of my graduate work. I thank present and past members of the Duronio, Sekelsky, and Marzluff labs for great discussions and friendships. I would also like to recognize the technical assistance provided by Dr. Tony Perdue, Brandon Burch, Dr. Xiao-cui Yang, and members of the Peifer and Rogers labs, all whom without which this work would not have been possible. I especially thank Dr. Nasser Rusan for scientific and professional advice. Finally, I would like to acknowledge several collaborators that were crutial for this work: Dr. Brian Calvi, Dr. Joe Gall, Sarada Bulchand, and Dr. Greg Matera. This work was partly funded through a fellowship awarded by the UNC Developmental training program and by a Doctoral Research Award from the Canadian Institutes of Health Research.

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Act5C	actin 5C
ATAC	Ada2A containing
CB	Cajal body
CDK	Cyclin-dependent kinase
Cy	cyanine
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
ECL	enhanced chemiluminescence
EMS	ethyl methanesulfonate
E2F	E2-Factor
FISH	fluorescent *in situ* hybridization
FLASH	FLICE-associated huge protein
GFP	green fluorescent protein
HA	hemagglutinin
HAT	histone acetyltransferase
HLB	histone locus body
HRP	horseradish peroxidase
H3	histone H3
IP	immunoprecipitate
ISWI	Imitation SWI
Lsm	Sm-like

LSM	laser scanning microscope
MPM-2	Mitotic protein monoclonal 2
MS	mass spectroscopy
Mute	Muscle wasted
Mxc	Multi sex combs
NE	nuclear extract
NPAT	Nuclear Protein Ataxia-Telangiectasia locus
IP	immunoprecipitate
PBS	phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PH3	phospho-histone H3
PMSF	phenylmethanesulphonylfluoride
pRb	Retinoblastoma protein
PTB	Polypyrimidine Tract Binding protein
RNA	ribonucleic acid
RNAi	RNA interference
RSC	Chromatin structure remodeling complex
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S2	Schneider 2
SLBP	stem loop binding protein
SnRNP	small nuclear ribonucleoprotein
stg	string
SWI/SNF	Switch/sucrose nonfermentable

TOF	time of flight
ubi	ubiquitin
WT	wild-type
YY1	Yin Yang 1

CHAPTER I

INTRODUCTION

The set of genes expressed and silenced by a cell is fundamental to determining its identity. In order to better appreciate how cellular identity is achieved, it is essential to understand the mechanisms controlling gene expression. Broadly, genome function is organized on three major levels: the spatial and temporal coordination of gene expression events, the organization of chromatin environments, and the arrangement of genes and chromosomes within the nucleus (Misteli, 2007). The spatial and temporal organization of interactions between proteins modulating gene expression and chromatin within the nucleus is predominantly influenced by nuclear architecture (Matera et al., 2009; Misteli, 2007). The nuclear landscape is comprised of nuclear compartments called nuclear bodies that concentrate factors regulating gene expression. Nuclear bodies are thought to facilitate the assembly of macromolecular complexes necessary for gene expression. Some are located near the genomic locus that they regulate, such as nucleoli, Cajal bodies, and histone locus bodies (HLBs) (Matera, 1999; Matera et al., 2009). Nuclear bodies are very dynamic structures that can modulate gene expression in response to changes in cellular signals through changes in their size, composition, and activities (Matera et al., 2009). For example, nucleoli closely associate with the genes encoding the 35S pre-ribosomal RNA and their size is a function of rRNA transcriptional activity (Haaf et al., 1991; Scheer et al., 1984). Nuclear body activity can be also regulated by

the cell cycle. For example, mammalian HLBs and nucleoli disassemble during mitosis and reform during the subsequent interphase (Hernandez-Verdun, 2006; Ma et al., 2000; Zhao et al., 2000). Thus, understanding how nuclear bodies control gene expression by responding to various cellular inputs, such as cell cycle inputs, will increase our understanding of how genome function is modulated. This dissertation uses histone locus bodies (HLBs) as a paradigm of nuclear body function to explore how nuclear bodies are regulated by the cell cycle, assemble, and contribute to gene expression.

Histone locus bodies as paradigms of nuclear bodies

Histone locus bodies (HLBs) were first discovered in *Drosophila* as nuclear bodies enriched with factors necessary for efficient histone gene expression, such as U7 snRNP and FLASH (Liu et al., 2006; Yang et al., 2009). HLBs associate with the replication-dependent histone genes, which are encoded in a 5-kb repeat present in approximately 100 tandem copies in *Drosophila* (Liu et al., 2006). They are distinct from and frequently situated next to Cajal bodies (CBs) in *Drosophila* (Liu et al., 2006; Liu et al., 2009). CBs are involved in many aspects of RNA metabolism, including small nuclear ribonucleoprotein (snRNP) maturation (Cioce and Lamond, 2005; Kiss, 2004; Matera and Shpargel, 2006; Stanek and Neugebauer, 2006). In mammalian cells and *Xenopus* oocytes, CBs contain the U7 snRNP and can transiently associate with the snRNA, small nucleolar RNA (snoRNA), and histone genes (Matera et al., 2009).

The presence of U7 snRNP in human CBs and fly HLBs initially gave the impression that HLBs were a unique feature of *Drosophila*. However, recent emerging evidence suggests that human cells also possess HLBs that are distinct from CBs. Studies

performed in primary human cells revealed that U7 snRNP co-localizes with histone gene expression-specific factors FLASH and NPAT (Ghule et al., 2008). The ubiquitous presence of U7 snRNP in CBs seen in human cancer cell lines, therefore, may be a consequence of transformation and an exception to the norm.

HLBs serve as a good model for understanding nuclear body function, because they assemble at a discrete nuclear location, the histone genes, and are likely a conserved feature of the metazoan nucleus. In addition, the cell cycle is a critical cellular input regulating HLB function, which limits replication-dependent histone gene expression to S phase when DNA is being replicated. For these reasons, studying HLBs will both increase our understanding of nuclear body function and how the cell cycle modulates gene expression.

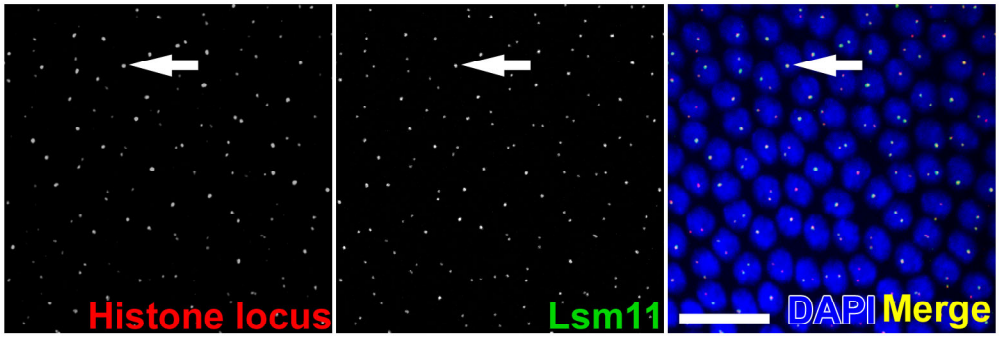

Figure 1.1. Histone locus bodies in *Drosophila*. Syncytial blastoderm (cycle 13) embryos were hybridized with an H3/H4 gene probe (red in merge) and stained with anti-Lsm11 (green in merge) antibodies and the DNA labeling dye DAPI (blue in merge). Arrows indicate a single histone locus body. Scale bar: 20 μm.

Cell cycle regulation of replication-dependent histone biosynthesis

Replication-dependent histone production is under tight cell cycle control, such that most histone biosynthesis occurs only during S phase. This ensures that nascent DNA is correctly assembled into chromatin and will be faithfully segregated when the

cell divides during mitosis. The coupling of histone biosynthesis to S phase is crucial for cell viability. DNA replication without concomitant histone protein synthesis is lethal in budding yeast (Han et al., 1987; Kim et al., 1988). Further, *in vitro* reconstitution experiments have shown that production of histones in excess to DNA can cause insoluble histone-nucleic acid aggregates (Carruthers et al., 1999; Detke et al., 1979; Dyer et al., 2004), which could lead to incorrect DNA segregation should these conditions arise in the cell. Thus, the timing and amount of histone protein biosynthesis must be carefully controlled to ensure that cell division is successful.

Cyclin-dependent kinase (Cdk) complexes coordinate the major events of cell division. In animal cells Cyclin E/Cdk2 phosphorylates proteins, such as pRb, that mediate changes in gene expression associated with the onset of S phase, including proteins that regulate histone gene expression (Du and Pogoriler, 2006). Two Cyclin E/Cdk2 substrates, NPAT and HIRA, activate and repress, respectively, histone gene expression in human cell culture (Hall et al., 2001; Ma et al., 2000; Miele et al., 2005; Nelson et al., 2002; Zhao et al., 2000). How the activity of such factors is modulated by Cyclin E/Cdk2 and integrated into cell cycle-regulated histone gene expression is incompletely understood. Even less is known about how histone gene expression is regulated during animal development, because studies have mainly been done in cell culture.

For most organisms the amount of replication-dependent histone protein synthesized depends almost entirely on the rate of histone mRNA biosynthesis (Marzluff and Duronio, 2002). Histone mRNA levels increase 35-fold as mammalian cells enter S phase (Borun et al., 1975; Breindl and Gallwitz, 1973; DeLisle et al., 1983a; Detke et al.,

1979; Harris et al., 1991; Heintz et al., 1983; Parker and Fitschen, 1980). This sharp rise in mRNA levels is achieved by increases in the rate of transcription initiation, pre-mRNA processing, and half-life of the mature mRNA (Marzluff and Duronio, 2002). Whether all of these mechanisms are under cell cycle control is unknown.

Transcription of the histone genes in mammalian cells is stimulated by the Cyclin E/Cdk2 substrate NPAT (Ma et al., 2000; Wei et al., 2003; Ye et al., 2003; Zhao et al., 2000). NPAT is found in HLBs associated with the histone genes in both primary human cells and cancer cells (Ma et al., 2000; Zhao et al., 2000). Phosphorylated NPAT acts with histone subtype-specific transcription factors, such as HiNF-P and OCA-S, which bind subtype-specific regulator elements in the promoters of the histone H4 and H2B genes, respectively (Miele et al., 2005; Mitra et al., 2003; Ye et al., 2003; Zheng et al., 2003). While several phosphorylation sites on NPAT have been mapped, how this phosphorylation regulates NPAT activity is incompletely understood (Ma et al., 2000; Zhao et al., 2000). It was recently proposed that NPAT plays a role in promoting histone H4 acetylation by recruiting the TRRAP-Tip60 complex to the histone genes (DeRan et al., 2008). A connection between NPAT and histone pre-mRNA processing has also been made, whereby NPAT interacts with CDK9 at the histone genes. CDK9 is a component of the P-TEFb transcription elongation complex that also monoubiquitinates histone H2B, which in turn recruits the histone pre-mRNA processing machinery (Pirngruber and Johnsen, 2010). Cyclin E/Cdk2 could also promote histone mRNA biosynthesis by regulating HLB dynamics as well as the activity of other HLB proteins.

Cyclin E/Cdk2 might also regulate the histone pre-mRNA processing reaction. Replication-dependent histone mRNAs are the only eukaryotic mRNAs that are not

polyadenylated (Marzluff and Duronio, 2002; Marzluff et al., 2008). Instead they end in a conserved stem loop structure that regulates all aspects of replication-dependent histone mRNA metabolism, including biosynthesis, translation, and stability (Marzluff, 2005). Binding of Stem Loop Binding Protein (SLBP) to the stem loop in the 3' UTR and U7snRNP to the purine-rich downstream element recruits a cleavage endonuclease complex. This complex cleaves the histone pre-mRNA 4-5 nucleotides after the stem loop producing the unique 3' end of the mature histone mRNA (Dominski and Marzluff, 1999; Marzluff, 2005). In mammalian cells SLBP protein levels are cell cycle regulated, yet this mode of regulation is not universal as SLBP protein is expressed uniformly through the cell cycle in *Drosophila* cells (Lanzotti et al., 2004b; Marzluff et al., 2008). Moreover, it is uncertain whether other components of the histone pre-mRNA processing machinery are cell cycle regulated. Cyclin E/Cdk2 does, however, regulate pre-mRNA splicing in cultured mammalian cells. Three subunits of the essential splicing factor SF3, SAP114, SAP145, and SAP155, as well as the U2 snRNA and the snRNP core protein B/B' have been shown to immunoprecipitate with cyclin E (Seghezzi et al., 1998). Of these proteins, Cyclin E/Cdk2 has been shown to phosphorylate SAP155 *in vitro* (Seghezzi et al., 1998). Similarly, Cyclin E/Cdk2 may interact with and/or phosphorylate proteins specifically involved in histone pre-mRNA processing.

Studying histone mRNA biosynthesis regulation in *Drosophila melanogaster*

Drosophila has provided fundamental insights into the regulation of the cell cycle, as it undergoes a stereotypic and well-characterized developmental program (Lee and Orr-Weaver, 2003; Swanhart et al., 2005). For these reasons it provides an excellent

model system to explore how histone mRNA biosynthesis is regulated. As part of normal fly development, the first 13 embryonic cycles after fertilization consist only of rapid, synchronous S-M nuclear division cycles that occur in a common cytoplasm without cytokinesis. These early cycles depend on maternally contributed factors. During interphase 14 nuclei pause in G2 for the first time and cellularization takes place. Groups of cells then enter mitosis 14 asynchronously in a manner dependent on zygotic transcription of an activator of mitosis encoded by the *string*^{cdc25} (*stg*) gene (Edgar and O'Farrell, 1989a; Edgar and O'Farrell, 1990a). String^{cdc25} is a phosphatase that activates the mitotic Cdk, Cdc2. The spatial and temporal pattern of these divisions is controlled by the patterning genes (O'Farrell et al., 1989). G1 is introduced in embryonic cycle 17 and at this point all cell types require zygotic *Cyclin E* expression for DNA replication and cell cycle progression. Previously it was shown that string^{cdc25} and Cyclin E are required for histone gene expression at cycles 14 and 17, respectively (Lanzotti et al., 2004a). The exact mechanism of this regulation, however, has not been elucidated.

We sought new tools to study Cyclin E/Cdk2 regulation of histone mRNA biosynthesis. One such reagent was the monoclonal antibody MPM-2, which has been previously used as a marker of Cyclin E/Cdk2 activity in *Drosophila* cells (Calvi et al., 1998). MPM-2 was generated using a mitotic HeLa cell extract and recognizes conserved cell cycle–dependent phospho-epitopes present in a variety of proteins across many species (Davis et al., 1983). One epitope recognized by MPM-2 is a consensus Cdk phosphorylation site (Westendorf et al., 1994). Consequently, MPM-2 has been used extensively to study mitotic phospho-proteins in a variety of systems (Albert et al., 2004; do Carmo Avides et al., 2001b; Hirano and Mitchison, 1991; Kuang et al., 1989;

Lange et al., 2005a; Logarinho and Sunkel, 1998a; Yaffe et al., 1997). In *Drosophila* salivary glands and ovarian follicle cells MPM-2 labels a discrete spherical nuclear structure whose cell cycle appearance in S phase is dependent on Cyclin E/Cdk2 activity (Calvi et al., 1998). Moreover, our collaborator Brian Calvi observed that MPM-2 only labels the histone genes of polytene chromosomes from larval salivary glands (Chapter II, Figure 2.1A). Based on these results, Brian Calvi and we hypothesized that the protein(s) detected by MPM-2 are Cyclin E/Cdk2 substrates that co-localize with HLBs at the histone genes and activate histone mRNA biosynthesis. A major emphasis of this dissertation was to identify and characterize MPM-2 reactive HLB proteins.

Dissertation goals

This dissertation describes work done examining how histone mRNA biosynthesis is regulated by components of the histone locus body (HLB). In particular, it focuses on the molecular mechanisms regulated by Cyclin E/Cdk2. Chapter II presents data showing that the MPM-2 antibody detects protein(s) that are part of the histone locus body. The MPM-2 epitope present at HLBs is under both cell cycle and developmental control, such that Cyclin E/Cdk2 activity is necessary for production of the MPM-2 epitope and mature HLBs are formed coincidentally with zygotic histone gene transcription. Surprisingly we found that although HLBs are closely associated with the histone loci, they can form in nuclei lacking the histone genes suggesting that HLBs assemble independently of histone gene transcription. Together, work detailed in this chapter supports our hypothesis that MPM-2 recognizes a Cyclin E/Cdk2 substrate that regulates histone gene expression.

To further understand how Cyclin E/Cdk2 might regulate histone gene expression we sought to identify MPM-2 antigen(s) that are part of HLBs. Chapter III describes biochemical and genome-wide functional genomics screens performed to pinpoint MPM-2 antigen(s). Both approaches identified novel components of HLBs, including two MPM-2 reactive proteins (Mxc and Mute-S). Mxc is necessary for HLB assembly and histone pre-mRNA processing in S2 cells. HLBs are highly dynamic, such that core components begin assembling one nuclear cycle prior to the formation of mature HLBs and zygotic gene transcription. Likewise, this "core" HLB remains assembled throughout mitosis, unlike mammalian HLBs and other types of nuclear bodies. Chapter IV explores the significance of the data presented in this dissertation and elaborates on a few outstanding questions in the field brought out by this work.

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CHAPTER II

DEVELOPMENTAL AND CELL CYCLE REGULATION OF THE *DROSOPHILA* HISTONE LOCUS BODY

Preface

This work was published in *Molecular Biology of the Cell* and featured on the journal cover. Michelle Leslie performed the experiment shown in Figure 3.10. Julie Norseen, a student in Dr. Brian Calvi's lab, performed MPM-2 staining on polytene chromosomes from larval salivary glands. I performed all other experiments. My advisor Dr. Robert Duronio designed the project. He, Dr. William Marzluff, and I wrote the manuscript and analyzed the data.

White, A.E., Leslie, M.E., Calvi, B.R., Marzluff, W.F., and Duronio, R.J. (2007) Developmental and cell cycle regulation of the *Drosophila* histone locus body. Mol. Biol. Cell. 18:2491-502.

Abstract

Cyclin E/Cdk2 is necessary for replication-dependent histone mRNA biosynthesis, but how it controls this process in early development is unknown. We show that in *Drosophila* embryos the MPM-2 monoclonal antibody, raised against a phosphoepitope from human mitotic cells, detects Cyclin E/Cdk2-dependent nuclear foci that co-localize with nascent histone transcripts. These foci are coincident with the histone locus body (HLB), a Cajal body-like nuclear structure associated with the histone

locus and enriched in histone pre-mRNA processing factors such as Lsm11, a core component of the U7 snRNP. Using MPM-2 and anti-Lsm11 antibodies, we demonstrate that the HLB is absent in the early embryo and appears when zygotic histone transcription begins during nuclear cycle 11. Whereas the HLB is found in all cells after its formation, MPM-2 labels the HLB only in cells with active Cyclin E/Cdk2. MPM-2 and Lsm11 foci are present in embryos lacking the histone locus, and MPM-2 foci are present in *U7* mutants, which cannot correctly process histone pre-mRNA. These data indicate that MPM-2 recognizes a Cdk2-regulated protein that assembles into the HLB independently of histone mRNA biosynthesis. HLB foci are present in histone deletion embryos, although the MPM-2 foci are smaller, and some Lsm11 foci are not associated with MPM-2 foci, suggesting that the histone locus is important for HLB integrity.

Introduction

Cell cycle-regulated histone protein biosynthesis is controlled primarily through the regulation of histone mRNA abundance, which in cultured mammalian cells increases 35-fold at the G1-S transition (Borun et al., 1975; Breindl and Gallwitz, 1973; DeLisle et al., 1983; Detke et al., 1979; Harris et al., 1991; Heintz et al., 1983; Parker and Fitschen, 1980). This rapid rise in mRNA is achieved by increases in the rate of transcription initiation and pre-mRNA processing as cells enter S phase, followed by rapid degradation of histone mRNA at the end of S-phase (Marzluff and Duronio, 2002). How these various aspects of histone mRNA metabolism are linked to other events that drive progression through the cell cycle by regulating the activity of the cyclin-dependent kinases (Cdk) remains incompletely understood.

In animal cells Cyclin E/Cdk2 promotes the G1 to S transition in part by phosphorylating proteins that mediate changes in gene expression associated with the onset of DNA replication (e.g. pRb; (Du and Pogoriler, 2006)). These include proteins that regulate histone expression. For instance, human NPAT and human HIRA are Cyclin E/Cdk2 substrates that act to stimulate and repress, respectively, histone gene transcription in cell culture experiments (Hall et al., 2001; Ma et al., 2000; Miele et al., 2005; Nelson et al., 2002; Zhao et al., 2000). How the activity of such factors is modulated by Cyclin E/Cdk2 and integrated into cell cycle-regulated histone gene expression *in vivo* is not known.

Cyclin E/Cdk2 may also regulate features of histone mRNA biosynthesis other than transcription, such as pre-mRNA processing. Rather than being polyadenylated, histone mRNAs terminate in a conserved stem loop structure that regulates all aspects of replication-associated histone mRNA metabolism, including biosynthesis, translation, and stability (Marzluff, 2005). This unique mRNA 3' end is formed through a pre-mRNA processing reaction that cleaves the histone pre-mRNA 4-5 nucleotides after the stem loop producing mature histone mRNA (Dominski and Marzluff, 1999; Marzluff, 2005). The cleavage endonuclease complex is recruited to histone pre-mRNA by Stem Loop Binding Protein (SLBP), which binds the stem loop in the 3' UTR, and U7 snRNP, which binds a purine rich sequence located downstream of the cleavage site.

In mammalian cells and *Xenopus* oocytes U7 snRNP localizes to Cajal bodies (CBs), which are sub-nuclear organelles involved in several aspects of RNA metabolism, including snRNP maturation (Cioce and Lamond, 2005; Kiss, 2004; Matera and Shpargel, 2006; Stanek and Neugebauer, 2006). Histone mRNA biosynthesis is thought

to occur within or near a subset of Cajal bodies. Unlike U7 snRNP which is found in all Cajal bodies (Frey and Matera, 1995), NPAT localizes to the subset of Cajal bodies associated with histone genes (Ma *et al.*, 2000; Zhao *et al.*, 2000). Thus, Cyclin E/Cdk2 may regulate Cajal body function or the activity of proteins that act within Cajal bodies to regulate histone mRNA biosynthesis.

Here we examine the connection between Cyclin E/Cdk2 activity and cell cycle-regulated histone mRNA biosynthesis in *Drosophila* embryos, which have provided fundamental insights into the regulation of the cell cycle and how this regulation is coordinated with development (Lee and Orr-Weaver, 2003; Swanhart *et al.*, 2005). *Drosophila* nuclei contain both Cajal bodies and a distinct nuclear body that is often observed in proximity to the Cajal body called the histone locus body (HLB) (Liu *et al.*, 2006). The HLB is associated with the histone genes (which are contained in a 5-kb sequence present in approximately 100 tandemly repeated copies) and is enriched in U7 snRNP particles (Liu *et al.*, 2006). Cyclin E/Cdk2 activity is necessary for histone gene expression during embryogenesis (Lanzotti et al., 2004), but how this occurs is not known. In this report we demonstrate that the HLB contains a cell cycle-regulated, Cyclin E/Cdk2-dependent phospho-epitope recognized by the MPM-2 monoclonal antibody.

The MPM-2 antibody was generated using a mitotic HeLa cell extract and recognizes conserved cell cycle–dependent phospho-epitopes present in a variety of proteins across many species (Davis *et al.*, 1983). One epitope recognized by MPM-2 is a consensus Cdk phosphorylation site (Westendorf *et al.*, 1994). MPM-2 has been used extensively to study mitotic phospho-proteins in a variety of systems (Albert et al., 2004;

do Carmo Avides et al., 2001a; Hirano and Mitchison, 1991; Kuang et al., 1989; Lange et al., 2005b; Logarinho and Sunkel, 1998a; Yaffe et al., 1997). In *Drosophila* ovarian cells, MPM-2 labels a spherical nuclear body whose cell cycle appearance is dependent on Cyclin E/Cdk2 activity (Calvi *et al.*, 1998). Here we show that MPM-2 nuclear foci are coincident with the HLB and exploit this finding to characterize the connection between Cyclin E/Cdk2 activity, nuclear organization, and histone mRNA biosynthesis during early *Drosophila* development.

Materials and Methods

Drosophila stocks. *Slbp*^{15} (Sullivan *et al.*, 2001), *stg*^{4} (Edgar and O'Farrell, 1989), *cyclin E*^{AR95} (Knoblich *et al.*, 1994), *U7*^{14} (Godfrey *et al.*, 2006), *Df(3R)stg*^{AR2} (Lehman *et al.*, 1999), the histone locus deletion *Df(2L)Ds6* (Moore, 1983), *cyclin E*^{hsp70} (Richardson *et al.*, 1995), *UAS:YFP-Lsm11* (Liu *et al.*, 2006) and *daGAL4* (Wodarz *et al.*, 1995) were all previously described. *cyclin E* mutant embryos were unambiguously identified using a *CyO P[wg-lacZ]* balancer chromosome. *w*^{1118} flies were used as wild type control, except in Fig. 6A where a sibling embryo of the *stg* mutant was used as control.

Immunostaining and in situ hybridization. Embryos were dechorionated, fixed in a 1:1 mixture of 5% formaldehyde:heptane for 25 minutes or 20% formaldehyde:heptane for 10 minutes, and incubated with primary and secondary antibodies each for 1 hour at 25^{o}C or overnight at 4^{o}C. YFP-Lsm11 embryos were fixed in a 1:1 mixture of 4% formaldehyde:heptane for 20 minutes. Fat bodies were dissected in Schneider's media, fixed in 5% formaldehyde for 25 minutes, permeabilized with 0.3% Triton X-100

(ACROS, New Jersey) for 45 minutes, blocked with 1% BSA, and incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 hour at 25°C. The following primary antibodies were used: monoclonal mouse anti-Ser/Thr-Pro MPM-2, (1:1000, Upstate, Lake Placid, NY); monoclonal mouse anti-phospho-histone H3 (Ser10) (1:1000, Upstate); polyclonal rabbit anti-phospho-histone H3 (Ser10) (1:1000, Upstate); polyclonal rabbit anti-phospho-tyrosine (1:100, Upstate); chicken anti-GFP (1:2000, Upstate); monoclonal rat anti-phospho-tyrosine (1:100, R & D systems, Minneapolis, MN); chicken anti-beta-gal (1:1000, ProSci, Poway, CA); rabbit anti-GFP (1:2000, Abcam, Cambridge, MA); and affinity-purified polyclonal rabbit anti-Lsm11 (1:1000, gift of Joe Gall (Liu *et al.*, 2006)). Embryos that were hybridized with H3/H4 DNA probes, *cycE*^{AR95}, *hsp70::cycE* and its control embryos were incubated overnight with anti-Lsm11 antibodies at 37°C. The following secondary antibodies were used: goat anti-mouse IgG labeled with Oregon Green 488 (Molecular Probes, Eugene, OR), Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA) or Cy5 (Jackson); goat anti-rabbit IgG labeled with Rhodamine Red (Molecular Probes, Eugene, OR), Cy2 (Jackson), or Cy5 (Abcam); goat anti-rat IgG labeled with Cy3 (Jackson), donkey anti-rat Cy5 (Jackson); donkey anti-chicken IgY labeled with Cy2, Cy3, or Cy5 (all from Jackson). DNA was detected by staining embryos with DAPI (1:2000 of 1mg/ml stock, DAKO Corporation, Carpinteria, CA) for 30 seconds.

Histone H3 transcripts were detected by fluorescent *in situ* hybridization using digoxigenin-labeled H3 coding or H3-ds probes as described previously (Lanzotti *et al.*, 2002). Hybrids were detected using the fluorescein tyramide signal amplification fluorescence system (Perkin Elmer, Boston, MA).

The histone locus was detected by fluorescent *in situ* hybridization with ạbiotin-labeled DNA probe (125 pg/ul) as previously described (Dernburg and Sedat, 1998). The probe was derived from a clone containing both the H3 and H4 genes (Lanzotti *et al.*, 2002) that was digested with *MaeIII*, *RsaI*, *MseI*, and *HaeIII* to generate fragments of an average length of 50 bp. DNA fragments were biotin-labeled using the BrightStar psoralen-biotin labeling kit (Ambion, Austin, TX). Prior to hybridization embryos were stained with MPM-2 and anti-Lsm11 antibodies using methods described above. Biotinylated probe was detected using the Cyanine 5 tyramide signal amplification fluorescence system (Perkin Elmer, Boston, MA).

Cultured cell immunostaining and RNAi. *Dmel-2* cells were grown in Sf-900 II SFM serum-free media using standard techniques. dsRNAs were made by *in vitro* transcription using a PCR product as template and T7 polymerase. The following primer pairs were used to amplify *Lsm11* and *PTB* (control), respectively: 5'-GGTAATACGACTCACTAT AGATGGAATCGAGGGACCGGAAAAC-3,'5'-GGTAATACGACTCACTATAGCAA CAGTTCACCCTCGACACTGCC-3' and 5'-GGTAATACGACTCACTATAGTGGAA TGAATTGTTCTTTGTGAA-3', 5'-GGTAATACGACTCACTATAGGCCCATAGCG ACTACAGC-3'. 2 x 10^6 cells were plated in 6-well plates and treated with 10□g of dsRNA daily for five days, and were split 1:1 on days 3 and 5. Knockdown was confirmed by Western blot (not shown). Cells were fixed directly to coverslips in 10% formaldehyde for 10 minutes, extracted using 0.1% Triton X-100 for 15 minutes, and blocked with 5% normal goat serum in PBST for 20 minutes. The same antibodies and incubation times used to stain embryos were used to

stain cells.

Microscopy. Confocal images were taken at a zoom of 1.0-2.0 with a 63x (NA 1.40) Plan Apochromat objective on a Zeiss 510 laser scanning confocal microscope using the LSM data acquisition software (Carl Zeiss, Germany). YFP-Lsm11 embryo images were acquired on a Zeiss 410 laser scanning confocal microscope (Carl Zeiss, Germany). Image false coloring and contrast was adjusted using Photoshop (Adobe Systems Inc.).

Measurement of MPM-2 focus size. MetaMorph software (Molecular Devices, Sunnyvale, CA) was used to characterize HLB structure from confocal images. Measurements were made using a 4□m deep compilation of confocal images from the tip of the extended germ band in three embryos per genotype. Both the length and width of MPM-2 foci were measured using the linescan function of MetaMorph. Two perpendicular, one pixel-thick lines from 10 to 30 pixels long were drawn across the nuclear MPM-2 focus in cells that contained only one MPM-2 focus. The lines were long enough to include pixels that were outside of the MPM-2 focus. The average background fluorescence was calculated by taking the mean of six randomly chosen pixels from each linescan that were outside of the MPM-2 focus. The half-max value for each linescan was determined by taking 50% of the difference of the peak of fluorescence in the MPM-2 focus and average background fluorescence. The number of pixels whose fluorescence fell within or equaled the half-max was summed and multiplied by a conversion factor of 0.12 □m/pixel, which was obtained from the Zeiss LSM Image Browser software. The

sum of the length and width measurements in □m was calculated for 75 cells from wild-type and 75 cells from *Df(2L)Ds6* homozygous mutant embryos. Data is reported as the average of these sums ± standard deviation. P value was obtained by conducting a student's t test with two tails and unequal variance. The standard error was +/-0.043 for wild-type and +/-0.031 for *Df(2L)Ds6*.

Results

MPM-2 labels the histone locus body

MPM-2 labels a discrete spherical body in the nuclei of polyploid cells of the salivary gland and ovary (Calvi et al., 1998; Millar et al., 1987). To determine whether this MPM-2 body preferentially accumulates at a particular genomic locus, polytene chromosomes from squashed and whole mount salivary glands of third instar larvae were stained with MPM-2 antibodies (Figure 2.1A-A' and data not shown). MPM-2 strongly labels a single locus near the chromocenter on the left arm of chromosome 2. Interpretation of the cytogenetic banding pattern of these chromosomes indicated that this label is near or coincident with the chromosomal location of the histone gene cluster (data not shown). Cyclin E /Cdk2 activity is required for MPM-2 labeling of this subnuclear body, and for histone gene expression (Calvi et al., 1998; Lanzotti et al., 2004). We therefore hypothesized that MPM-2 recognizes a phospho-protein(s) that localizes to the histone locus body.

To test this hypothesis, we used confocal microscopy to analyze embryos labeled with both MPM-2 and anti-Lsm11 antibodies. Liu et al. (2006) originally visualized the HLB primarily by detecting Lsm11, an Sm-like protein that is a core component of the

U7 snRNP. *Drosophila* embryos undergo a stereotyped cell cycle program that is characterized by distinct modes of cell cycle progression that appear at successive stages of embryogenesis. During the first two hours of embryogenesis, maternally contributed factors drive thirteen rapid and synchronous nuclear cycles, consisting only of S-M phases, in a syncytial cytoplasm. At the end of this period (i.e. nuclear cycle 13), MPM-2 labels both the nucleoplasm and distinct foci within the nucleus that co-localize with Lsm11 foci (Figure 2.1B-B'). MPM-2 foci also co-localize with the histone locus, which was detected by FISH using a labeled DNA probe containing the H3 and H4 genes (Figure 2.1B''-B'''). These data indicate that an MPM-2 epitope is associated with the HLB. By western blot analysis, MPM-2 is known to react with at least a dozen targets in *Drosophila* (Lange et al., 2005a; Logarinho and Sunkel, 1998b)(M.E.L. and R.J.D., unpublished), and thus the nucleoplasmic staining may not be the same protein that concentrates in the HLB.

Cellularization takes place during cycle 14, when after the completion of S phase the nuclei pause for the first time in G2. Then, as gastrulation ensues, groups of cells in different regions of the embryo enter mitosis 14 together. These groups of cells, called "mitotic domains", enter into mitosis 14 at different times generating a reproducible and well described pattern of mitosis (Foe, 1989). Entry into mitosis 14 requires zygotic transcription of the *string*^{cdc25} (*stg*) gene, which encodes a Cdc25-type phosphatase that stimulates mitotic Cdk1 activity by removing inhibitory Y15 phosphorylation from Cdc2 (Edgar and O'Farrell, 1989). Cycles 15 and 16 are also regulated at the G2-M transition by developmentally controlled pulses of *stg* transcription, and still lack G1 phase as in the early syncytial cycles (Edgar et al., 1994; Edgar and O'Farrell, 1990; Lehman et al.,

1999). MPM-2 and Lsm11 nuclear foci co-localize and are continuously present during interphase of these "post-blastoderm" cell cycles (Figure 2.1C-C''). MPM-2 foci are continuously present most likely because Cyclin E/Cdk2 activity is also ubiquitous at this time, including during G2 phase (Sauer *et al.*, 1995).

G1 phase first appears during cycle 17, and subsequent entry into S phase in all cell types requires zygotic expression of *cyclin E* (Knoblich *et al.*, 1994). Consistent with previous observations that MPM-2 foci are cyclin E dependent, MPM-2 only labels replicating cells where Cyclin E/Cdk2 is active (Figure 2.1D-D'', arrow). Cells that have exited the cell cycle, such as those in the amnioserosa (Figure 2.1D-D'', yellow arrowhead) and epidermal cells arrested in G1 (Figure 2.1D-D'', white arrowhead), do not contain MPM-2 foci. In contrast, Lsm11 foci can be detected in all cells (Figure 2.1, arrows). Thus, the HLB is present ubiquitously, as previously described for post embryonic stages of development (Liu *et al.*, 2006), but the MPM-2 epitope appears at the HLB only in cells that contain active Cyclin E/Cdk2.

One obvious feature of Lsm11 and MPM-2 staining of blastoderm embryos is that each nucleus contains either one or two foci (Figure 2.1B-B''', 2.1C-C'', arrows and arrowheads, respectively). Homologous chromosomes are often paired in *Drosophila* cells, and we therefore hypothesized that one and two foci results from paired and unpaired homologous chromosomes, respectively. In the early embryo, the pairing of homologous chromosomes is dynamic, such that the frequency of pairing at any particular locus increases as the embryo ages (Fung *et al.*, 1998). We counted the number of cells with one or two MPM-2 foci in blastoderm embryos during interphase of cell cycle 14. At this stage, 71% of cells contained one MPM-2 focus and 29% contained

two MPM-2 foci (n = 293). Fung et al. (1998) reported a pairing frequency for the histone locus of 71% and 84% at 2.5 and 4 hours of development, respectively, which encompasses the cycle 14 cells we analyzed (Fung *et al.*, 1998). By the time of germ band retraction during cycle 17, most cells contain a single focus (Figure 2.1D). These data are consistent with the HLB specifically associating with the histone locus (Fig. 2.1B) (Liu *et al.*, 2006). Also in support of this interpretation is the observation that polyploid nurse cells in the ovary have partially unpaired sister chromatids at the histone locus and contain multiple MPM-2 foci (data not shown) (Calvi et al., 1998; Hammond and Laird, 1985). Taken together, these results indicate that MPM-2 recognizes an epitope at the histone locus that is a component of the Lsm11-containing HLB.

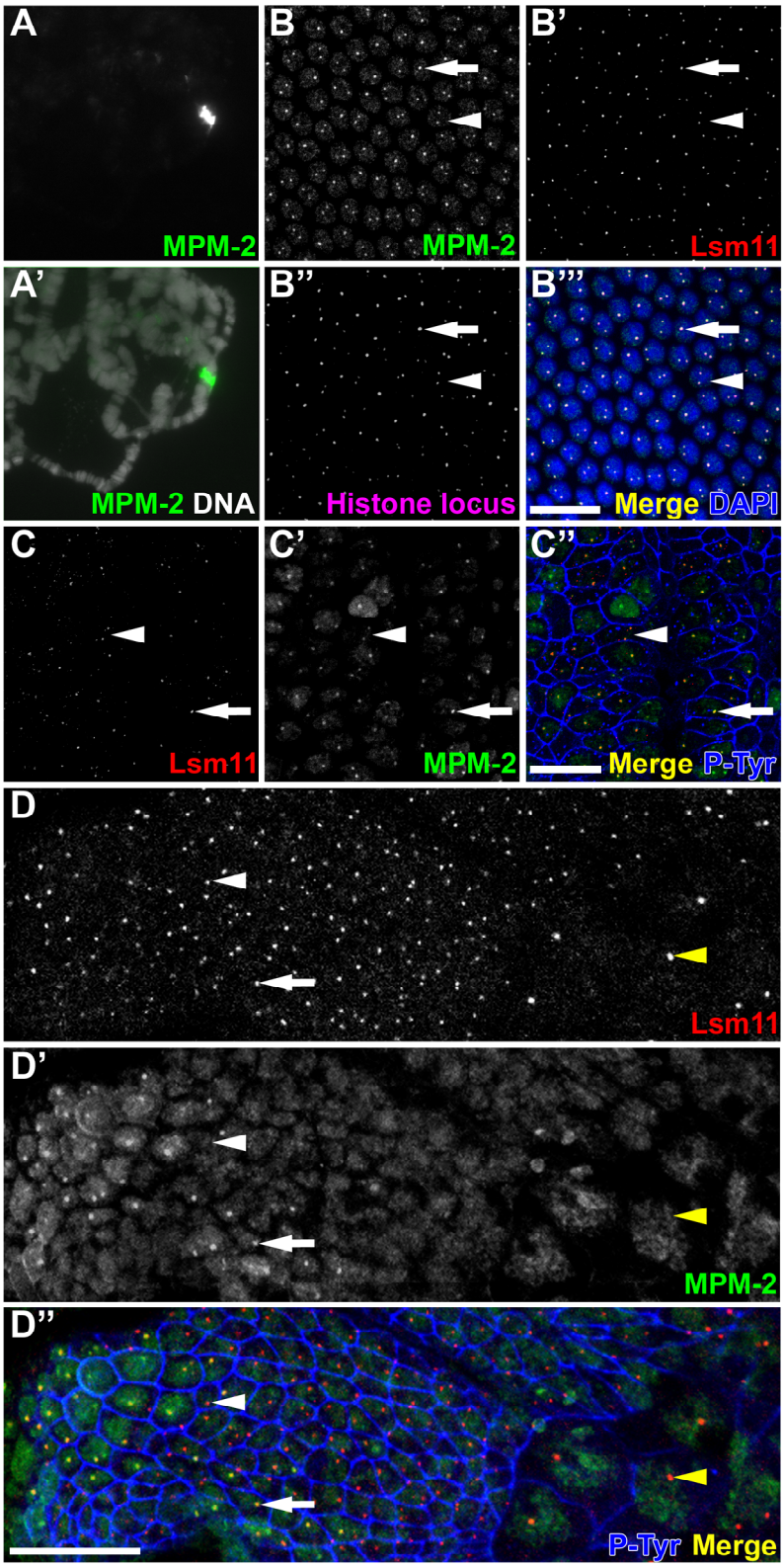

Figure 2.1: MPM-2 labels the histone locus body. (A and A') Squashed polytene chromosomes from CyO/+ third instar larva salivary glands were stained with MPM-2 (A; green in A') and the DNA binding

dye DAPI (A'). (B-B''') w^{1118} syncytial blastoderm (cycle 13) embryos were stained with MPM-2 (B; green in B'''), α-Lsm11 (B', red in B'''), and hybridized with an H3/H4 gene probe (B'', magenta in B'''). (C-C'') Germ band extended w^{1118} embryos were stained with α-Lsm11 (C, red in C''), MPM-2 (C', green in C'') and α-phospho-tyrosine (P-Tyr; blue in C'') to visualize cell boundaries. Arrows and arrowheads in B and C panels indicate nuclei with one or two HLB, respectively. (D-D'') Stage 12 w^{1118} embryo stained with MPM-2 (green in D''), α-Lsm11 (red in D''), and α-P-Tyr (blue in D''). Arrows indicate an S_{17} cell containing an MPM-2 focus that co-localizes with Lsm11. White arrowheads indicate a $G1_{17}$ cell lacking MPM-2 foci but containing Lsm11 in the HLB. Yellow arrowheads indicate amnioserosal cells, which have permanently exited the cell cycle in $G2_{14}$. Anterior is to the top and ventral to the left. Bars: 20 μm.

Embryonic MPM-2 foci depend on Cyclin E/Cdk2 activity

If the embryonic MPM-2 foci are related to the previously described MPM-2 foci in follicle cells, then their presence should depend on Cyclin E activity. To test this we characterized MPM-2 staining in stage 13 (cycle 17 in the epidermis) *cyclin E* mutant embryos relative to phenotypically wild type sibling controls. Wild type embryos at this developmental stage contain proliferating diploid cells in the central and peripheral nervous systems as well as endoreduplicating cells in various tissues (e.g. midgut, salivary gland, posterior spiracles). *cyclin E* mutant embryos develop normally until this stage because of maternal deposition of Cyclin E, and then arrest in G1 of cycle 17. Consequently, DNA synthesis in both proliferating neuronal cells and endoreduplicating cells is severely compromised in *cyclin E* mutants (Knoblich *et al.*, 1994). We focused on endoreduplicating cells in the posterior spiracle primordium, since they are near the surface and relatively easy to image. In control embryos these cells contain robust MPM-2 foci (Figure 2.2A-A', arrow). In contrast, the posterior spiracle cells of stage matched *cyclin E* mutants lack detectable MPM-2 foci (Figure 2.2B-B', arrow) while they still

contain Lsm11 foci (Figure 2.2C-C', arrow). These data indicate that Cyclin E is not required for maintenance of the HLB, but rather for the appearance of an MPM-2 epitope at the HLB. We also performed the reciprocal experiment of Cyclin E over-expression. Epidermal cells arrest in G1 of cycle 17, and contain Lsm11 foci but not MPM-2 foci (Fig. 2.2D). Ubiquitous expression of Cyclin E with a heat inducible promoter (*hsp70::cyclin E)* drives these $G1_{17}$ cells into S phase (Duronio and O'Farrell, 1995; Knoblich et al., 1994). This treatment also results in the appearance throughout the epidermis of MPM-2 foci that co-localize with Lsm11 foci (Figure 2.2E, arrowheads). We conclude from these data that Cyclin E/Cdk2 activity in the embryo is both necessary and sufficient to produce nuclear MPM-2 foci that co-localize with the HLB. Because histone gene expression is lost in *cyclin E* mutant embryos when they undergo cell cycle arrest in G1 of cycle 17, we hypothesize that MPM-2 recognizes a Cyclin E/Cdk2 substrate that contributes to histone mRNA biosynthesis. We therefore characterized embryonic MPM-2 foci in more detail.

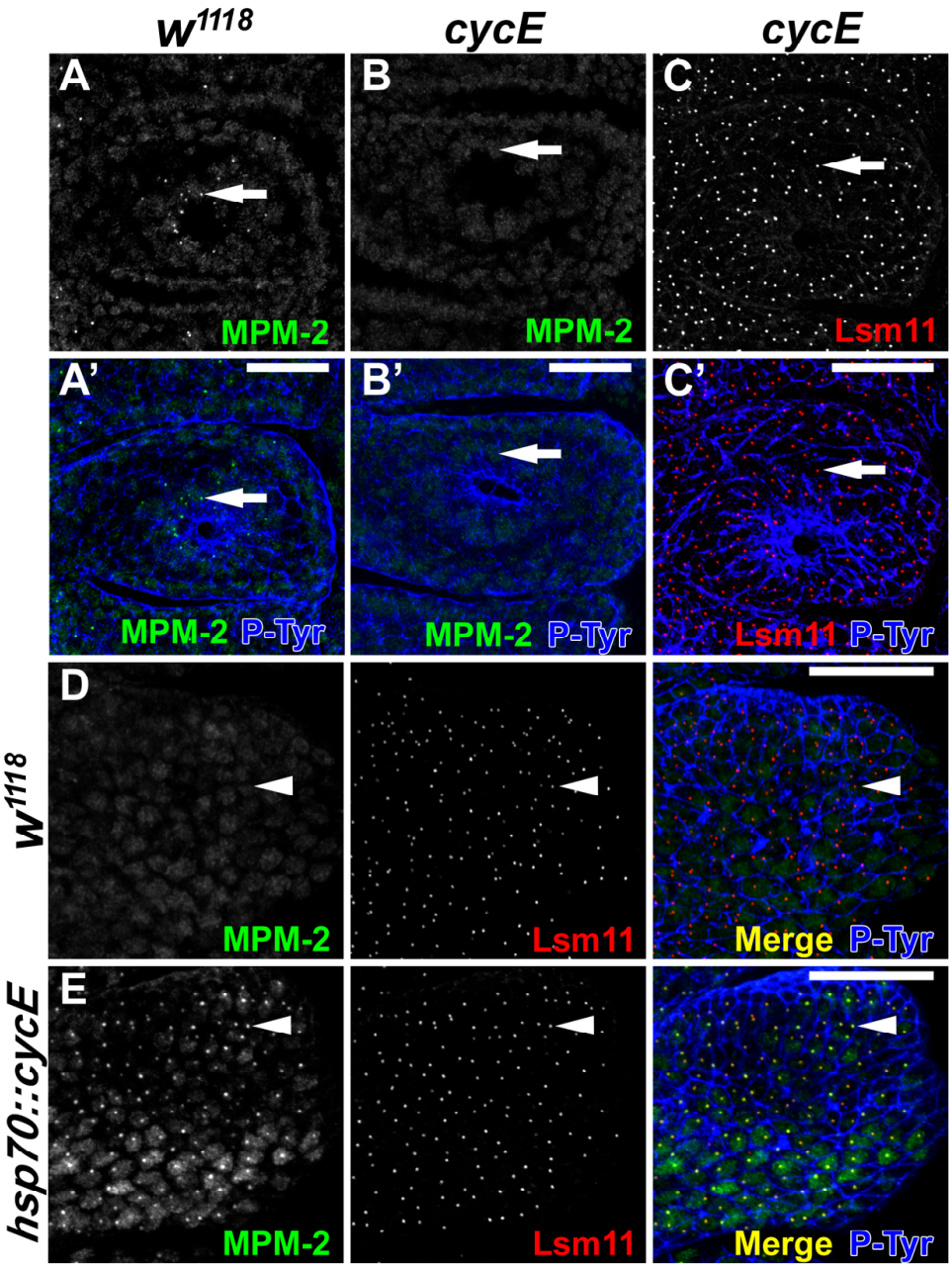

Figure 2.2: Embryonic MPM-2 foci depend on Cyclin E activity. (A-C) Images of the posterior spiracles of germ band retracted (stage 13) embryos. (A) w^{1118} embryos stained with MPM-2 (A, green in A') and α-P-Tyr (blue in A'). (B,C) $cycE^{AR95}$ homozygous mutant embryos stained with MPM-2 (B, green in B') or α-Lsm11 (C, red in C') and α-P-Tyr (blue in B' and C'). Arrows indicate a posterior spiracle cell. w^{1118} control (D) or *hsp70::cyclin E* transgenic (E) embryos were given a 37°C heat shock for 30 minutes prior to fixation and then stained with MPM-2 (green), α-Lsm11 (red), and α-P-Tyr (blue). Epidermal cells of the first thoracic segment are shown. Arrowheads indicate the HLB. Anterior is to the top. Bar: 20 μm.

MPM-2 foci co-localize with nascent histone transcripts

If MPM-2 recognizes an HLB phospho-protein involved in histone gene expression, then it should label sites of histone mRNA biosynthesis. We previously developed a cytological assay that detects nascent, chromatin-associated histone mRNA transcripts in intact *Slbp* mutant embryos (Lanzotti *et al.*, 2002). *Slbp* mutants are defective in normal histone pre-mRNA processing, and accumulate inappropriately long, polyadenylated histone mRNAs (Sullivan *et al.*, 2001). These aberrant mRNAs result from the use of cryptic polyadenylation signals located downstream of the normal pre-mRNA processing site in each of the 5 replication-associated histone genes (Lanzotti *et al.*, 2002). Because the 3' UTR of these polyadenylated histone mRNAs contain sequences not found in the wild type mRNA, we were able to develop a probe (H3-ds) that specifically recognizes the polyadenylated form of histone H3 mRNA. Cryptic polyadenylation is less efficient than the normal 3' end processing, causing nascent pre-mRNA to accumulate on the chromatin template in *Slbp* mutants. These nascent transcripts can be visualized as a nuclear focus by fluorescent whole mount *in situ* hybridization with the H3-ds probe (Figure 2.3, A and B) (Lanzotti et al., 2002; Lanzotti et al., 2004). In addition, nascent histone transcripts appear soon after zygotic transcription of the histone genes begins, because there is no maternal SLBP in the early embryo. We previously showed that nascent H3 transcripts arise in very late $G2_{14}$ immediately preceding each mitosis and persist into S phase of cycle 15 (Lanzotti et al., 2004). Fluorescent *in situ* hybridization of *Slbp* mutant embryos with the H3-ds probe in conjunction with MPM-2 staining showed that MPM-2 foci co-localize with these nascent H3 transcripts (Figure 2.3, A and B, arrows). Thus, MPM-2 foci co-localize with

sites of active histone mRNA biosynthesis in embryonic cells. MPM-2 also stains foci in cells in early $G2_{14}$ that lack nascent H3 transcripts but that are known to contain active Cyclin E/Cdk2 (Figure 2.3A, C, arrowhead). This suggests that MPM-2 foci can be present without ongoing active histone gene transcription (see below, Fig. 2.6).

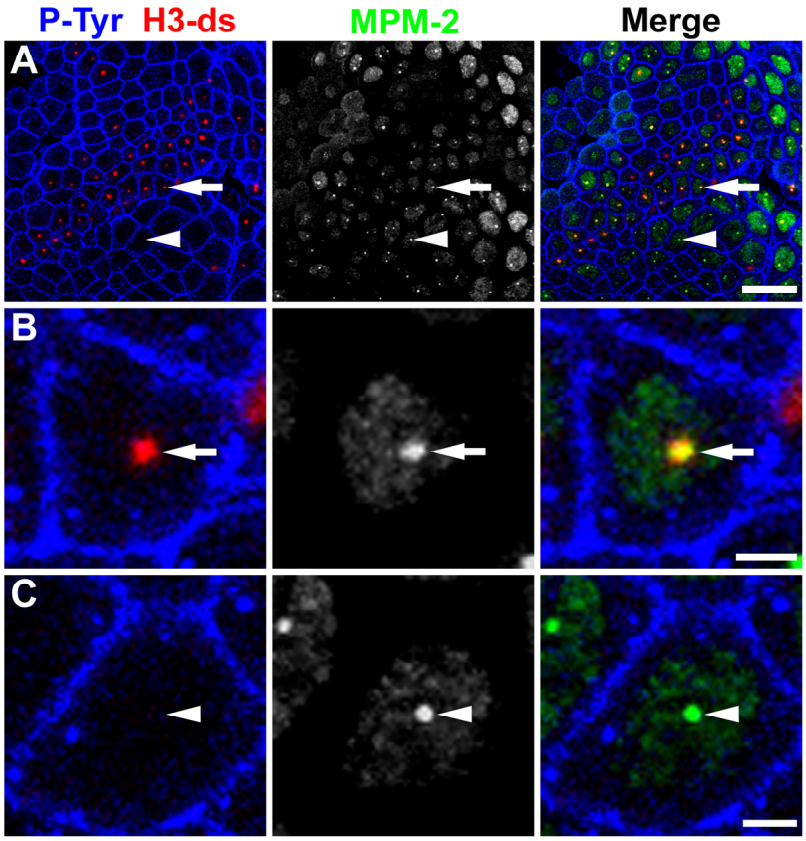

Figure 2.3: MPM-2 foci co-localize with the histone locus body in replicating cells. (A) An Slbp[15] homozygous mutant post-blastoderm embryo was stained with α-P-Tyr (left panel in blue), MPM-2 (green in merge), and hybridized with a fluorescent probe that recognizes misprocessed, polyadenylated H3 mRNA as nascent transcripts in the nucleus (H3-ds; left panel in red). Arrows indicate a cell in S_{15}, which contains an MPM-2 focus that co-localizes with nascent H3 transcripts. Arrowheads indicate an MPM-2 focus in a $G2_{14}$ cell that lacks nascent H3 transcripts. Anterior is to the top and ventral to the right. Bar: 20 μm. (B) S_{15} cell marked by arrows in (A). Bar: 2 μm. (C) $G2_{14}$ cell marked by arrowheads in (A). Bar: 2 μm.

The HLB disassembles during mitosis

Components of the mammalian Cajal body such as NPAT disassemble at the metaphase-anaphase transition and reassemble in the following interphase (Ma *et al.*, 2000). To determine whether HLBs behave similarly, embryos were stained with MPM-2 or anti-Lsm11 antibodies and anti-phospho-histone H3 antibody (PH3) to mark mitotic cells. MPM-2 staining was examined in the early mitotic domains of cell cycle 14 in gastrulating embryos (Figure 2.4). Because the groups of cells comprising a mitotic domain do not enter mitosis synchronously, we could examine all stages of mitosis within a single domain. The nuclear MPM-2 foci present in $G2_{14}$ persist into early mitosis, and all M_{14} prophase cells examined contained MPM-2 foci associated with condensing chromosomes (n=125, Figure 2.4A, arrow). In contrast, only 77% of metaphase cells (n=106, Figure 2.4A, arrowhead) and none of the anaphase cells (n=102, Figure 2.4A, yellow arrowhead) contain detectable chromosome-associated MPM-2 foci. At the following interphase the nuclear MPM-2 foci reappear. In syncytial blastoderm embryos where progression through mitosis occurs synchronously, we also failed to detect MPM-2 foci during anaphase and some metaphase embryos (Figure 2.5). These data indicate that the MPM-2 phospho-epitope begins to decline during metaphase and becomes undetectable by anaphase, either because the HLB disassembles or because the protein containing the MPM-2 epitope is destroyed or dephosphorylated. A similar analysis was performed in cycle 14 embryos with Lsm11 antibodies. As with MPM-2 foci, Lsm11 foci are present in prophase, become undetectable by anaphase, and return in interphase (Figure 2.4B). These data suggest that components of the HLB disassemble during mitosis and are similar to what has been previously reported for NPAT in fibroblast cell

lines (Ma *et al.*, 2000; Zhao *et al.*, 2000). *Drosophila* centrosomes have been reported to contain proteins with phospho-epitopes that are recognized by MPM-2 during mitosis (do Carmo Avides et al., 2001b; Lange et al., 2005a; Logarinho and Sunkel, 1998b). With the fixation conditions we used, MPM-2 staining increases throughout the cell during mitosis, but we did not observe specific staining of centrosomes.

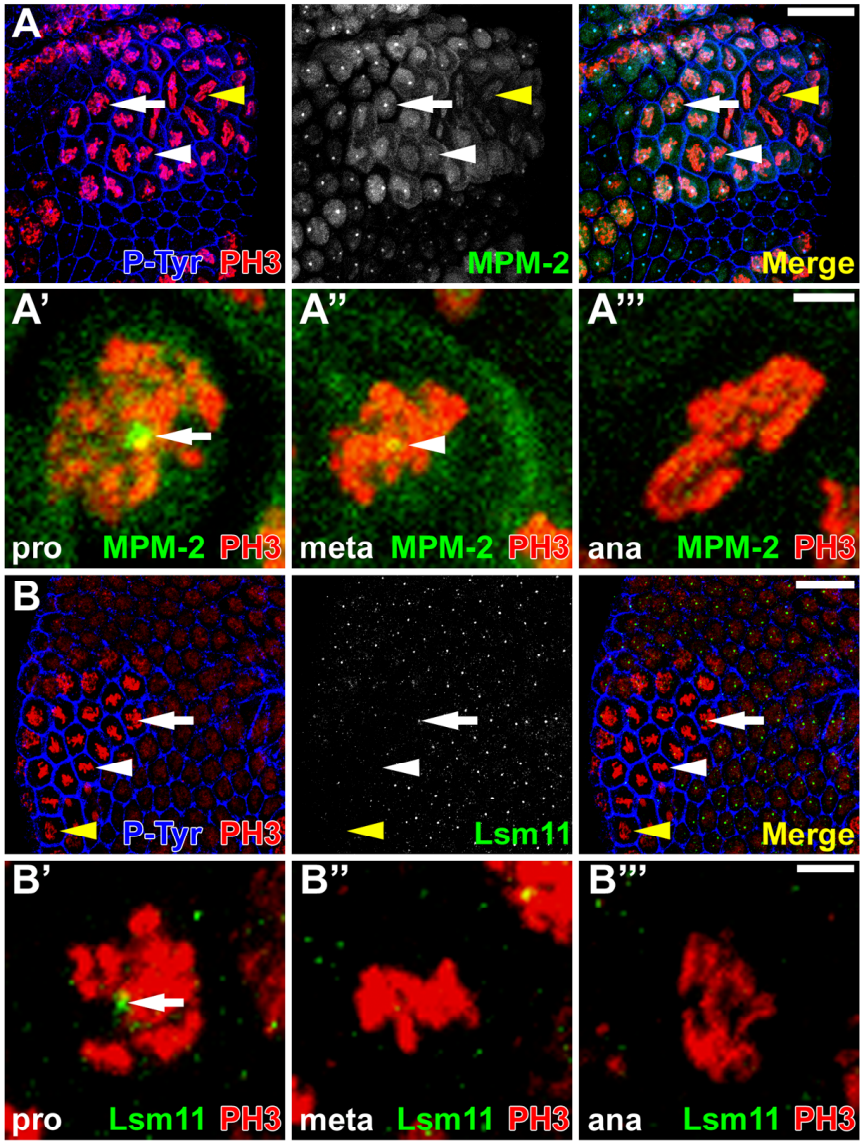

Figure 2.4: MPM-2 and Lsm11 foci are cell cycle dependent. Post-blastoderm w^{1118} embryos in cell cycle 14 were stained with α-P-Tyr (blue), α-PH3 (red), and MPM-2 (A) or α-Lsm11 (B) (middle panels, green in merge). Arrows indicate prophase cells, arrowheads indicate metaphase cells, and yellow

arrowheads indicate anaphase cells. Bar: 20 μm. Single prophase, metaphase, and anaphase cells are shown in (A') and (B'), (A'') and (B''), and (A''') and (B''') respectively. Bars: 2 μm. Mitotic domain 5 is shown in (A) and mitotic domain 1 is shown in (B). Note that the rat α-P-Tyr used here recognizes an antigen at the HLB and the mouse α-PH3 used in (B) stains interphase cells weakly.

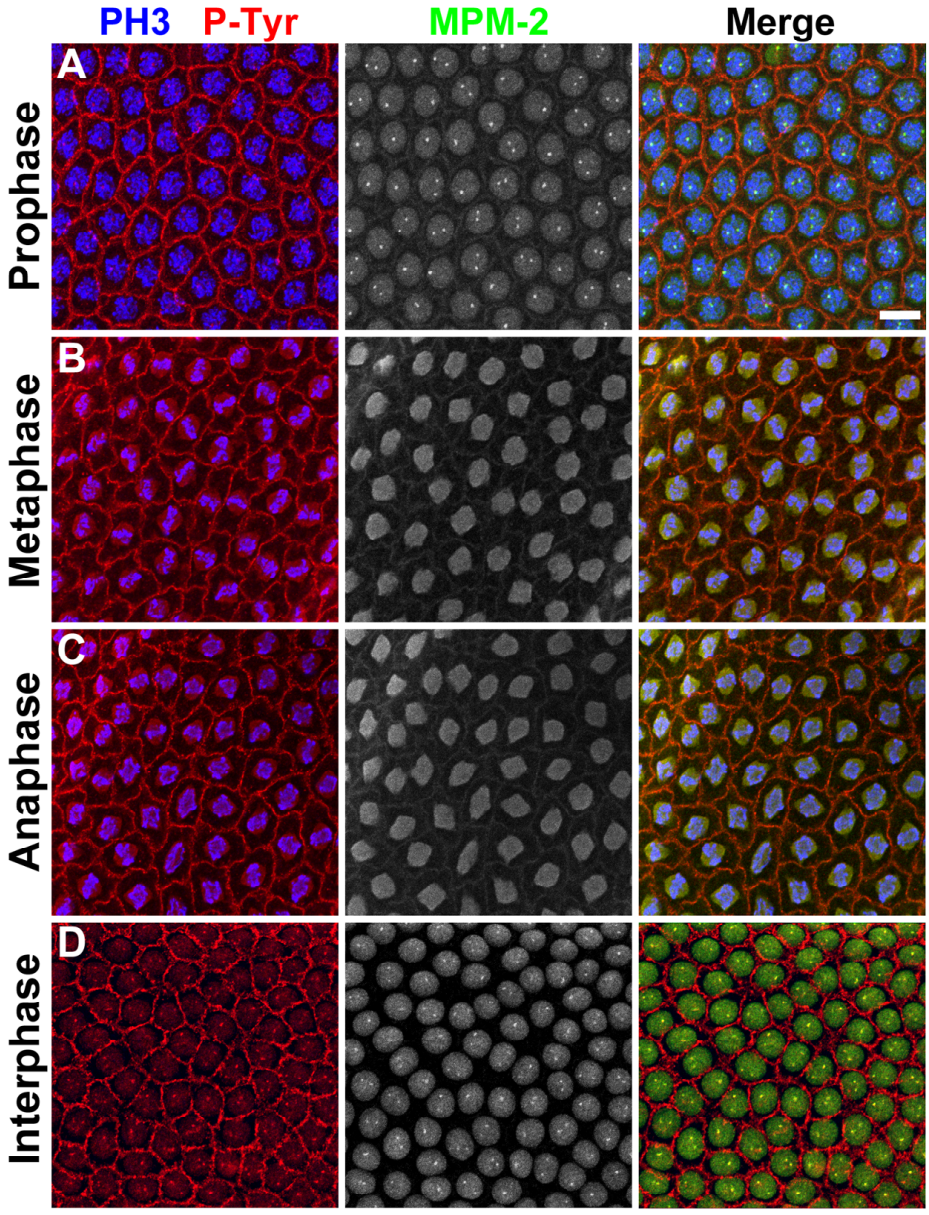

Figure 2.5: MPM-2 foci depend on the cell cycle. w^{1118} syncytial blastoderm embryos were stained with α-PH3 (left panels in blue), α-P-Tyr (left panels in red), and MPM-2 (middle panels, green in merge).

Shown are cycle 12 nuclei in (A) prophase, (B) metaphase, and (C) anaphase. (D) Cycle 13 interphase nuclei. Bar: 10 μm.

MPM-2 foci assemble in the absence of the histone pre-mRNA processing factors SLBP and U7 snRNP

Our analysis thus far suggests that MPM-2 recognizes a Cyclin E/Cdk2 substrate found in the HLB that could be involved in histone mRNA biosynthesis. One possibility is that one or more components of the histone pre-mRNA processing machinery are required for the formation of MPM-2 foci. To test this, we stained *Slbp* mutants with MPM2 antibodies. As shown above (Figure 2.3), MPM-2 foci are observed in *Slbp* mutant embryos, which are deficient in SLBP because there is no maternal store of SLBP protein (Lanzotti *et al.*, 2002). This indicates that SLBP does not contain the primary MPM-2 epitope in the HLB, and suggests that components of the HLB assemble independently of SLBP. Consistent with this, Lsm11 foci are readily detected in *Slbp* mutant embryos, and co-localize with sites of nascent histone H3 transcription (data not shown).

To test whether the U7snRNP is required for either MPM-2 foci or the HLB to assemble, we stained tissues from *U7* snRNA mutant third instar larvae. Because of maternal loading of U7 snRNA, the third larval instar is the earliest time during development when U7 snRNA is depleted and the *U7* mutant phenotype appears (Godfrey *et al.*, 2006). We confirmed the disruption of the U7 snRNP by assaying for Lsm11, a U7 snRNP-specific protein that no longer accumulates in the HLB in *U7* mutant cells (Figure 2.6, B and D). MPM-2 foci form in *U7* mutant salivary gland-associated fat bodies and eye discs (Figure 2.6, A and C), as they do in *SLBP* mutant

embryos. Similarly, the MPM-2 antigen, but not Lsm11, localizes to the HLB in *U7* mutant follicle cells in the adult ovary (J. Gall, personal communication). Therefore, the HLB with an associated protein recognized by MPM-2 assembles independently of the U7 snRNP. To confirm this result, we performed RNAi knockdown of Lsm11 in *Dmel-2* cells and observed that MPM-2 staining was unperturbed following *Lsm11 dsRNA* treatment although the Lsm11 foci were no longer detectable (Figure 2.7). In sum, our results indicate that the MPM-2 phospho-epitope and Lsm11 are detected in the HLB whether or not SLBP is present in the cell. Unlike Lsm11, however, the detection of the MPM-2 phospho-epitope in the HLB does not depend on U7 snRNA, suggesting that at least a partial HLB can form independently of an intact U7 snRNP complex.

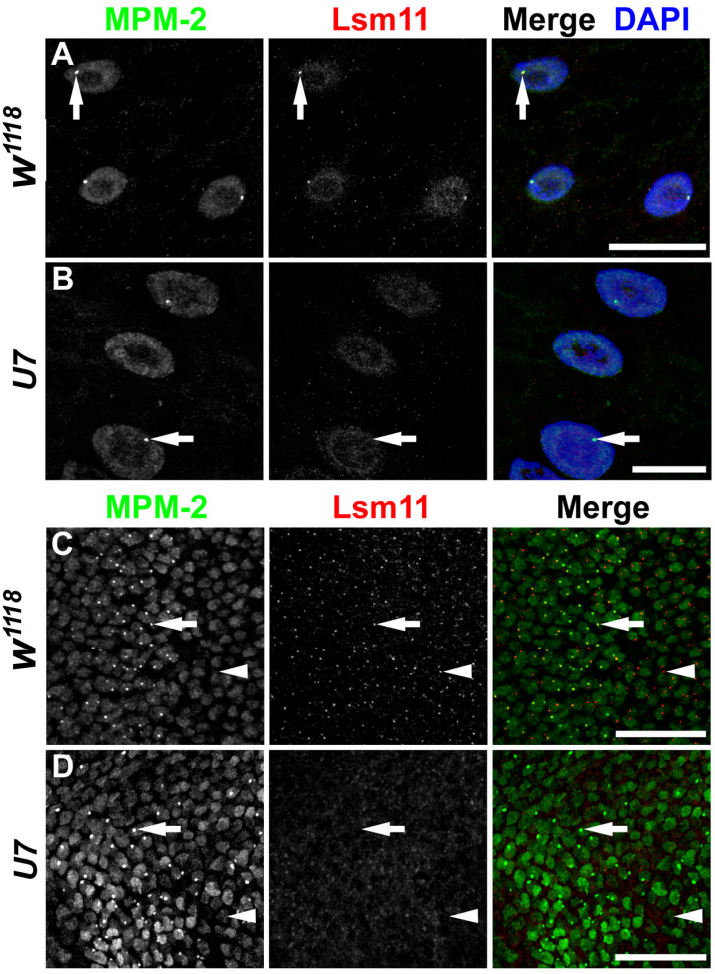

Figure 2.6: MPM-2 foci form independently of the U7 snRNP. Fat bodies (A and B) and eye discs (C and D) dissected from w^{1118} and $U7^{14}$ homozygous mutant third instar larvae were stained with MPM-2 (left panels, green in merge) and α-Lsm11 (middle panels, red in merge). Fat bodies were also stained with DAPI (blue in merge). Arrows and arrowheads indicate nuclei with and without MPM-2 foci, respectively. Note that in wild type Lsm11 foci are present in cells without MPM-2 (arrowhead in C) and that Lsm11 foci are absent in the *U7* mutant cells. Bars: 20 μm.

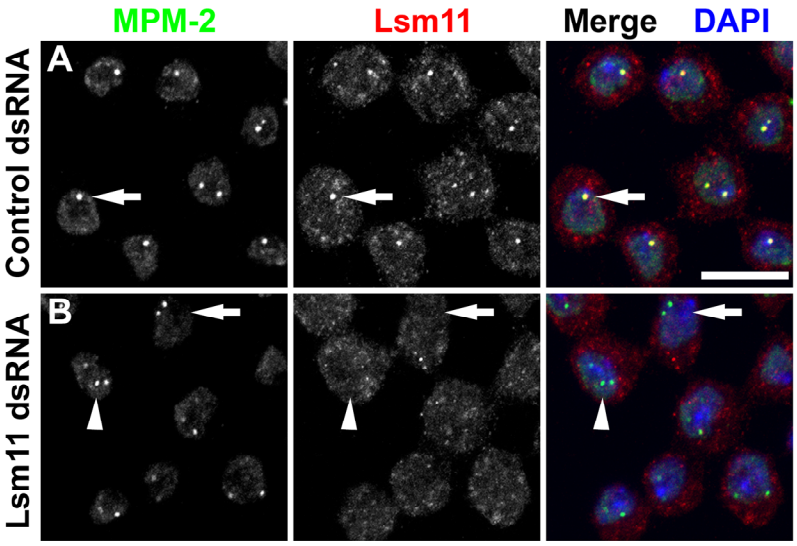

Figure 2.7: MPM-2 foci are not dependent on Lsm11. *Dmel-2* cells treated with either (A) control dsRNA or (B) Lsm11 dsRNA stained with MPM-2 (left panels in green), α-Lsm11 (middle panels in red), and DAPI (right panels in blue). Arrow in (A) indicates a cell with a single HLB. Arrow and arrowhead in (B) indicates cells with two or three, respectively, MPM-2 foci and no Lsm11 foci. Bar:10 μm.

Embryonic MPM-2 foci do not require string^{cdc25} or histone transcription

Cyclin E/Cdk2 phosphorylation of the protein containing the MPM-2 epitope may regulate, or even result from, the transcription of histone genes. To test this, we examined MPM-2 staining in *string* mutant embryos. In late $G2_{14}$, histone gene transcription occurs in a pattern that precisely correlates with the mitotic domain pattern, and this transcription is lost in *string* mutants, which arrest in $G2_{14}$ after destruction of

maternal *string* mRNA and protein (Figure 2.8, A and B) (Lanzotti et al., 2004). MPM-2 foci can be readily detected in *string Slbp* null mutant embryos that lack nascent histone transcripts (Figure 2.8, A and C). This result indicates that formation of MPM-2 foci does not require histone gene transcription or the function of mitotic Cdks activated by string^{cdc25}. Consistent with this interpretation, MPM-2 foci are present in all cells throughout interphase 14 (*i.e.* both S phase and G2 when Cyclin E/Cdk2 is active) at times when neither string^{cdc25} is active nor histone transcription occurs (Figure 2.3C). These data indicate that the formation of MPM-2 foci occurs independently of string^{cdc25} (and thus by inference Cdk1 activity) and histone transcription.

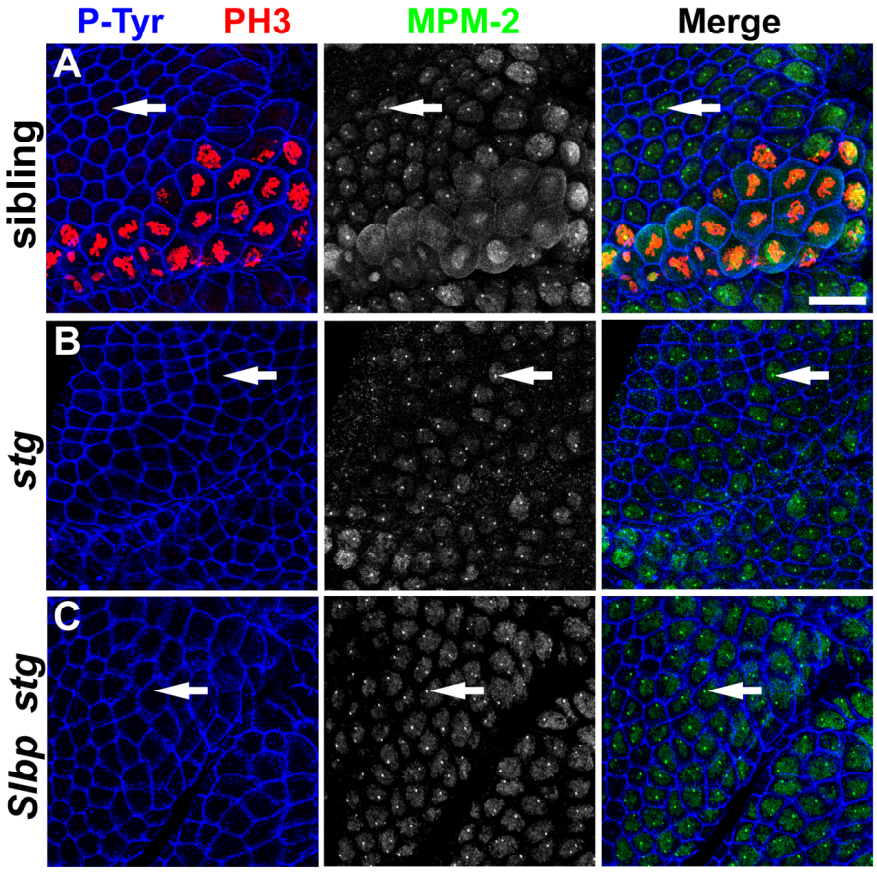

Figure 2.8: MPM-2 foci do not depend on *string* or *Slbp*. All embryos were stained with α-P-Tyr (blue) α-PH3 (red), and MPM-2 (middle panels, green in merge). (A) Stage-matched sibling control, (B) *stg*^{4} homozygous mutant, and (C) *stg Slbp* double (*Df(3R)stg*^{AR2}) homozygous mutant. Lack of PH3 staining

was used to distinguish *stg* mutants from siblings. Anterior is to the top and ventral to the right. Bar: 20 μm.

MPM-2 foci appear coincidentally with activation of zygotic histone gene expression

Most zygotic gene expression begins at the blastoderm stage during cycle 14 (Edgar and Schubiger, 1986). Histone genes are an exception, and become transcriptionally active precisely in nuclear cycle 11 (Edgar and Schubiger, 1986). If the protein containing the MPM-2 epitope regulates some aspect(s) of histone mRNA biosynthesis, then we expect MPM-2 foci to be present once zygotic expression of the histone genes has begun. To determine whether there is a relationship between the onset of zygotic histone transcription, the assembly of the HLB, and the appearance of the MPM-2 epitope at the HLB, we carefully analyzed syncytial blastoderm embryos stained with MPM-2 and anti-Lsm11 antibodies. Nuclear density was used to accurately stage the embryos with respect to each nuclear cycle. Uniform MPM-2 staining throughout the nucleus was detectable in all syncytial blastoderm cycles, perhaps because maternal Cyclin E/Cdk2 is present throughout the early embryo. MPM-2 foci appear for the first time precisely during nuclear cycle 11, concomitantly with the onset of zygotic histone gene transcription (Figure 2.9). Similarly, nuclear Lsm11 staining is undetectable prior to cycle 11, and Lsm11 foci first appear during nuclear cycle 11 and co-localize with MPM-2 foci (Figure 2.9). The appearance of Lsm11 foci in cycle 11 is not the result of new, zygotic synthesis, since Lsm11 protein is present in 0-30 min embryos (data not shown), prior to the onset of zygotic gene expression. In addition, maternal expression of a YFP-Lsm11 fusion protein in da-GAL4/UAS-YFP-Lsm11 females results in appearance of YFP-Lsm11 foci in embryonic cycle 11 (Figure 2.10). These data indicate

that the onset of zygotic histone gene transcription and formation of the MPM-2-positive HLB during early development are temporally and spatially coincident.

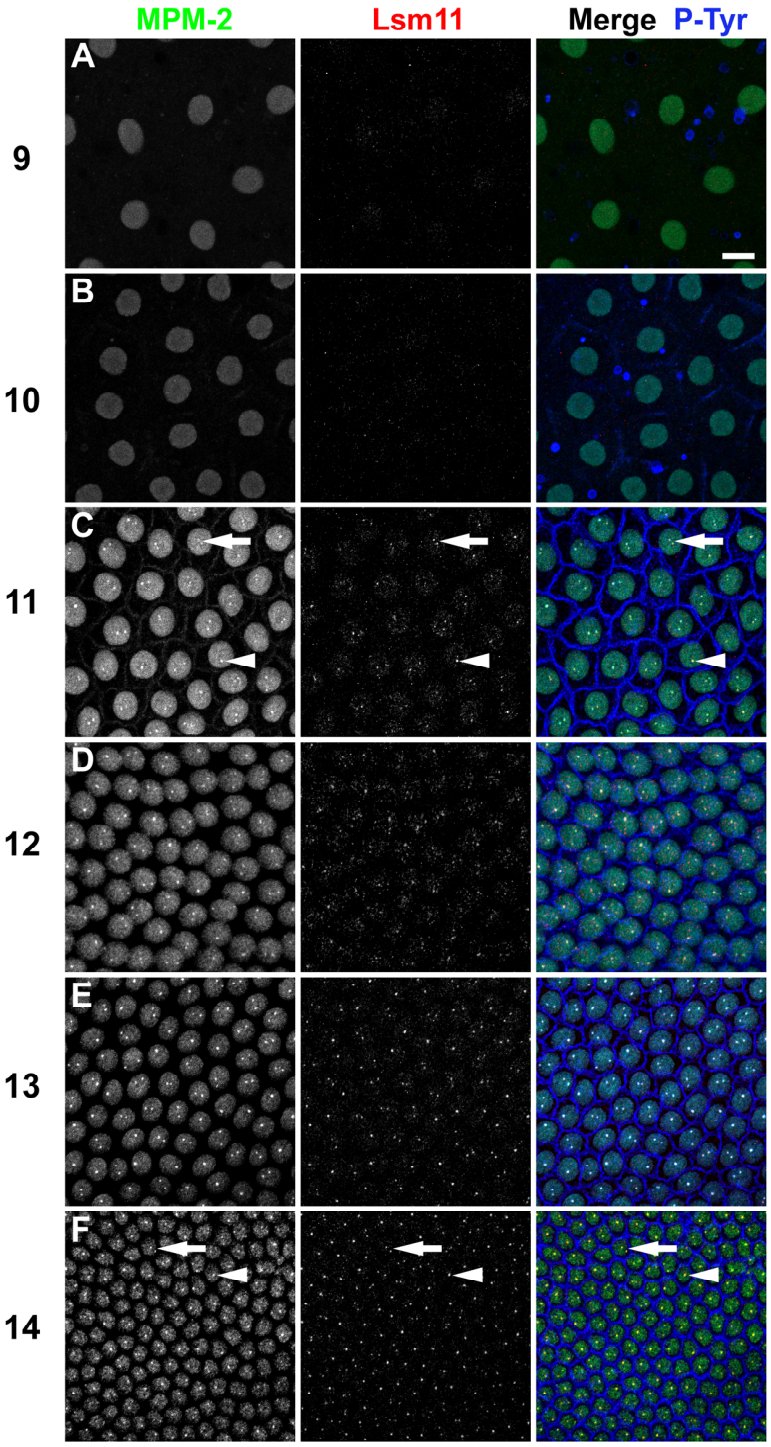

Figure 2.9: The histone locus body forms for the first time during nuclear cycle 11. w^{1118} syncytial blastoderm embryos were stained with MPM-2 (left panels, green in merge), α-Lsm11 (middle panels, red

in merge), and α-P-Tyr (right panels in blue). Interphase of nuclear cycles 9 to 14 are shown, as indicated on the left. Bar: 10 μm.

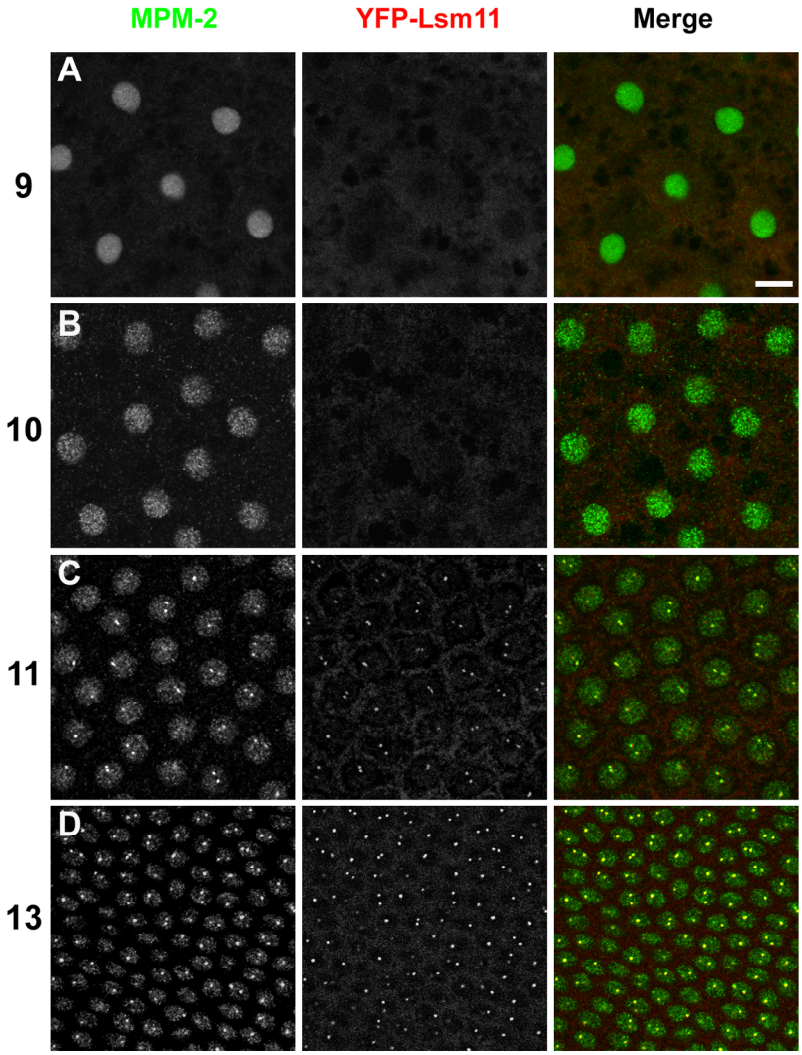

Figure 2.10: The histone locus body first forms and co-localizes with MPM-2 foci during nuclear cycle 11. Syncytial blastoderm embryos with a maternally provided supply of YFP-Lsm11 were stained with MPM-2 (red) and α-GFP (YFP-Lsm11; green). Interphase of nuclear cycles 9, 10, 11 and 13 are shown, as indicated on the left. Bar: 10 μm.

MPM-2 foci form independently of the histone locus

The coincidence in developmental timing of the onset of zygotic histone transcription and formation of the HLB suggests two possibilities. Histone gene

transcription may directly result in the nucleation of the HLB at the histone locus, or *de novo* assembly of the HLB may trigger the initiation of histone transcription. To attempt to distinguish between these possibilities, we stained embryos containing a homozygous deletion of the entire histone locus (*Df(2L)Ds6*) with MPM-2 and anti-Lsm11 antibodies. We hypothesized that chromatin at or near the histone locus is necessary for the assembly of the HLB, as it might function as a scaffold on which the HLB is assembled to carry out histone mRNA biosynthesis. Surprisingly, both MPM-2 and Lsm11 foci are detected in *Df(2L)Ds6* homozygous embryos lacking the histone genes (Figure 2.11, arrows), which were distinguished from siblings containing histone genes by in situ hybridization of histone H3 mRNA (Figure 2.11, left panels). Moreover, the distribution of nuclei with one versus two MPM-2 foci is similar between wild type and *Df(2L)Ds6* embryos, such that the percentage of cells containing one MPM-2 focus in wild type and *Df(2L)Ds6* embryos is 89% and 84%, respectively, and the percentage of cells containing two MPM-2 foci in wild type and *Df(2L)Ds6* embryos is 11% and 7.8%, respectively. This result indicates that the histone locus, and therefore histone transcription, is not required for the initial nucleation of the HLB. This is consistent with our analysis of *string* mutant embryos, which also indicated that histone transcription is not required to maintain MPM-2 foci.

Although HLBs form in the absence of the histone locus, we observed differences in MPM-2 and Lsm11 staining between wild type and *Df(2L)Ds6* embryos. First, the lack of the histone gene cluster altered the high incidence of MPM-2 and Lsm11 co-localization (Table 2.1). Using fluorescent images, nuclei were assigned to a category based on the type of MPM-2 and Lsm11 foci present in the nucleus of stage 11 wild type

or *Df(2L)Ds6* embryos. Whereas in wild type embryos only 5% of interphase nuclei contained Lsm11 foci that did not co-localize with MPM-2, in *Df(2L)Ds6* mutants the proportion of this class of nuclei increased to ~35% (Table 2.1). In addition, 8% of the nuclei contained more than one Lsm11 focus that did not co-localize with MPM-2 foci (Table 2.1). These data indicate that full association of the U7 snRNP and the protein containing the MPM-2 epitope requires the histone locus. Second, the HLBs in *Df(2L)Ds6* embryos are smaller than in controls as revealed by measuring the size (see Materials and Methods) of the MPM-2 nuclear focus in 75 cells from two stage-matched, stage 11 embryos. The average size (defined as length plus width) of the MPM-2 focus in control and *Df(2L)Ds6* embryos is 1.48 ± 0.37 and 1.06 ± 0.27 μm, respectively ($p<0.001$). Thus, while the histone locus is not absolutely essential for the formation of the HLB, as assessed by co-localization of MPM-2 and Lsm11 foci, full, stable assembly of the HLB requires the histone locus.

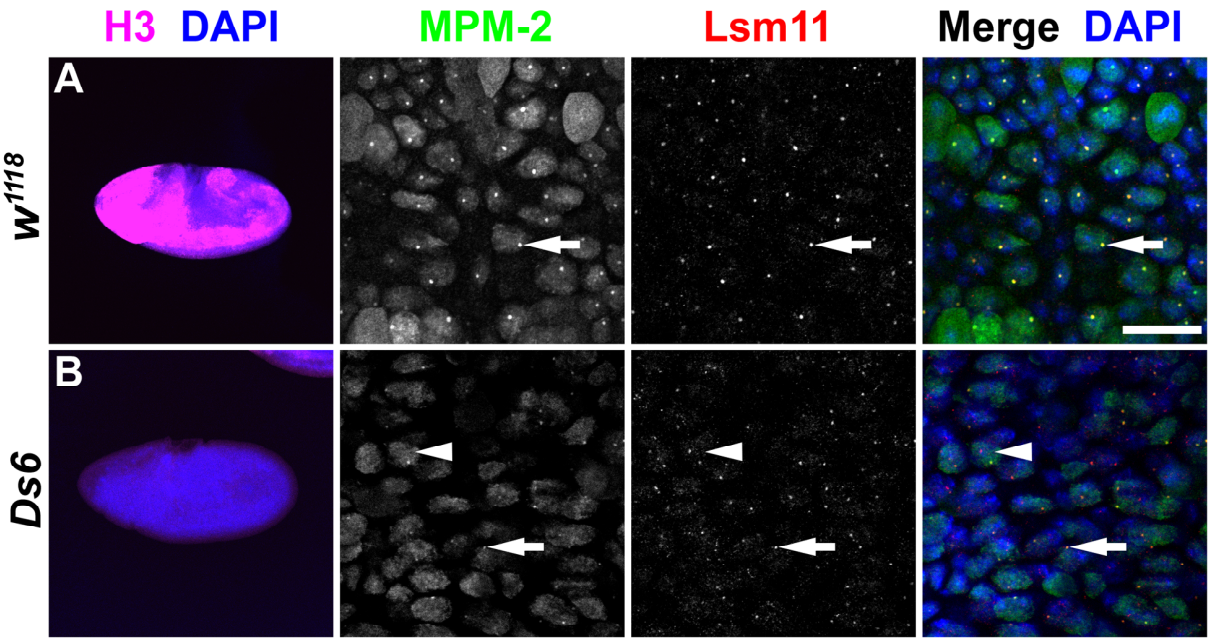

Figure 2.11: Aberrant histone locus bodies form in the absence of the histone locus. Post-blastoderm embryos hybridized with an H3 RNA probe (purple) were stained with MPM-2 (green), α-Lsm11 (red),

and DAPI (blue). *Df(2L)Ds6* homozygous mutant embryos that lack the histone gene cluster (B) were distinguished from control siblings (A) by the lack of zygotic histone H3 mRNA. Arrows indicate foci of MPM-2 and α-Lsm11 that co-localize. Arrowheads indicate cells containing a focus of co-localizing MPM-2 and α-Lsm11 as well as a focus of just α-Lsm11. Anterior is to the top. Bar: 20 μm.

Table 2.1 Characterization of the HLB in histone deletion embryos

	1 ML*	2 ML	1 ML + 1 L	2 L	1 L	1 ML + 2 Ls	2 M	Other
w^{1118} (n=191)	81.2	11.0	5.2	0	0.5	0	0	2.1
Ds6 (n=192)	54.7	4.2	20.3	3.1	3.6	7.8	0.5	5.7

*ML indicates a focus of co-localized MPM-2 and Lsm11 staining, L indicates a focus containing only Lsm11, and M indicates a focus containing only MPM-2. "Other" refers to individual patterns of staining that are not represented in the indicated categories and that each comprise less than 0.5% of cells.

Discussion

Using the MPM-2 monoclonal antibody as a tool, we have presented evidence that a Cyclin E/Cdk2 substrate localizes to the HLB, a recently described *Drosophila* nuclear organelle likely to be directly involved in histone mRNA biosynthesis (Liu et al. 2006). MPM-2 foci co-localize with the histone locus, nascent histone transcripts, and the U7 snRNP-specific protein Lsm11. We propose that the MPM-2 antibody recognizes an activator of histone mRNA biosynthesis that either stimulates histone transcription and/or pre-mRNA processing during S phase.

The identity of the protein containing the MPM-2 epitope is unknown, and there are few if any known *Drosophila* proteins with the properties one would predict for an MPM-2 target involved in histone mRNA biosynthesis. In contrast, the human NPAT protein has several of these properties and provides an example of the type of protein that may be recognized by MPM-2. NPAT is a Cyclin E/Cdk2 substrate that functions as a general activator of transcription of the replication-dependent histone genes that localizes to those Cajal bodies that are associated with histone loci during S phase (Ma et al., 2000;

Miele et al., 2005; Wei et al., 2003; Zhao et al., 1998; Zhao et al., 2000; Zheng et al., 2003). The NH_2-terminus of NPAT contains a LisH domain that is necessary for H4 transcription (Wei *et al.*, 2003). Although there are fifteen predicted LisH-containing proteins in *Drosophila*, no ortholog of NPAT has yet been identified. Coilin, which functions to maintain the integrity of the Cajal body, can be phosphorylated by Cyclin E/Cdk2 *in vitro* (Hebert et al., 2001; Liu et al., 2000; Tucker et al., 2001). However, MPM-2 does not recognize Cajal bodies in HeLa cells (A.E.W. and R.J.D., unpublished), and the *Drosophila* Cajal body in *Drosophila* cells is distinct from the HLB (Liu *et al.*, 2006). The orthologue of coilin has also not been reported in *Drosophila*.

HLB behavior during early Drosophila development

Our cytological analysis of MPM-2 staining of the HLB during early *Drosophila* embryogenesis indicates that the HLB is a dynamic, cell cycle-regulated structure. As detected by MPM-2 and anti-Lsm11 staining, the HLB assembles for the first time in development during S phase of nuclear cycle 11 in the early syncytial embryo. HLB nucleation occurs during the exact same cycle that zygotic histone gene transcription begins. In frog oocytes, increasing U7 snRNA expression can induce formation of Cajal body like structures (Tuma and Roth, 1999), suggesting that in vertebrate cells U7 snRNP or nascent histone transcripts may trigger formation of Cajal bodies associated with the histone locus. Interestingly, however, HLB assembly does not require U7 snRNA or the histone locus, and therefore occurs independently of histone gene expression. We thus favor a model in which developmentally controlled HLB formation is essential for the onset of zygotic histone gene transcription.

Although the HLB is capable of forming independently of the histone locus, the histone locus contributes to the structural integrity of the HLB. In histone deletion embryos, HLBs are smaller and some interphase nuclei contain Lsm11 foci that do not co-localize with MPM-2. The size of the nucleolus is determined by the amount of ribosomal gene transcription (Hernandez-Verdun, 2006). Thus, the size and overall composition of the HLB may be similarly dependent on transcription of the histone genes. Consistent with this, MPM-2 and Lsm11 foci are present at maximal size during prophase of cycle 14, just after the initiation of histone transcription in late $G2_{14}$. Nascent histone transcripts are likely aborted during mitosis (Shermoen and O'Farrell, 1991), which correlates with the loss of MPM-2 and Lsm11 staining we observe in anaphase. Alternatively, reduced HLB size may be a secondary consequence of cell cycle arrest resulting from lack of histone biosynthesis (Smith *et al.*, 1993).

In wild type embryos we observe either one or two MPM-2 or Lsm11 foci per cell at frequencies similar to the known pairing frequencies of the histone loci on homologous second chromosomes (Fung *et al.*, 1998). This is also consistent with the association of the HLB with histone genes (Liu *et al.*, 2006). Surprisingly, we often detect one or two MPM-2 foci in histone deletion embryos, suggesting that HLB number is not dictated solely by homologous pairing at the histone locus. HLB components may associate with another chromosomal locus in the absence of the histone genes. Alternatively, the de novo formation of one or two HLB's may actually drive homologous pairing at the histone locus.

Cell cycle regulation of the HLB

Histone mRNA is greatly depleted in *cyclin E* mutant embryos (Lanzotti et al., 2004). Since cyclin E mutant embryos arrest in G1 phase, it is difficult to know this observation is indicative of a direct involvement of cyclin E/Cdk2 in histone gene expression, or arises as a secondary consequence of cell cycle arrest. Because interphase MPM-2 foci are coincident with the HLB, are present in cells where Cyclin E/Cdk2 is active, and are absent in cells that lack Cyclin E/Cdk2 activity, we favor the interpretation that Cyclin E/Cdk2 phosphorylates a protein directly involved in histone mRNA biosynthesis. How Cyclin E/Cdk2 participates in this process is not known, but it is not required for recruitment of U7 snRNP to the HLB, since Lsm11 remains a stable component of the HLB in wild type cells arrested in G1 and in cells of *cyclin E* mutant embryos.

During the early embryonic cell cycles that have constitutive Cyclin E/Cdk2 activity, MPM-2 foci disappear as cells progress through mitosis and are undetectable by anaphase. Focal Lsm11 staining is also lost during mitosis, suggesting the disassembly of HLB components into the cytoplasm subsequent to nuclear envelope breakdown rather than simply dephosphorylation or destruction of the MPM-2 target(s). The HLB rapidly reforms during the subsequent interphase. These behaviors are similar to the behavior of NPAT foci during mitosis in cultured mammalian fibroblasts (Ma *et al.*, 2000; Zhao *et al.*, 2000). The nucleolus also disassembles during mitosis (Hernandez-Verdun, 2006)**,** suggesting that dynamic disassembly/re-assembly of specific nuclear compartments involved in gene expression is a general feature of nuclear behavior during metazoan mitosis.

Summary

The HLB is a dynamic structure capable of receiving input from both the histone locus and the cell cycle. Our analysis raises many questions, including how HLB assembly occurs at a specific time in a syncytial cytoplasm from abundant maternal components-even in the absence of a histone locus template, and how Cyclin E/Cdk2 regulates HLB function and histone mRNA biosynthesis. Determining the identity of the HLB protein(s) recognized by MPM-2 antigen will help answer such questions, and will provide a useful tool to examine how the regulation of such a fundamental process as histone mRNA biosynthesis is modulated by developmental programs.

Acknowledgements

We are grateful to Joe Gall for his generous sharing of reagents, especially Lsm11 antibodies, and of unpublished information. We thank Julie Norseen for contributing to MPM-2 polytene staining. We thank Mark Peifer for critical reading of the manuscript. This work was supported by NIH grant GM057859 to R.J.D.

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CHAPTER III

IDENTIFICATION OF NOVEL CELL CYCLE-REGULATED COMPONENTS OF THE *DROSOPHILA* HISTONE LOCUS BODY

Preface

This work represents a manuscript currently in preparation. My advisor Dr. Robert Duronio and I designed the experiments. Dr. Robert Duronio, Dr. William Marzluff, and I analyzed the data. Brandon Burch, a graduate student in Dr. Marzluff's lab, helped with the RNAi knockdowns shown in Figure 3.8A, performed the histone reporter assay shown in Figure 3.8B, and assisted in the analysis of the histone GFP reporter secondary screen. I performed all other experiments and wrote this manuscript.

Abstract

Histone mRNA biosynthesis is cell cycle regulated by Cyclin E/Cdk2; however, the underlying molecular mechanisms are incompletely understood. In *Drosophila* the monoclonal antibody MPM-2 detects epitopes that co-localize with the histone locus body (HLB), a nuclear body enriched with factors regulating histone mRNA biosynthesis, and depend on Cyclin E/Cdk2 activity. Using a genome-wide RNAi screen and mass spectroscopy, we aimed to uncover MPM-2 reactive HLB components and discover how histone mRNA biosynthesis is regulated. This work identified two novel HLB components: Mxc and Spt6. We show that Spt6, a transcription elongation factor, co-localizes with HLBs only when histone gene transcription and Cyclin E/Cdk2 are

active. Importantly, we demonstrate that Mxc is recognized by MPM-2. Mxc and the histone pre-mRNA processing factor FLASH establish a core HLB that forms before other HLB factors are localized during early embryogenesis and remains assembled throughout mitosis. Further, we show that Mxc is necessary for the correct localization of all HLB components tested, including FLASH, and for histone pre-mRNA processing. We propose that Mxc may represent a functional homolog to human NPAT.

Introduction

The majority of replication-dependent histone biosynthesis occurs exclusively during S phase, concomitant with DNA replication to facilitate the assembly of chromatin. Cell cycle control of metazoan histone protein synthesis is mainly regulated at the level of mRNA production (Borun et al., 1975; Breindl and Gallwitz, 1973; DeLisle et al., 1983; Detke et al., 1979; Harris et al., 1991; Heintz et al., 1983; Parker and Fitschen, 1980). For example, CHO cells accumulate 35-fold more histone mRNAs in S phase than in other phases of the cell cycle (Harris et al., 1991). Three features of histone gene expression contribute to high histone production in S phase: activation of transcription, increased efficiency of histone pre-mRNA processing, and increased stability of mature histone mRNAs (Marzluff and Duronio, 2002; Marzluff et al., 2008). Precisely how the cell cycle regulates these aspects of histone mRNA biosynthesis is not fully known.

Transcription of the histone genes and progression through S phase in mammalian cells is stimulated by the Cyclin E/Cdk2 substrate NPAT (Ma et al., 2000; Wei et al., 2003; Ye et al., 2003; Zhao et al., 2000). NPAT is a component of a specialized nuclear

structure called the histone locus body (HLB), which contains factors dedicated to the production of histone mRNAs (Ghule et al., 2008). Specifically how Cyclin E/Cdk2 regulates NPAT activity, however, is incompletely understood (Ma et al., 2000; Zhao et al., 2000). NPAT was also recently implicated in recruiting components of the histone pre-mRNA processing machinery (Pirngruber and Johnsen, 2010). Whether Cyclin E/Cdk2 promotes histone mRNA biosynthesis by regulating HLB dynamics or other aspects of histone gene transcription has not yet been determined.

Histone mRNAs are not polyadenylated and undergo a specialized pre-mRNA processing reaction controlled by the 3' end of histone pre-mRNAs (Marzluff and Duronio, 2002; Marzluff et al., 2008). An endonuclease is recruited to cleave the 3' end of histone pre-mRNAs through the activities of several specialized factors (Marzluff et al., 2008). Several of these factors, including FLASH and Lsm11, a core component of U7 snRNP, co-localize in *Drosophila* HLBs (Liu et al., 2006; Yang et al., 2009).

HLBs were first discovered in *Drosophila* as foci of Lsm11 associated with the histone genes, which are clustered in a 5 kb sequence present in ~100 tandemly repeated copies (Liu et al., 2006). Our previous work revealed that an HLB component regulated by Cyclin E/Cdk2 is recognized by the mouse monoclonal antibody MPM-2 (White et al., 2007), an antibody that marks Cyclin E/Cdk2 activity in *Drosophila* cells (Calvi et al., 1998). Based on our earlier work, we hypothesized that MPM-2 recognizes a Cyclin E/Cdk2 substrate. We hypothesized that identifying the MPM-2 reactive component of HLBs would help us to understand the mechanism(s) that Cyclin E/Cdk2 regulates histone mRNA biosynthesis and reveal clues about how histone gene expression is regulated in *Drosophila* cells.

None of the previously known histone mRNA biosynthesis factors was necessary for the MPM-2 epitope(s) co-localizing with HLBs. We thus performed a genome-wide RNAi screen in S2 cells to discover gene products necessary for localization of these MPM-2 epitope(s). Here we report 140 genes that, when reduced in expression, lowered the percentage of cells containing nuclear MPM-2 foci. Two of these genes encode recently described HLB components: the histone pre-mRNA processing factor FLASH and Mute, which is required for muscle specification (Bulchand and Chia, personal communication). A third high scoring hit was the homeotic gene Mxc, which contains some sequence similarity to human NPAT and was previously shown to be necessary for cell proliferation (Docquier et al., 1996; Remillieux-Leschelle et al., 2002). Studies of the novel HLB component Mxc reveal that it is MPM-2 reactive and necessary for histone pre-mRNA processing and for the correct localization of all known HLB markers. We also report that HLB assembly is ordered and begins with Mxc and FLASH in nuclear cycle 10, followed by Mute and the MPM-2 epitope during nuclear cycle 11 when zygotic histone gene transcription initiates. This core HLB remains assembled throughout mitosis, unlike other nuclear bodies such as nucleoli and Cajal bodies. We also report results from validation and secondary screens performed on the top 95 hits that identified genes required for the correct localization of HLB components and for efficient histone pre-mRNA processing. Using mass spectroscopy, we identified 11 proteins that co-immunoprecipitate with MPM-2 from S phase-arrested S2 cell nuclear extracts, including the transcription elongation factor Spt6. We show that Spt6 is a novel HLB component that co-localizes with HLBs only when histone gene transcription is active.

Materials and Methods

Cell culture, RNA interference, and cell staining. S2 cells were grown in Sf-900 II SFM serum-free media (Gibco) using standard techniques. S phase arrest was induced by treating cells with 10 μM aphidicolin and 1 μM hydroxyurea (HU) for 24-36 hours. For the genome-wide screen a library of 15,680 dsRNAs was obtained from Open Biosystems (Thermo Scientific). 25 x 10^4 cells were plated in 96-well plates containing 1μg of dsRNAs and grown at 25^{o}C for six days. Cells were treated with aphidicolin and HU during the last 24 hours of incubation with dsRNAs. Cells were plated to tissue culture grade plastic 96 well plates that were pre-treated with concanavalin A and allowed to spread for 4-6 hours. For the secondary screens, cells were plated to Whatman glass 96 well plates pre-treated with concanavalin A. Cells were fixed in 4% paraformaldehyde for 10 minutes, extracted using 0.1% Triton X-100 for 15 minutes, and blocked with 5% normal goat serum in PBST for 30 minutes. The following primary antibodies were used: monoclonal mouse anti-Ser/Thr-Pro MPM-2, (1:10000, Millipore), affinity-purified polyclonal rabbit anti-FLASH (1:5000, gift of Zbigniew Dominski (Yang et al., 2009)), polyclonal guinea pig anti-Mute-LS (1:2000, gift of William Chia), and polyclonal guinea pig anti-Mxc (1:5000). The same secondary antibodies and DAPI were used as described for embryo staining and all were diluted at 1:1000. Plates were stored at 4^{o}C until they were imaged. For the validation screen dsRNAs were obtained from the *Drosophila* RNAi Screening Center at Harvard Medical School. SK dsRNAs were made by *in vitro* transcription using pBluescript as template and T7 polymerase. For all other RNAi experiments, dsRNAs were synthesized using a PCR product as a

template.

Screen imaging, analysis, and candidate selection for secondary screening. Screen plates were imaged using the Cellomics Arrayscan VTI (Thermo Scientific) platform and Spotdetector plate protocol provided with the accompanying software. For each screen, cells were identified as objects by DAPI staining and within each object (nucleus) the algorithm identified fluorescent spots and quantified the percentage of objects containing at least one fluorescent spot (MPM-2 or spot index) for each well. Data was acquired for at least 400 objects for each well when possible and a well containing fewer than 200 objects was not scored. MPM-2 indices were plotted for each plate and normalized based on the mean MPM-2 index of the plate. A well with an MPM-2 index of more than 3 standard deviations below the plate average was considered a hit. To select candidates for validation, images corresponding to the top 150 dsRNAs were visually confirmed as lacking MPM-2 nuclear foci to eliminate false positives. Genes considered indirect hits were not selected, such as genes previously reported to be required for cell cycle progression (Bjorklund et al., 2006). For the top ninety-five verified candidates, dsRNAs with different targets than in the primary screen were selected. Data acquisition and analysis was performed in the same manner as in the primary screen, except that the MPM-2 index was compared to control SK and no dsRNA controls. For anti-Mxc, anti-FLASH, and anti-Mute stainings, an index for each factor was determined in the same manner as for MPM-2. For MPM-2 and Mxc, average indices from two separate experiments were analyzed. Indices (%) for each marker were plotted from lowest to highest and values smaller than the control wells were binned into

three categories based on the index reduction relative to controls: severe, moderate, slight.

Histone GFP reporter assay. Dmel-2 cells stably expressing the Act5Cp/H3/GFP reporter described previously (Yang et al., 2009) were plated to a 96 well plate containing candidate dsRNAs and imaged after 5 days. Wells were manually scored for the presence of GFP on a relative scale of 2-5 (weak-very strong) based on two replicate experiments. Imaging was performed using an Olympus IX81 microscope equipped with a 10x objective.

Constructs and transfections. Two overlapping PCR products amplified from clone LD32107 and S2 cDNA were ligated together to generate full-length Mxc. Mxc and CG2247 open reading frames were cloned into pENTR/D-TOPO (Invitrogen), confirmed by sequencing, and recombined with pAVW (*Actin5C* promoter, N-term GFP) and pAWV (*Actin5C* promoter, C-term GFP). pENTR-Mxc was also recombined with pAFW (*Actin5C* promoter, N-term FLAG), pAWF (*Actin5C* promoter, C-term FLAG), and pUGW (*Ubiquitin* promoter, N-term GFP). pUGW was a generous gift from Stephanie Nowotarski. Actp-dSpt6FH (*Actin5C* promoter, C-term FLAG/His) was a generous gift from John Lis (Andrulis et al., 2002).

S2 cells were transfected in 6 well plates using the Amaxa Nucleofector apparatus and Nucleofector V kit (Lonza) as directed by the manufacturer. Stables cell lines were generated by co-transfection with pCoHygro (Invitrogen) and growing cells in 250 μg/ml Hygromycin B (Invitrogen) for >14 days.

Immunoprecipitations and Western blotting. Nuclear extracts were made as described in (Sullivan et al., 2009), except that cells were lysed with 40 strokes of a dounce homogenizer and no dialysis was performed. Antibodies were covalently cross-linked to protein G beads with dimethyl pimelimidate as previously described (Rogers et al., 2009). Proteins were immunoprecipitated by incubating nuclear extracts with antibody-coupled beads overnight at 4^{o}C in NET buffer (50 mM Tris, pH 7.5, 400 mM NaCl, 5 mM EDTA, 1% NP-40) supplemented with inhibitors of proteases (0.5 μg/ml leupeptin, 0.25 μg/ml pepstatin A, 1.5 μg/ml aprotinin, 575 μM phenylmethanesulfonyl fluoride) and phosphatases (1 μM microcystin-LR and 312.5 ηM okadaic acid). Immunocomplexes were washed four times with NET buffer supplemented with phosphatase inhibitors before being boiled for 5 minutes in SDS-PAGE sample buffer. The following primary antibodies were used: affinity purified monoclonal mouse anti-FLAG M2 (1:2000, Sigma). Secondary antibodies used were: ECL sheep anti-mouse HRP (1:1000-2000, GE Healthcare), ECL donkey anti-rabbit HRP (1:2000, GE Healthcare), and goat anti-guinea pig HRP (1:2000, Jackson Immunoresearch Laboratories). Blots were developed using the ECL plus detection system (GE Healthcare) following manufacturers directions.

Protein identification by mass spectroscopy. Proteins were immunoprecipitated from 10.4 mg S-phase arrested S2 nuclear extract diluted to 1mg/ml in NET buffer, supplemented with protease and phosphatase inhibitors, with 25 μg MPM-2 or anti-HA antibodies pre-cross-linked to 50 μl of protein G beads. Immunocomplexes were washed

four times, boiled in SDS-PAGE sample buffer, and run on a 4-15% Tris-HCL mini-gel (BioRad). Proteins were stained with Coomassie G-250 (Invitrogen) overnight at 4^{o}C. Proteins of interest were excised from the gel, digested with trypsin, and analyzed by ABI 4800 MALDI TOF/TOF MS at the UNC-Duke Michael Hooker Proteomics Center.

***Drosophila* strains.** *Mxc*^{G48} was previously described (Santamaria and Randsholt, 1995). *w*^{1118} flies were used as wild type control. The Spt6 protein trap line (Spt6-PT), *P{PTT-GA}Spt6*^{CA07692}, contains an in-frame EGFP insertion between exons 2 and 3 of Spt6 and was previously described (Buszczak et al., 2007). *GFP::Mxc* transgenic flies were created by injecting *y*^{1} *w*^{1118}*; PBac{y*$^{+}$*-attP-3B}VK00033* embryos with pUGW vectors containing Mxc. Best Gene Inc. (Chino Hills, CA) performed embryo injections.

Immunofluorescence of embryos and larval brains. Embryos were dechorionated, fixed in a 1:1 mixture of 5% formaldehyde:heptane for 20 minutes and incubated with primary and secondary antibodies each for 1 hour at 25^{o}C or overnight at 4^{o}C. The following primary antibodies were used: monoclonal mouse MPM-2, (1:1000, Millipore); monoclonal mouse anti-phospho-histone H3 (Ser10) (1:1000, Millipore); polyclonal rabbit anti-phospho-histone H3 (Ser10) (1:1000, Millipore); polyclonal rabbit anti-phospho-tyrosine (1:100, Millipore); monoclonal rat anti-phospho-tyrosine (1:100, R & D systems, Minneapolis, MN); mouse and rabbit anti-GFP (1:2000, Abcam, Cambridge, MA); affinity-purified polyclonal rabbit anti-FLASH (1:1000), polyclonal ginea pig anti-Mute-LS (1:1000), affinity purified rabbit and guinea pig anti-Mxc (1:2000), and affinity-purified polyclonal guinea pig anti-Spt6 (1:1000, gift of Fred

Winston (Kaplan et al., 2000)). The following secondary antibodies were used: goat anti-mouse IgG labeled with Oregon Green 488 (Molecular Probes, Eugene, OR), Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA) or Cy5 (Jackson); goat anti-rabbit IgG labeled with Rhodamine Red (Molecular Probes, Eugene, OR), Cy2 (Jackson), or Cy5 (Abcam); goat anti-rat IgG labeled with Cy3 (Jackson), donkey anti-rat Cy5 (Jackson); donkey anti-chicken IgY labeled with Cy2, Cy3, or Cy5 (all from Jackson). DNA was detected by staining embryos with DAPI (1:1000 of 1mg/ml stock, DAKO Corporation, Carpinteria, CA) for 2 minutes.

Confocal Microscopy. Confocal images were taken at a zoom of 1.0-8.0 with a 63x (NA 1.40) Plan Apochromat objective on a Zeiss 510 laser scanning confocal microscope using the LSM data acquisition software (Carl Zeiss, Germany). Image false coloring and contrast was adjusted using Photoshop (Adobe Systems Inc.).

Results

A genome-wide screen for MPM-2 reactive proteins co-localizing with HLBs

We previously reported that the monoclonal antibody MPM-2 detects a protein(s) that co-localizes with histone locus bodies (HLBs), and that this epitope depends on Cyclin E/Cdk2 activity (White et al., 2007). In order to identify MPM-2 reactive components of HLBs, we developed a high-throughput microscope-based screen in *Drosophila* S2 cells (Figure 3.1A). The screen relied on the premise that knocking-down an MPM-2 reactive component of HLBs would reduce the percentage of cells that contain nuclear MPM-2 foci, called the MPM-2 index. An asynchronously dividing

population of cells has an MPM-2 index of 51%, n=298 cells. In order to increase the baseline MPM-2 index of our assay and thus our ability to detect decreases in the MPM-2 index, cells were arrested in S phase by treating them with aphidicolin and hydroxyurea during the final 24 hours of treatment with dsRNA. This treatment increased the MPM-2 index of a control population of cells to 98% (n=53 cells).

cyclin E was chosen as a positive control for our screen, because it is known to be required for MPM-2 foci formation (White et al., 2007). In our pilot studies, cells treated with *cyclin E* double-stranded (ds) RNAs for 6 days and aphidicolin/hydroxyurea for 24 hours have an MPM-2 index of 17.92% (n=519) as compared to 88.72% (n=532) for cells treated with SK dsRNAs, a negative control dsRNAs containing pBluescript sequences. Using *cyclin E* dsRNAs treatment as a guide, we expected that dsRNAs targeting gene expression of an MPM-2 reactive protein should give a comparably low MPM-2 index.

For the genome-wide screen, S2 cells were plated to 174 96-well plates containing a library of 15,680 dsRNAs, grown at 25^{o}C for six days, and aphidicolin and hydroxyurea were added to the cultures during the last 24 hours. Cells were plated, fixed, and stained with MPM-2 and DAPI (see Materials and Methods). MPM-2 indices were obtained for each dsRNA treatment automatically, using the SpotDetector plate protocol (see Materials and Methods), which was optimized to discriminate between a nucleus containing MPM-2 foci and a nucleus containing diffuse MPM-2 staining throughout (often observed in positive wells lacking MPM-2 foci; Figure 3.1A). Diffuse nuclear MPM-2 staining may be the consequence of other epitopes being more highly recognized by MPM-2 in the absence of the HLB epitope or the mislocalization of the HLB epitope throughout the nucleus. The screen yielded 140 genes that when knocked down in

expression generated MPM-2 indices that were 3 standard deviations lower than the plate mean index (see Figure 3.1B-C, Table 3.1, and Materials and Methods). We then selected 95 genes from this original 140 to validate by re-screening using a set of dsRNAs that target a different region of each gene than in the primary screen. To select these 95, we eliminated genes (e.g. ribosomal proteins) whose depletion is known to arrest the cell cycle outside of S phase. The validation screening of the selected 95 candidates is described with the secondary screens in a later section in this chapter. RNAi treatments that reduced the MPM-2 index during the validation screening are indicated in Table 3.1.

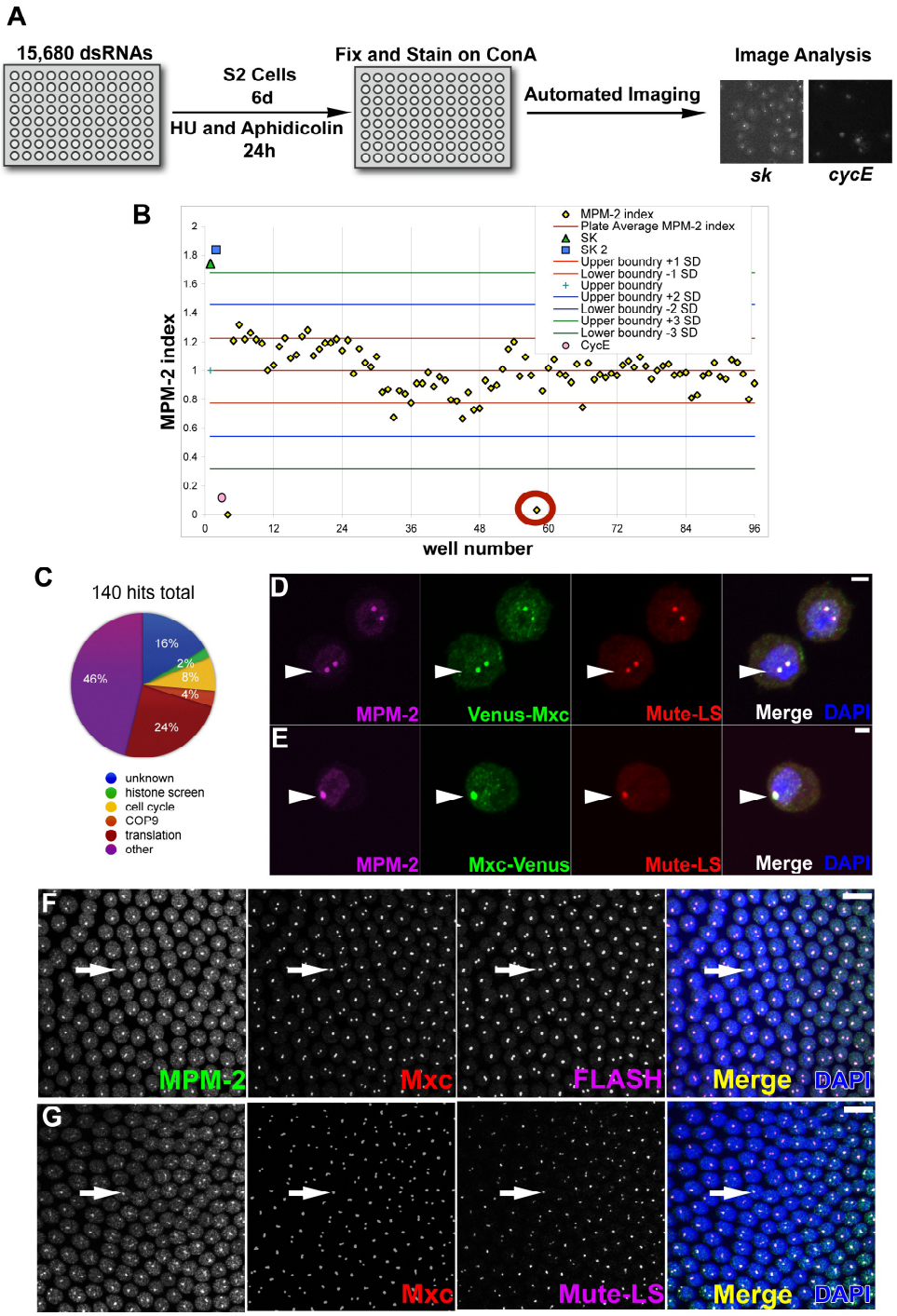


Figure 3.1: Genome-wide RNAi screen for MPM-2 co-localization with HLBs. (A) Overview of the screen, see text for details. (B) Representative graph of the MPM-2 indices of a plate from the screen referenced to the average MPM-2 index of the plate (dark red line). Wells with an MPM-2 index below three standard deviations (bottom green line) from the plate average MPM-2 were considered a hit (well 58 is an example of a hit, circled in red). (C) Functional categories represented by the top 140 hits. (D)

Venus-Mxc and (E) Mxc-Venus expressing S2 cells stained with MPM-2 (magenta), α-GFP to detect Venus-tagged Mxc (green), α-Mute-LS (red), and DAPI (blue in merge). Arrowheads indicate foci of MPM-2, Venus-tagged Mxc, and Mute-LS that co-localize. (F) and (G) w^{1118} syncytial blastoderm (cycle 13) embryos were stained with MPM-2 (green in merge), α-Mxc (red in merge), DAPI (blue in merge), and α-FLASH (magenta in merge) in (F) and α-Mute-LS (magenta in merge) in (G). Arrows indicate foci of MPM-2, Mxc, and FLASH in (F) or Mute-LS in (G) that co-localize. Bars: 2 μm in (D) and (E) and 10 μm in (F) and (G).

Table 3.1: Top hits from primary screen.

CG	Z value	Gene Name	Function or pathway (Flybase)	Hit in related screens?	Selected for validation	Validated hits
HDC9857	-7.695	HDC9857	unknown		Y	N
CG4616	-6.297	FLASH	histone pre-mRNA processing		Y	Y
CG10161	-5.615	eIF-3p66	translation initiation factor	Bjorklund et al. 2006	Y	Y
CG17841	-5.593	CG17841	unknown		Y	N
CG15081	-5.491	l(2)03709	unknown		Y	N
CG14884	-5.464	CSN5	COP9 complex homolog subunit	Bjorklund et al. 2006	Y	Y
CG10498	-5.149	cdc2c	Cdk2, S phase	Bjorklund et al. 2006; Wagner et al. 2007	N	ND
CG12324	-5.144	RpS15Ab	ribosome	Bjorklund et al. 2006	N	ND
CG8171	-5.029	dup	DNA replication, S phase	Bjorklund et al. 2006	N	ND
HDC14221	-5	HDC14221	expressed in posterior endoderm anlage		Y	N
CG3479	-4.976	outspread	unknown		Y	N
CG18332	-4.901	CSN3	COP9 complex homolog subunit	Bjorklund et al. 2006	Y	Y
CG11522	-4.857	RpL6	ribosome	Bjorklund et al. 2006	N	ND
CG6141	-4.843	RpL9	ribosome		N	ND
CG9476	-4.661	a-Tub85E	cytoskeleton, mitotic spindle assembly	Bjorklund et al. 2006	N	ND
CG8522	-4.645	HLH106	fatty acid biosynthesis, transcription	Bjorklund et al. 2006	Y	N
CG18041	-4.578	dgt1	phosphate metabolism		Y	N
CG13623	-4.506	CG13623	iron-sulfur cluster assembly		Y	N
CG17737	-4.31	CG17737	translation initiation factor		Y	N
CG12124	-4.279	mxc	similar sequence to NPAT		Y	Y
CG8725	-4.262	CSN4	COP9 complex homolog subunit		Y	Y
CG7726	-4.251	RpL11	ribosome	Bjorklund et al. 2006	N	ND
CG10324	-4.247	CG10324	nucleic acid binding		Y	N
CG33455	-4.236	Mute	regulation of transcription		Y	N
CG4453	-4.188	Nup153	nuclear pore, zinc ion binding		Y	N
CG11985	-4.118	CG11985	spliceosome		Y	Y
HDC15896	-4.116	HDC15896	unknown		Y	N
CG11397	-4.107	gluon	mitotic spindle organization		Y	N
CG8636	-4.057	CG8636	translation initiation factor		N	ND

CG6050	-4.038	EfTuM	translation elongation		N	ND
CG8745	-4.003	CG8745	arg to glu catabolism		Y	N
CG16983	-4.002	skpA	proteolysis, cell cycle	Bjorklund et al. 2006	N	ND
CG5739	-3.979	CG5739	unknown		Y	N
CG9556	-3.972	alien	CSN2, protein stabilization	Bjorklund et al. 2006	Y	Y
CG8129	-3.969	CG8129	metabolic process		Y	N
CG1891	-3.937	sax	TGFbeta receptor activity,		Y	N
CG17077	-3.929	pnt	transcription factor		Y	Y
CG32000	-3.923	CG32000	ATP biosynthesis	Bjorklund et al. 2006	Y	N
CG18789	-3.836	CG18789	unknown		Y	N
CG10652	-3.823	RpL30	ribosome	Bjorklund et al. 2006	N	ND
CG18495	-3.819	Prosa1	threonine-type endopeptidase activity		N	ND
CG4963	-3.807	CG4963	mitochondrial iron ion transport	Wagner et al. 2007	Y	N
HDC16921	-3.797	HDC16921	unknown		Y	N
CG9677	-3.795	Int6	translation initiation factor		Y	Y
CG18591	-3.767	CG18591	spliceosome		Y	Y
CG8610	-3.76	Cdc27	mitosis	Bjorklund et al. 2006	N	ND
CG2038	-3.758	CSN7	COP9 complex homolog subunit		N	ND
CG18009	-3.752	Trf2	transcription factor		Y	N
CG15693	-3.722	RpS20	ribosome		N	ND
CG1475	-3.722	RpL13A	ribosome		N	ND
CG18740	-3.716	moira	chromatin remodeling, transcription		Y	N
CG11092	-3.703	CG11092	transport		Y	Y
HDC18575	-3.702	HDC18575	unknown		Y	N
CG3157	-3.692	g-Tub23C	mitotic spindle organization		N	ND
CG4046	-3.677	RpS16	ribosome	Bjorklund et al. 2006	N	ND
CG7483	-3.668	eIF4AIII	translation initiation factor	Bjorklund et al. 2006	N	ND
CG10042	-3.666	MBD-R2	binding	Wagner et al. 2007	Y	Y
CG1676	-3.655	cactin	dorsal-ventral axis specification		Y	N
CG5844	-3.629	CG5844	phagocytosis		Y	Y
CG1135	-3.627	Rcd5/MCRS1	centriole duplication	Wagner et al. 2007	Y	N
CG6817	-3.622	foi	zinc ion transmembrane transporter		Y	N
CG15365	-3.603	CG15365	unknown		Y	N
CG8053	-3.591	eIF-1A	translation initiation factor		N	ND
CG12775	-3.59	RpL21	ribosome	Bjorklund et al. 2006	N	ND
CG12113	-3.584	l(1)G0095	binding		Y	Y
CG8857	-3.569	RpS11	ribosome		N	ND
CG8542	-3.562	Hsc70-5	protein folding		N	ND
CG10423	-3.558	RpS27	ribosome		N	ND
CG7808	-3.556	RpS8	ribosome	Bjorklund et al. 2006	N	ND
CG3661	-3.549	RpL23	ribosome	Bjorklund et al. 2006	N	ND
CG10370	-3.539	Tbp-1	proteolysis	Bjorklund et al. 2006	Y	Y
CG9423	-3.513	Kap-a3	protein import in nucleus		Y	N
CG4320	-3.509	raptor	response to DNA damage		Y	Y
CG11132	-3.505	DMAP1	negative regulator of transcription		Y	N
HDC19023	-3.503	HDC19023	unknown		Y	N

CG5859	-3.5	CG5859	unknown		Y	Y
CG10944	-3.486	RpS6	ribosome	Bjorklund et al. 2006	N	ND
CG6610	-3.476	CG6610	spliceosome		Y	N
CG14981	-3.476	maggie	intracellular protein transport		Y	Y
CG15319	-3.465	nej	histone acetyltransferase		Y	N
CG10480	-3.464	Bj1	chromatin binding, Ran GEF, mitosis		Y	N
CG13387	-3.451	emb	nuclear import/export	Bjorklund et al. 2006	Y	Y
CG9075	-3.448	eIF-4a	translation initiation factor	Bjorklund et al. 2006	N	ND
CG8198	-3.448	l(1)G0136	iron-sulfur cluster assembly		Y	N
CG7171	-3.437	Uro	metabolic process		Y	N
CG10333	-3.421	CG10333	spliceosome		Y	N
CG9805	-3.418	eIF3-S10	translation initiation factor	Bjorklund et al. 2006	N	ND
CG15793	-3.416	Dsor1	MAP kinase kinase kinase cascade		Y	N
CG14111	-3.413	CG14111	unknown		Y	N
CG5827	-3.382	RpL37A	ribosome	Bjorklund et al. 2006	N	ND
CG11154	-3.373	ATPsyn-B	ATP synthesis		N	ND
CG18176	-3.37	deflated	binding		Y	N
CG8190	-3.355	eIF2B-g	translation initiation factor		N	ND
CG8922	-3.338	RpS5a	ribosome		N	ND
CG6253	-3.326	RpL14	ribosome		N	ND
CG5119	-3.32	pAbp	poly(A) binding, translation	Bjorklund et al. 2006	N	ND
CG14206	-3.309	RpS10b	ribosome	Bjorklund et al. 2006	N	ND
CG6641	-3.306	Pbprp5	pheromone binding		Y	N
CG9769	-3.298	CG9769	translation initiation factor		Y	Y
CG7490	-3.296	RpLP0	ribosome	Bjorklund et al. 2006	N	ND
CG13128	-3.274	CG34109	unknown		Y	N
CG5147	-3.267	CG5147	transcription from RNA pol III promoter		Y	N
CG7597	-3.257	CG7597	CDK and RNA pol subunit kinase activity	Bjorklund et al. 2006	Y	Y
CG9772	-3.257	CG9772	ubiquitin-dependent protein catabolic process	Bjorklund et al. 2006	Y	N
CG9121	-3.257	CG9121	cytoskeletal anchoring		Y	N
CG32715	-3.255	CG32715	unknown		Y	N
CG6932	-3.233	CSN6	COP9 complex homolog subunit	Bjorklund et al. 2006	N	ND
CG7977	-3.216	RpL23A	ribosome	Bjorklund et al. 2006	N	ND
CG31111	-3.191	CG31111	DNA binding		Y	N
CG17420	-3.19	RpL15	ribosome	Bjorklund et al. 2006	N	ND
CG11992	-3.178	Relish	RNA pol II transcription factor		Y	N
CG9354	-3.177	RpL34b	ribosome	Bjorklund et al. 2006	N	ND
CG18787	-3.177	CG18787	nucleic acid binding		Y	N
CG13716	-3.176	CG13716	regulation of localization		Y	Y
CG14496	-3.175	CG34386	unknown		Y	N
CG4729	-3.17	CG4729	metabolic process		Y	N
CG7939	-3.165	RpL32	ribosome	Bjorklund et al. 2006	N	ND
CG10212	-3.163	SMC2	chromosome condensation, mitosis		N	ND
CG5714	-3.16	ecd	embryonic development, organelle organization		Y	N

CG17521	-3.15	Qm	ribosome		N	ND
CG1163	-3.134	RpII18	RNA pol II subunit, transcription		Y	Y
CG4849	-3.122	CG4849	translation elongation, nuclear mRNA splicing		Y	Y
CG8711	-3.119	cul-4	ubiquitin-dependent protein catabolic process	Bjorklund et al. 2006	N	ND
CG9412	-3.108	rin	Ras signaling		Y	N
CG8877	-3.081	prp8	spliceosome		N	ND
CG16982	-3.078	Roc1a	ubiquitin-dependent protein catabolic process	Bjorklund et al. 2006	N	ND
CG6741	-3.072	arc	binding		Y	N
CG13389	-3.072	RpS13	ribosome	Bjorklund et al. 2006	N	ND
CG12396	-3.071	Nnp-1	rRNA processing		Y	Y
CG11126	-3.067	CG42249	5' nucleotidase activity		Y	N
CG12852	-3.059	CG34354	mRNA binding		Y	N
CG6024	-3.055	CG6024	unknown		Y	N
CG15432	-3.049	CG15432	unknown		Y	N
CG2668	-3.045	Peb	post-mating behavior		Y	Y
CG10320	-3.036	CG10320	NADH dehydrogenase activity		Y	N
CG14792	-3.027	sta	ribosome		N	ND
CG16988	-3.027	Roc1b	ubiquitin-dependent protein catabolic process		N	ND
CG12199	-3.024	kekkon5	protein binding		Y	N
CG13129	-3.022	CG34109	unknown		N	ND
CG14630	-3.008	CG14630	electron carrier		Y	N
CG6420	-2.9987	CG6420	unknown		Y	N
CG11027	-2.989	Arf102F	ADP-ribosylation		Y	Y
CG14712	-2.9831	CG14712	aminoacyl-tRNA ligase, translation		N	ND
CG8142	-2.977	CG8142	DNA clamp loader, DNA replication		Y	N
CG15218	-2.9748	CycK	CDK regulator activity		Y	Y
CG8615	-2.9684	RpL18	ribosome		N	ND
CG5715	-2.9667	CG5715	metalloendopeptidase activity, phagocytosis		Y	N
CG7033	-2.9633	CG7033	ATP-dependent helicase, protein folding	Bjorklund et al. 2006	N	ND
CG11015	-2.9596	CG11015	mitochondrial electron transport		N	ND
CG17454	-2.9519	CG17454	spliceosome		N	ND
CG2013	-2.9501	UbcD6	ubiquitin ligase		N	ND
CG7769	-2.9486	DDB1	ubiquitin-dependent protein catabolic process		N	ND
CG9324	-2.9455	Pomp	proteolysis, cell proliferation		N	ND
CG9131	-2.9428	slmo	unknown		N	ND
CG6779	-2.9411	RpS3	ribosome	Bjorklund et al. 2006	N	ND
CG9183	-2.9269	plu	regulation of mitotic cell cycle, transcription		N	ND
CG6905	-2.9248	CG6905	spliceosome		N	ND
CG12357	-2.9242	Cbp20	RNA cap binding		N	ND
HDC11685	-2.8951	HDC11685	unknown		N	ND
CG6711	-2.8809	Taf2	general RNA pol II transcription factor		N	ND
CG3320	-2.879	Rab1	cell adhesion, transcription		N	ND

CG4717	-2.8604	knirps	transcription factor		N	ND
CG7014	-2.8479	RpS5b	ribosome		N	ND
CG12437	-2.8469	raw	cell death, morphogenesis	Bjorklund et al. 2006	N	ND
CG13900	-2.846	CG13900	spliceosome		N	ND
CG1554	-2.8427	RpII215	transcription	Bjorklund et al. 2006	N	ND
CG3241	-2.8423	msl-2	dosage compensation, X hyperactivation		N	ND
CG2938	-2.83	CG2938	unknown		N	ND
CG8222	-2.8291	Pvr	transmembrane receptor kinase activity		N	ND
CG8309	-2.8291	Tango7	Golgi organization		N	ND
CG12071	-2.8251	CG12071	phagocytosis, engulfment		N	ND
CG3671	-2.8201	Mvl	iron ion transport		N	ND
CG32334	-2.8106	CG32334	unknown		N	ND
CG32231	-2.8098	CG32231	unknown		N	ND
CG7664	-2.8051	crp	RNA pol II transcription factor	Bjorklund et al. 2006	N	ND
CG12254	-2.7997	MED25	RNA pol II transcription mediator activity		N	ND
CG32707	-2.7862	APC4	mRNA binding		N	ND
CG2021	-2.7807	CG2021	spliceosome		N	ND
CG12740	-2.7807	RpL28	ribosome	Bjorklund et al. 2006	N	ND
CG9862	-2.7747	Rae1	unknown		N	ND
CG7035	-2.7709	Cbp80	spliceosome		N	ND
CG3131	-2.7689	Duox	electron carrier, peroxidase activity		N	ND
CG11125	-2.766	CG11125	unknown		N	ND
CG10800	-2.7659	Rca1	G1/S transition, G2 phase		N	ND
CG6176	-2.7342	Grip75	mitotic spindle assembly, DNA repair		N	ND

Several proteins identified in the RNAi screen are novel components of HLBs

The RNAi screen should identify proteins required for assembly of the HLB and/or for phosphorylation of the MPM-2 epitope. Because our primary goal was to identify MPM-2 reactive HLB proteins, we focused on proteins known to localize to *Drosophila* HLBs or that are homologous to proteins known to localize to mammalian HLBs. Three hits satisfied these requirements: FLASH, Mute, and Mxc. FLASH is a histone pre-mRNA processing factor that co-localizes with NPAT in human cultured cells and is required for S phase progression (Barcaroli et al., 2006a; Barcaroli et al., 2006b). Very recently FLASH was shown to co-localize with HLBs in *Drosophila* cultured cells (Yang et al., 2009). *Muscle-wasted* (*Mute*) was recently discovered in a

screen for genes required for muscle specification during *Drosophila* development (S. Bulchand and W. Chia, personal communication). The *Mute* gene encodes two forms of Mute protein (Long and Short) that are produced from separate transcripts, but share common sequences that contain domains predicted to regulate gene transcription (S. Bulchand and W. Chia, personal communication). Curiously, anti-Mute-LS antibodies, which were raised against peptide sequences common to both forms of Mute, detect foci that co-localize with HLBs (S. Bulchand, personal communication and Figure 3.1E). *Multi sex combs* (*mxc*) is a polycomb group gene named for leg bristle duplications displayed by hypomorphic mutants (Saget et al., 1998; Santamaria and Randsholt, 1995). Moreover, *mxc* shares some sequence similarity to human NPAT. For this reason, we hypothesized that Mxc might function similarly to NPAT and co-localize with HLBs. We cloned and Venus-tagged *mxc* at both the N- and C- terminus and expressed these transgenes in S2 cells. Interestingly, both forms of Venus-tagged Mxc co-localize with HLBs (Figure 3.1D-E). The fact that several of our screen hits are components of HLBs makes it possible that other hits are additional HLB components.

FLASH, Mxc, and Mute localization in the embryo

Early *Drosophila* embryogenesis is marked by thirteen rapid and synchronous nuclear S-M phase cycles in a syncytial cytoplasm driven by maternally provided factors. HLBs containing Lsm11 and the MPM-2 epitope are detected during the synchronous nuclear cycles (11-14) following the activation of zygotic histone gene transcription (White et al., 2007). We wished to examine the localization of FLASH, Mxc, and Mute during the late syncytial cycles of the early embryo. To examine Mxc localization,

antibodies were raised against the C-terminal 169 amino acids in rabbits and guinea pigs. Foci of FLASH, Mxc, and Mute all co-localize with MPM-2 in syncytial blastoderm embryos (Figure 3.1F-G).

During embryonic cycle 14, cellularization occurs and nuclei pause for the first time in G2 after S phase. As gastrulation commences, groups of cells called "mitotic domains" enter into mitosis 14 at different times generating a reproducible and well described pattern of mitosis (Foe, 1989). Entry into mitosis 14 requires zygotic transcription of the *string*^{cdc25} (*stg*) gene, which encodes a Cdc25-type phosphatase that stimulates mitotic Cdk1 activity by removing inhibitory Y15 phosphorylation from Cdc2 (Edgar and O'Farrell, 1989). Cycles 15 and 16 are also regulated at the G2-M transition by developmentally controlled pulses of *stg* transcription, and still lack G1 phase as in the early syncytial cycles (Edgar et al., 1994; Edgar and O'Farrell, 1990; Lehman et al., 1999). We previously showed that foci of MPM-2 and Lsm11 co-localize during interphase of these "post-blastoderm" cell cycles and concluded that HLBs might be stable components of *Drosophila* nuclei (White et al., 2007). To test this hypothesis, we examined FLASH, Mxc, and Mute localization in post-blastoderm embryos. We find that antibodies against each of these proteins form nuclear foci that co-localize with MPM-2 (data not shown and Figure 3.4), suggesting that HLBs are a constant feature of the *Drosophila* nucleus.

G1 phase first appears during cycle 17, and subsequent entry into S phase in all cell types requires zygotic expression of *cyclin E* (Knoblich et al., 1994). Consistent with previous observations that MPM-2 foci are Cyclin E-dependent, MPM-2 only labels replicating cells where Cyclin E/Cdk2 is active ((White et al., 2007) and Figure 3.3).

Epidermal cells arrested in G1 that do not contain MPM-2 foci; however, they do contain foci of FLASH, Mxc, and Mute (Figure 3.3A-C). This same localization pattern was also observed for Lsm11, a component of the U7 snRNP (Liu et al., 2006; White et al., 2007). Taken together, these results support the idea that HLBs are present ubiquitously and that only the MPM-2 epitope depends on Cyclin E/Cdk2 activity.

A proteomic approach to identify MPM-2 reactive HLB proteins

In parallel to the genome-wide screen, we employed a proteomics approach to obtain additional candidate proteins that are MPM-2 reactive and present in S phase. For this approach, we made nuclear extracts from S2 cells arrested in S phase by aphidicolin and hydroxyurea treatment, performed immunoprecipitations (IPs) with either MPM-2 or control anti-HA antibodies, and analyzed proteins present only in the MPM-2 IPs by MALDI-TOF mass spectroscopy (Figure 3.2A). We identified MESR4, Spt6, Hcf, and CG2247 in three replicate experiments and decided to focus on these candidates further (see Figure 3.2 legend for a list of all proteins identified). MESR4 (Misexpression suppressor of ras 4) has been implicated in RAS1 signaling, but its precise function is not known (Huang and Rubin, 2000). Host Cell Factor (Hcf) is a transcriptional co-activator and part of the *Drosophila* ATAC (Ada2A containing) HAT (histone acetyltransferase) complex that promotes nucleosome sliding by ISWI, SWI-SNF, and RSC complexes (Guelman et al., 2006; Suganuma et al., 2008). Spt6 positively stimulates transcription by increasing the elongation rate of RNA polymerase II and through interactions with nucleosomes is thought to aid the elongation machinery to navigate around nucleosomes (Andrulis et al., 2000; Ardehali et al., 2009; Bortvin and Winston, 1996; Kaplan et al.,

2000). The function of CG2247 is completely unknown. The factors we identified in the RNAi screen were not detected by proteomics in the immunoprecipitation experiments likely due to their low abundance in cells.

As a first approach to assess whether these candidate proteins play a role in HLB function, we examined their subcellular localization by either using available specific antibodies or in the case of CG2247 creating a Venus-tagged fusion protein. MESR4 was not tested, because its size made cloning difficult and antibodies were not readily available. All proteins tested displayed nuclear localization (Figure 3.2B-E and data not shown). However, only Spt6 formed nuclear foci that co-localize with foci of MPM-2 and the U7 snRNP components Lsm10 and Lsm11 in S2 cells and embyos (Figure 3.2D-E and data not shown). We also observed anti-Spt6 staining throughout the nucleus as described previously (Kaplan et al., 2000), which is consistent with its role in promoting transcription elongation at many loci as part of a complex with active RNA polymerase II (Andrulis et al., 2000; Ardehali et al., 2009; Kaplan et al., 2000). Nuclear foci of Spt6 that co-localize with HLBs may reflect a high concentration of transcription elongation complexes needed to meet the high transcriptional demand placed by the histone genes.

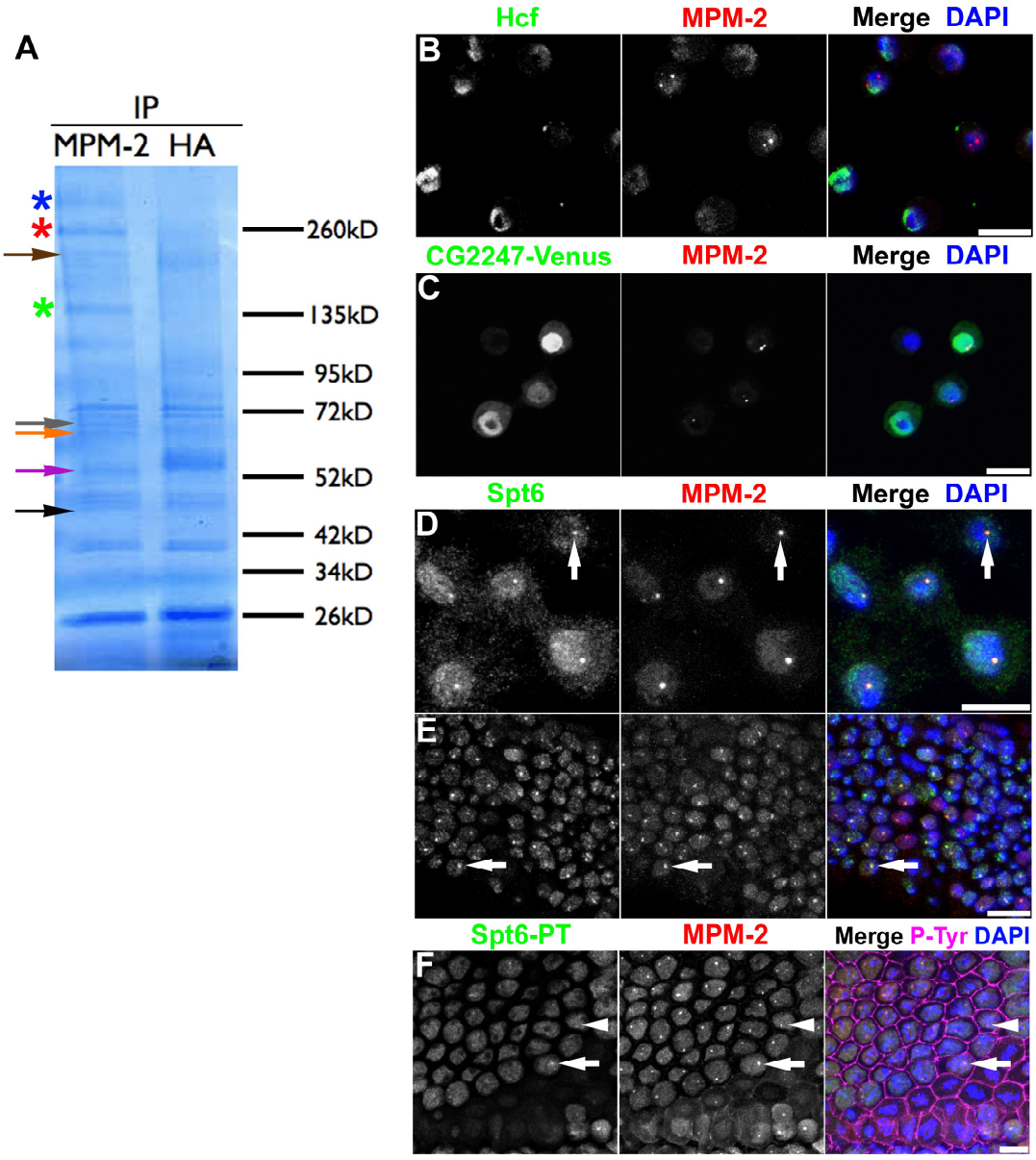


Figure 3.2: Spt6 is a novel HLB component identified by mass spectroscopy. Nuclear extracts made from S-phase arrested S2 cells were immunoprecipitated with MPM-2 or α-HA conjugated protein G beads, run on an SDS-PAGE gel, and stained with Coomassie brilliant blue. The following proteins were identified by mass spectroscopy: MESR4 (blue asterisk), Spt6 (red asterisk), Mus205 (brown arrow), CG2247 (green asterisk), fu2 and PH4αEFB (grey arrow), Hcf (orange arrow), αTub84B (purple arrow), Hrb27C, Act42A, and RpL4 (black arrow). (B) S2 cells were stained with α-Hcf (green in merge), MPM-2 (red in merge), and DAPI (blue in merge). (C) S2 cells transiently transfected with CG2247-Venus were stained with α-GFP (green in merge), MPM-2 (red in merge), and DAPI (blue in merge). S2 cells (D) and

post-blastoderm w^{1118} embryos in cell cycle 14 (E) were stained with α-Spt6 (green in merge), MPM-2 (red in merge), and DAPI (blue in merge). Only Spt6 forms foci that co-localize with MPM-2 (arrows). (F) Post-blastoderm *Spt6-PT* embryo entering mitosis in cell cycle 14 stained with MPM-2 (red in merge), α-GFP (green in merge), α-P-Tyr (to outline cells, magenta in merge), and DAPI (blue in merge). Prominent Spt6 foci form in late G2 cells (arrows) with active histone gene transcription, but not in early G2 cells (arrowheads). Bars: 10 μm.

Spt6 co-localization with HLBs correlates with active histone gene transcription

We examined stage 12 embryos to determine whether Spt6 co-localizes with HLBs in G1 phase cells, similar to FLASH, Mute, and Mxc. To perform this analysis, we took advantage of the availability of an Spt6 protein-trap line (Spt6-PT) that expressed a Spt6 fusion protein containing EGFP inserted in-frame between exons 2 and 3 (Buszczak et al., 2007). Spt6-PT foci were only detected in cells that were in S phase and contain MPM-2 foci and Cyclin E/Cdk2 activity (Figure 3.3D). Cells that are in G1 phase and have low histone gene transcription lacked Spt6 foci (Figure 3.3D). This localization pattern is consistent with the idea that Spt6 co-localizes with HLBs only when histone gene transcription occurs or Cyclin E/CDK2 is active. Observations made in post-blastoderm embryos further support this idea. At this stage, nascent histone transcripts are detected in cells in late G2 in anticipation of the upcoming S phase that occurs immediately after mitosis (Lanzotti et al., 2004). Prominent Spt6 foci were visible in late G2 cells poised to enter mitosis; however, Spt6 foci were undetectable in early G2 cells (Figure 3.2F). Because these cells contain MPM-2 foci and Cyclin E/Cdk2 activity, this data suggests that Cyclin E/Cdk2 activity is not sufficient for Spt6 localization to

HLBs. Rather, Spt6 co-localization with HLBs may depend on histone gene transcription.

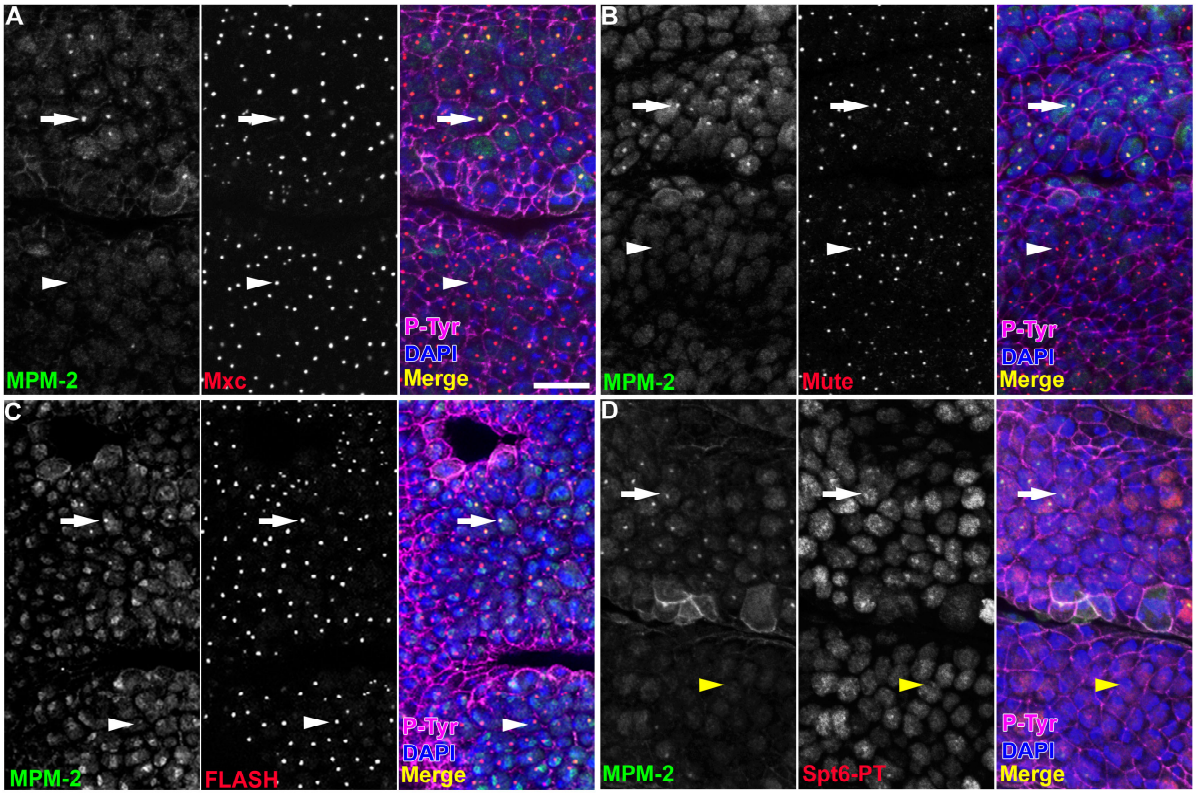


Figure 3.3: Spt6 co-localization with HLBs is cell cycle-dependent. Stage 12 w^{1118} embryos were stained with MPM-2 (green in merges), α-P-Tyr (magenta in merges), DAPI (blue in merges), and α-Mxc in (A) or α-Mute-LS in (B) or α-FLASH in (C) (red in merges). (D) Stage 12 *Spt6-PT* embryo were stained with MPM-2 (green in merge), α-GFP to detect the Spt6 protein trap fusion protein (red in merge), α-P-Tyr (magenta in merge), and DAPI (blue in merge). Arrows indicate S_{17} cells containing MPM-2 foci that co-localizes with Mxc (A), Mute-LS (B), FLASH (C), and Spt6-PT (D). White arrowheads indicate $G1_{17}$ cells lacking MPM-2 foci, but contain Mxc (A), Mute-LS (B), and FLASH (C) in the HLB. Yellow arrowheads indicate a $G1_{17}$ cell that lacks foci of MPM-2 and Spt6-PT. Anterior is to the top and ventral is to the left. Thoracic segments 1 and 2 are shown. Bar: 10 μm.

A core HLB remains assembled during mitosis

We reported previously that during the post-blastoderm cell divisions, foci of MPM-2 and Lsm11 disassemble at the metaphase-anaphase transition and reassemble in the following interphase (White et al., 2007), similar to the behavior of components of mammalian HLBs such as NPAT in fibroblast cell lines (Ma et al., 2000). To determine whether FLASH, Mxc, Mute, and Spt6 undergo the same disassembly-assembly cycle, we stained embryos entering mitosis of cell cycle 14 with anti-FLASH, anti-Mxc, anti-Mute-LS, or anti-GFP (to detect Spt6-PT), and anti-phospho-histone H3 antibody (PH3) to mark mitotic cells (Figure 3.4). During this embryonic stage, it is is possible to examine all stages of mitosis, because cells within a "mitotic domain" enter mitosis asynchronously. Surprisingly, both FLASH and Mxc form foci that are present in $G2_{14}$ and remained assembled throughout all phases of mitosis, even in telophase (Figure 3.4A and B). This behavior is different from that of other nuclear bodies, such as nucleoli and human HLBs, which disassemble at the metaphase-anaphase transition (Hernandez-Verdun, 2006; Ma et al., 2000; Zhao et al., 2000). In contrast, Mute foci follow a similar pattern to Lsm11 and MPM-2 localization, present in prophase and some metaphase cells, and undetectable in anaphase cells (Figure 3.4C). Spt6 foci were observed in prophase cells, but not in metaphase cells (Figure 3.4D). Even metaphase cells containing MPM-2 foci lacked Spt6 foci (Figure 3.4D and 3.2F). Loss of Spt6, Mute, Lsm11, and MPM-2 staining during mitosis correlates with the abortion of nascent histone transcripts (Shermoen and O'Farrell, 1991).

In sum, as cells enter mitosis Spt6 first disengages from HLBs before metaphase, followed by Mute and Lsm11 (U7 snRNP particles), and the disappearance of the MPM-

2 epitope during anaphase. Mxc and FLASH remain closely associated throughout anaphase and telophase, perhaps facilitating the rapid reassembly of HLBs in the following S phase.

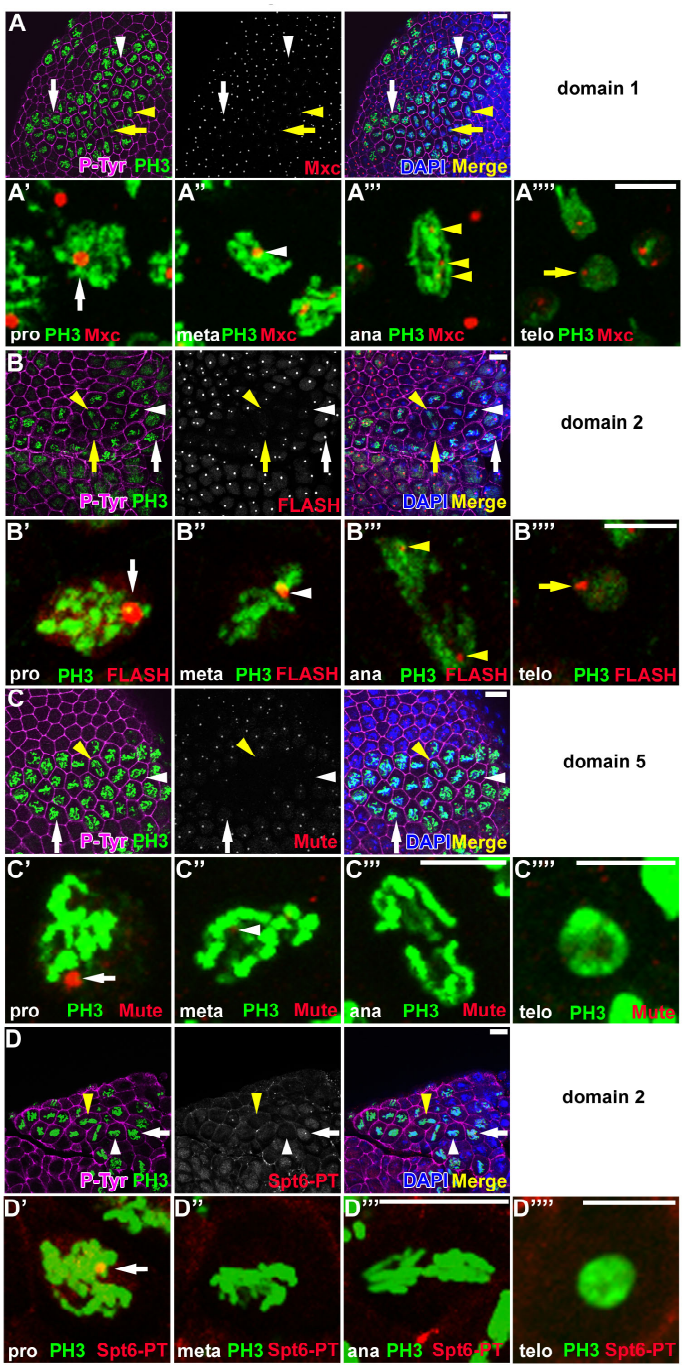


Figure 3.4: A core HLB remains assembled during mitosis. Post-blastoderm w^{1118} embryos in cell cycle 14 were stained with α-P-Tyr (magenta), α-PH3 (green), DAPI (blue) and α-Mxc (A), α-FLASH (B), α-

Mute (C), or α-GFP (D) (middle panels, red in merge). White arrows indicate prophase cells, white arrowheads indicate metaphase cells, yellow arrowheads indicate anaphase cells, and yellow arrows indicate telophase cells. Bar: 10 μm. Single prophase, metaphase, anaphase, and telophase cells are shown in (A'), (B'), (C') and (D'), (A''), (B''), (C''), and (D''), (A'''), (B'''), (C'''), and (D'''), and (A''''), (B''''), (C''''), and (D'''') respectively. Mitotic domains showns are 1 in (A), 2 in (B), 5 in (C), and 2 in (D). Note that the mouse α-PH3 used in (B) stains interphase cells weakly. Bars: 5 μm.

HLB assembly is ordered in the early embryo

During the early syncytial cycles MPM-2 and Lsm11 foci are first detected during nuclear cycle 11 (White et al., 2007), which is precisely when a high rate of zygotic histone gene expression begins (Edgar and Schubiger, 1986). To determine whether there is an order of assembly in which newly identified components of HLBs first assemble at the histone genes, we carefully analyzed syncytial blastoderm embryos stained with MPM-2, anti-FLASH, anti-Mxc, and anti-Mute-LS antibodies. Nuclear density was used to accurately stage the embryos with respect to each nuclear cycle. We hypothesized that the newly identified HLB components would first assemble into HLBs concomitantly with Lsm11 and the MPM-2 antigen. Unexpectedly, we first observed foci of FLASH and Mxc that co-localize in nuclear cycle 10 and when no obvious foci of MPM-2, Lsm11, or Mute-LS were detected (Figures 3.5 and 3.6). Interestingly, it is during nuclear cycle 10 that competence for transcriptional activation is first acquired (Edgar and Schubiger, 1986). This raises the possibility that the localization of Mxc and FLASH to HLBs might initiate histone gene transcriptional competence. In contrast, Mute-LS foci first appear during nuclear cycle 11 and co-localize with MPM-2 foci (Figure 3.6), suggesting that HLB assembly occurs in a stepwise fashion. Surprisingly in

cycle 10, pre-HLBs composed of FLASH and Mxc lack the MPM-2 epitope even though Cyclin E/Cdk2 is active at this time.

Taken together, these results suggest that HLB assembly is ordered during early development, beginning in nuclear cycle 10 with Mxc and FLASH before zygotic histone gene transcription begins. Concomitantly with histone gene transcription, the MPM-2 epitope is formed and Mute-LS and the U7snRNP are incorporated into HLBs.

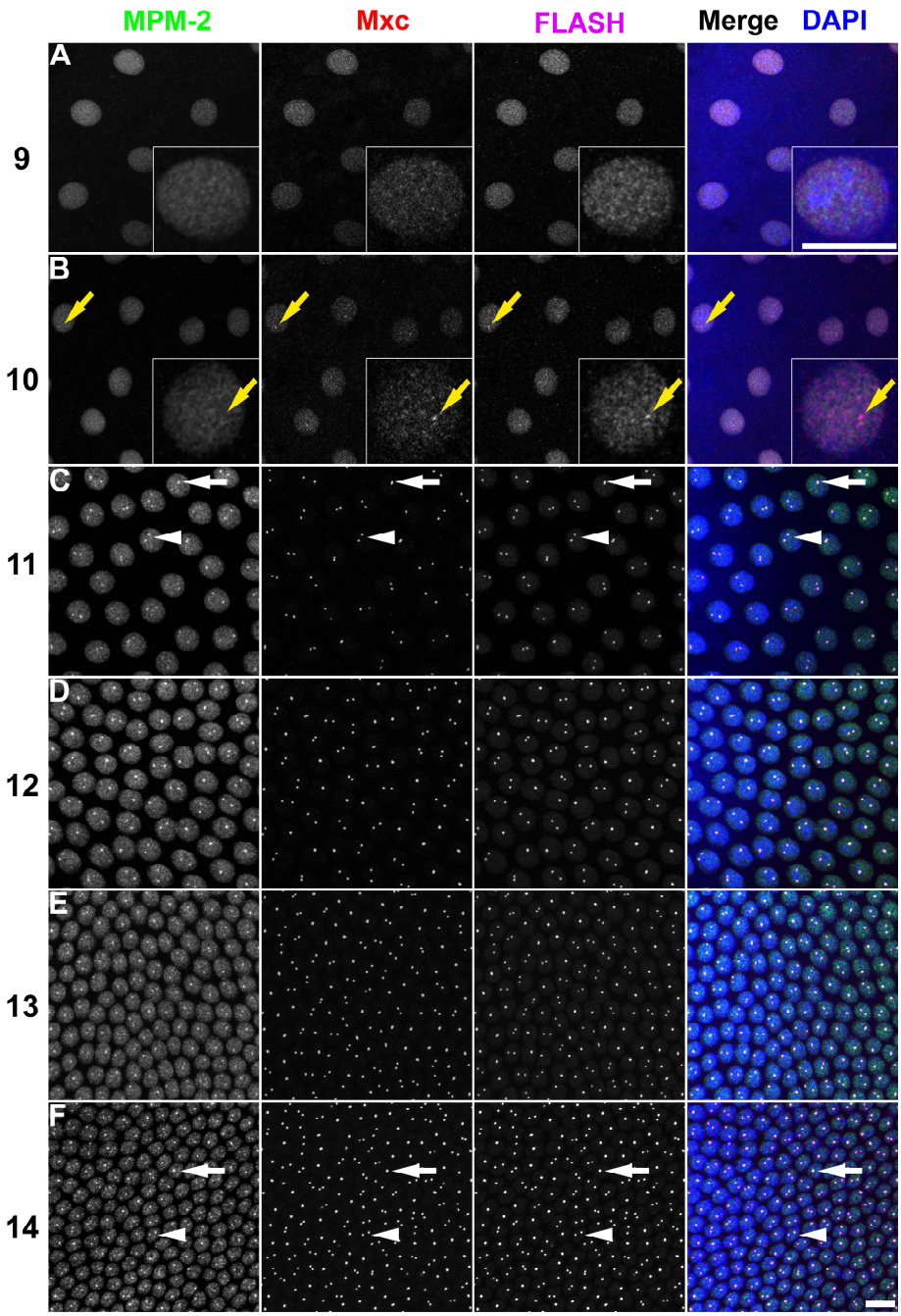


Figure 3.5: A core histone locus body begins assembling during nuclear cycle 10. w^{1118} syncytial blastoderm embryos were stained with MPM-2 (left panels, green in merge), α-Mxc (middle panels, red in merge), α-FLASH (right panels, magenta in merge), and DAPI (blue in merge). Interphase of nuclear cycles 9 to 14 are shown, as indicated on the left. Yellow arrows indicate a nucleus containing foci of Mxc and FLASH and lacking a MPM-2 focus. A nucleus with one focus (white arrows) and a nucleus with two foci (white arrowheads) of all three HLB markers are indicated. Bar: 10 μm.

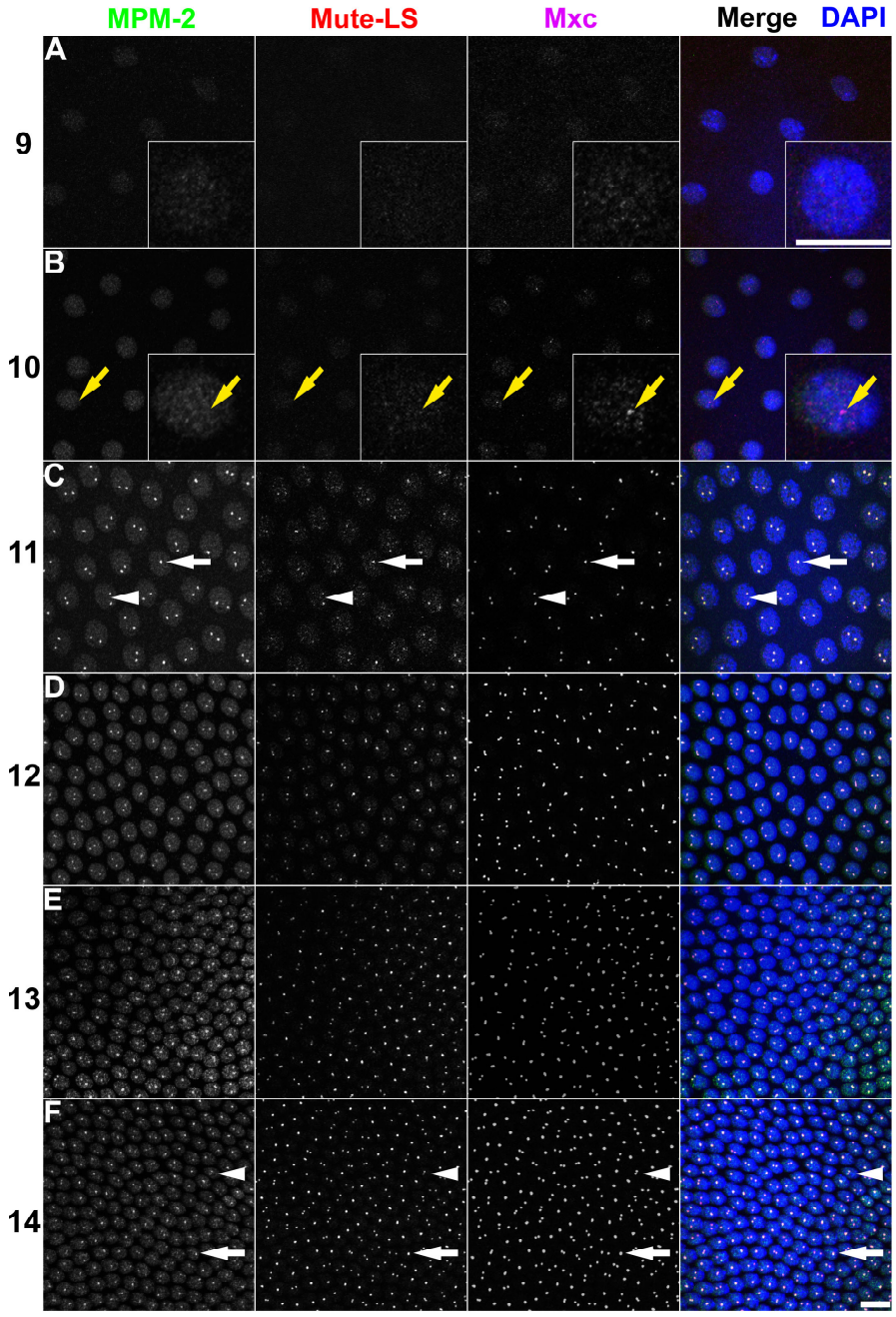


Figure 3.6: Histone locus bodies fully assemble during nuclear cycle 11. w^{1118} syncytial blastoderm embryos were stained with MPM-2 (left panels, green in merge), α-Mute-LS (middle panels, red in merge), α-Mxc (right panels, magenta in merge), and DAPI (blue in merge). Interphase of nuclear cycles 9 to 14 are shown, as indicated on the left. Yellow arrows indicate a nucleus containing a focus of Mxc and lacking MPM-2 and Mute foci. A nucleus with one focus (white arrows) and a nucleus with two foci (white arrowheads) of all three HLB markers are indicated. Bar: 10 μm.

Four HLB proteins co-immunoprecipitate with MPM-2, and Mxc is MPM-2 reactive

Because FLASH, Mxc, Mute, and Spt6 are all components of HLBs, we hypothesized that one might be a MPM-2 antigen. To first determine whether these factors can interact with MPM-2, either directly or indirectly, we immunoprecipitated (IPed) proteins from an S-phase arrested S2 nuclear extract with MPM-2, divided the IP into three samples to run on a SDS-PAGE gel, and each sample was blotted with Mxc, Mute, or Spt6 antibodies. MPM-2 co-IPed Mxc, Mute (both long and short forms), and Spt6 (Figure 3.7A-D). The amount of Mxc, Mute, and Spt6 that co-IPed with MPM-2 was greatly reduced when the nuclear extract was pre-treated with lambda phosphatase (Figure 3.7A-C), suggesting that the MPM-2 co-IPs depend on phosphorylation events. In contrast, we were unable to detect FLASH in MPM-2 IPs, even though were able to detect Spt6 in these IPs (Figure 3.7D).

We next assayed whether the proteins that co-IP with MPM-2 are directly recognized by MPM-2. Each protein was IPed from S-phase arrested S2 nuclear extracts and blotted with MPM-2. For Mxc, we overexpressed a C-terminal Venus- or FLAG-tagged fusion protein in S2 cells, and IPed Mxc-Venus and Mxc-FLAG using anti-GFP and anti-FLAG antibodies, respectively. Both tagged forms of Mxc co-IPed with MPM-2, just like endogenous Mxc (data not shown). MPM-2 recognized a band running at the same molecular weight as full-length Mxc-Venus as well as an additional band running immediately below the full-length protein, which may be a degradation product (Figure 3.7E, top panels). To further confirm the MPM-2 reactivity of Mxc-Venus, this MPM-2 blot was stripped and reprobed with anti-GFP antibodies, which recognized the same bands as MPM-2 (data not shown). Similar results were obtained for Mxc-FLAG (not

shown). Other faster migrating bands are detected in our Mxc IPs with both anti-GFP and anti-FLAG antibodies, making it likely that these bands are fragments of Mxc containing the FLAG or Venus tag and hence the C-terminal portion of Mxc. The fact that the Mxc fragments running faster than ~200 kD are not MPM-2 reactive, suggests that the MPM-2 epitope is probably near the N-terminus of Mxc (Figure 3.7E, bottom panels).

We also tested whether Mute-Long and Mute-Short are MPM-2 reactive. Because Mute-Short shares identical sequences to Mute-L, we tagged Mute-S with Venus at the N-terminus or FLAG at the C-terminus to be able to IP and detect Mute-S independently of Mute-L. To assess whether Mute-S is MPM-2 reactive, we overexpressed Venus-Mute-S in S2 cells, IPed it with anti-GFP antibodies, and blotted with MPM-2. By this method, we failed to detect any MPM-2 reactivity of Mute-S (Figure 3.7F). We also tested whether Mute-L is MPM-2 reactive by performing IPs using anti-Mute-L antibodies from cells overexpressing Mute-S-FLAG (Figure 3.7G). In these experiments, we did not detect any MPM-2 reactive bands at the same size as full-length Mute-L, however, some smaller bands were present in the MPM-2 Western that are specific to the Mute-L IP and are also present in the Mute-L Western (Figure 3.7G). These bands could represent degradation products of Mute-L that are MPM-2 reactive. Alternately, the faster migrating bands present in the MPM-2 and Mute-L Westerns could be non-specific bands. Further experiments are needed to be able to distinguish between these possibilities. Interestingly, Mute-S-FLAG was detected in the Mute-L IPs using anti-FLAG antibodies. This suggests that the two isoforms of Mute are part of a complex.

To assess whether Spt6 is MPM-2 reactive, we over-expressed a C-terminal FLAG-tagged fusion protein (Spt6-FLAG) in S2 cells and IPed Spt6-FLAG using anti-FLAG antibodies. We never detected any MPM-2 reactive bands from our Spt6-FLAG IPs, despite having IP-ed Spt6-FLAG (Figure 3.7H). It is unlikely that the FLAG-tag interferes with a potential MPM-2 epitope on Spt6, because Spt6-FLAG did co-IP with MPM-2 antibodies (data not shown). Rather, it appears that the MPM-2-Spt6 co-IP is due to an interaction between Spt6 and another protein containing the MPM-2 epitope.

In sum, these data indicate that Mxc and Mute-Short are MPM-2 reactive HLB components, and therefore could be directly regulated by Cyclin E/Cdk2. Spt6 and Mute-Long are not MPM-2 reactive and probably co-IP with MPM-2 because they interact with Mxc, Mute-Short, and/or another MPM-2 reactive component of HLBs.

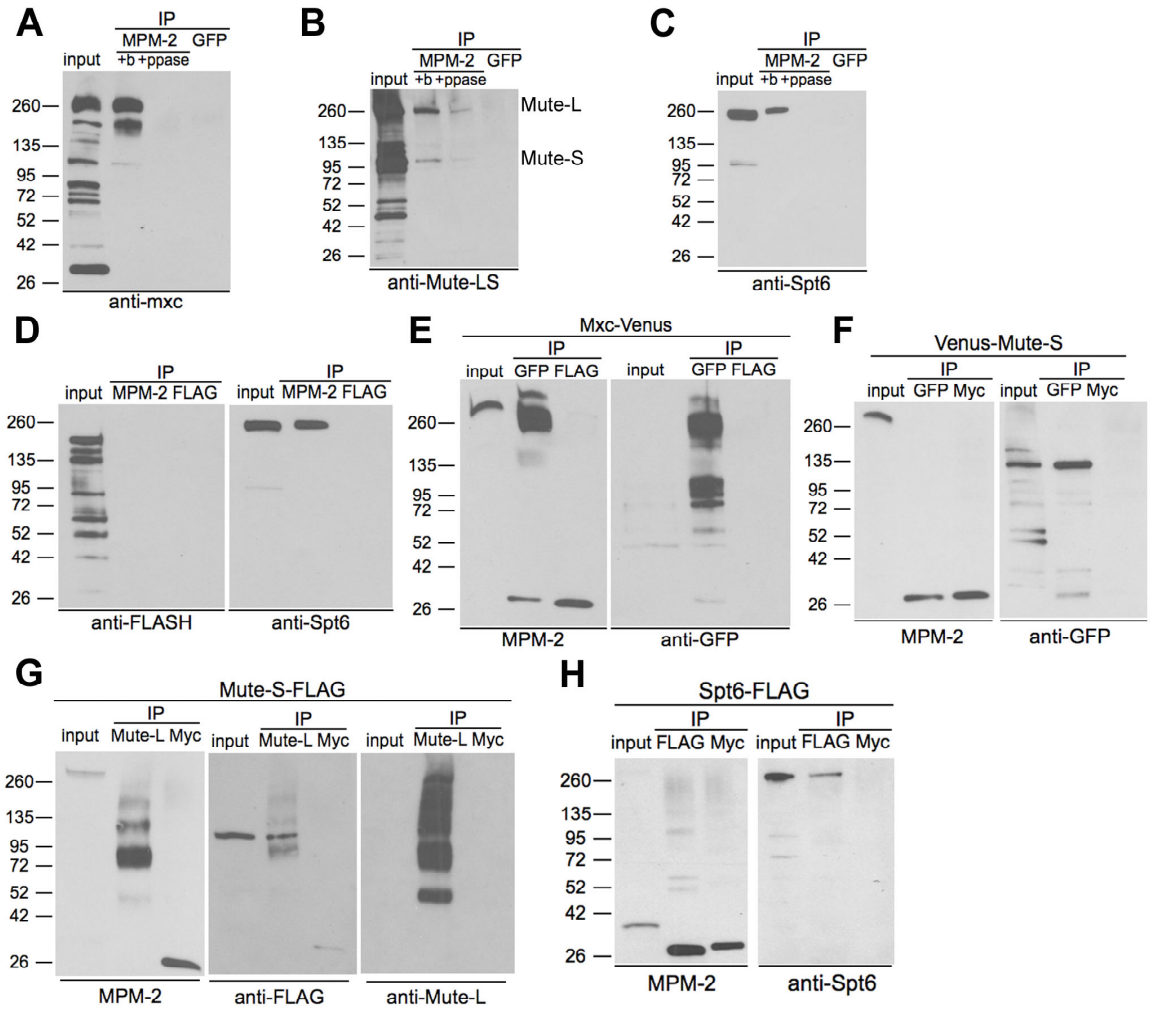


Figure 3.7 Mxc and Mute-S are MPM-2 reactive. (A-C) Nuclear extracts from S-phase arrested S2 cells were pre-treated with phosphatase buffer only (+b) or lambda phosphatase (+ppase) prior immunoprecipitation (IP) with MPM-2. α-GFP IP served as a negative control. The three IPs (+b MPM-2; +ppase MPM-2; GFP) were each divided into three and analyzed by Western blotting with α-Mxc (A), α-Mute-LS (B), or α-Spt6 (C). Note that α-Mute-LS detects both Mute-Long (Mute-L, ~260 kD) and Mute-Short (Mute-S, ~95 kD). (D) Western analysis of proteins precipitated with MPM-2 or α-FLAG (negative control) from an S-phase arrested S2 cell nuclear extract. IPs were split into two and analyzed by Western blotting with either α-FLASH or α-Spt6. (E) Cells stably expressing Mxc-Venus were subjected to IP with α-GFP and α-FLAG (negative control). IPs were divided in two, run on a gel, and blotted with either MPM-2 or α-GFP. (F) Cells stably expressing Venus-Mute-S were subjected to IP with α-GFP and α-Myc (negative control). IPs were divided into two, run on a gel, and blotted with either α-GFP or MPM-2. (G)

Cells stably expressing Mute-S-FLAG were subjected to IP with α-Mute-L and α-Myc (negative control). IPs were divided in three, run on a gel, and blotted with MPM-2, α-FLAG, or α-Mute-L. Note that the bands present in the MPM-2 Western are not the expected size of Mute-S, nor the size of full-length Mute-L, however, smaller MPM-2 reactive bands might represent degradation products of Mute-L. (H) Cells stably expressing Spt6-FLAG were subjected to IP with α-FLAG and α-Myc (negative control). IPs were divided into two, run on a gel, and blotted with α-Spt6 or MPM-2.

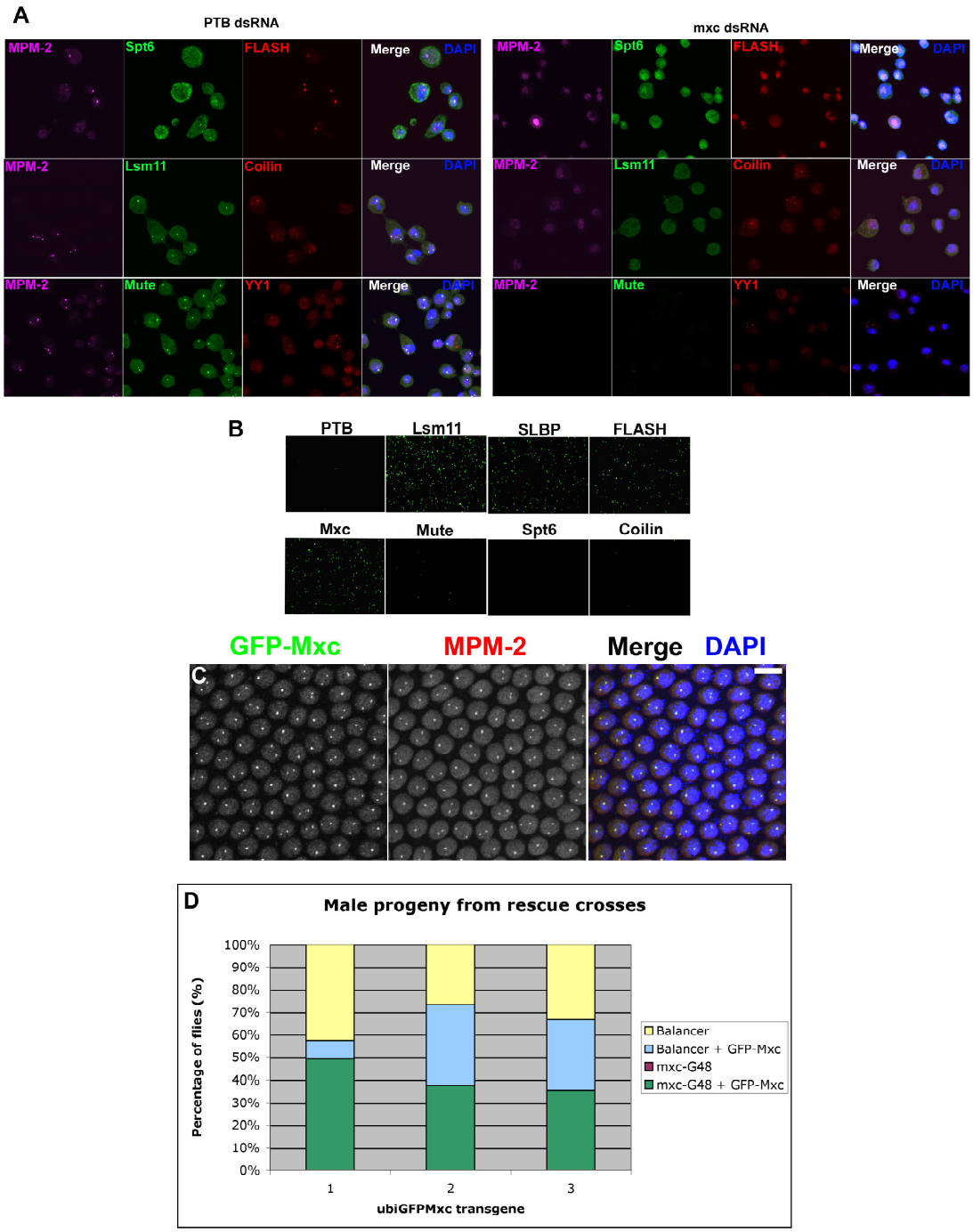
Mxc is a novel regulator of HLB assembly and histone gene expression

Mxc is a core component of HLBs that first assembles into HLBs prior to other factors and remains assembled during all of mitosis. For these reasons, we hypothesized that Mxc might be a structural component required for HLB formation and maintenance. To test this, we knocked down *mxc* expression in S2 cells by RNAi for 5 days, fixed, and stained the cells with MPM-2, anti-Lsm11, anti-FLASH, anti-Mute-LS, anti-Spt6, and anti-Mxc (to assess knockdown efficiency) antibodies. We also examined the localization of Coilin and YY1 in these cells. In *Drosophila*, Coilin is required for Cajal body formation and co-localizes with Cajal Bodies and at varying levels with HLBs, depending on the cell type (Liu et al., 2009). In S2 cells, Coilin co-localizes predominantly with HLBs ((Liu et al., 2009) and Figure 3.8). The transcription factor YY1 (Yin Yang 1) is a polycomb group member that together with its co-activator YY1-associated protein (YY1AP) regulates the expression of many genes involved in proliferation and development, including the histone genes (Castellano et al., 2009; Wang et al., 2004). We examined YY1 localization, because Mute and human YY1AP are 27% identical by BLAST search (S. Bulchand and W. Chia, personal communication). In cells treated with control (*PTB*) dsRNA, we observed foci of each HLB marker, except

for YY1, which displayed varying levels of staining throughout the nucleus. In contrast, foci of all HLB markers except Coilin were absent in *mxc* knockdown cells (Figure 3.8A). This data suggests that Mxc is necessary for proper HLB assembly. We hypothesize that Coilin may co-localize with Cajal bodies in *mxc* knockdown cells.

Because Mxc is necessary for proper HLB assembly, we hypothesized that it may also be required for efficient histone pre-mRNA processing *in vivo*. Cells defective in histone pre-mRNA processing accumulate long polyadenylated histone mRNAs resulting from the use of cryptic polyadenylation signals situated downstream of the normal histone pre-mRNA processing site (Lanzotti et al., 2002; Sullivan et al., 2001). A histone misprocessing reporter transgene has been developed that utilizes a fragment of the *histone H3* gene containing all necessary histone processing sequences and GFP sequences located after the normal histone processing site (Wagner et al., 2007; Yang et al., 2009). Thus, cells only express GFP from this reporter when histone mRNAs are misprocessed. To evaluate whether Mxc is needed for histone pre-mRNA processing, we knocked down *mxc* expression in Dmel-2 cells stably expressing the histone GFP reporter expressed under the *actin5C* promoter. Using this reporter, we find that *mxc* knockdown cells strongly misprocess histone mRNAs when qualitatively scored using the same classification scale that was used in a screen done searching for histone pre-mRNA processing factors (Figure 3.8B and Table 3.3) (Wagner et al., 2007). Reduction in *FLASH* expression scored more strongly than *mxc*, consistent with its role as a histone pre-mRNA processing factor (Yang et al., 2009). In contrast, reduction in either Mute or Spt6 expression did not score with the reporter (Figure 3.8B).

The *mxc* gene is a member of the polycomb group and mutant alleles cause homeotic transformations that mimic the ectopic gain of function of *BX-C* and *ANT-C* genes (Saget et al., 1998; Santamaria and Randsholt, 1995). In addition, some *mxc* mutants have a grandchildless phenotype, which is caused by mutant mothers that lay eggs lacking germ cells. Because mutations in the classic *mxc* alleles were recently re-annotated from the *CG12058* locus to the *CG12124* locus (which we have called *mxc* throughout this chapter), both located on the X chromosome, we wished to genetically confirm that mutations contained in the *mxc* alleles map to *CG12124*. We generated flies expressing a transgene containing GFP-tagged Mxc under the control of the ubiquitin promoter on the third chromosome (*ubiGFPmxc*). GFP-Mxc is expressed in the early syncytial embryo and co-localizes with MPM-2 at HLBs (Figure 3.8C). The mxc^{G48} allele was created by ethyl methanesulfonate (EMS) treatment and is lethal by larval stages (Saget et al., 1998; Santamaria and Randsholt, 1995). To confirm that *CG12124* is disrupted in these mutants, we crossed male flies carrying our transgene to mxc^{G48} females to determine whether it could rescue the lethality. The only mxc^{G48} males that eclosed were brown in body color indicating that they carry the chromosome containing the *ubiGFPmxc* transgene, which also contains the gene necessary to produce brown body color. mxc^{G48} males without the *ubiGFPmxc* transgene would be yellow in color and we did not see any flies from this class. Thus, the lethality caused by mxc^{G48} is rescued by GFP-Mxc (Figure 3.8D).



3.8: Mxc is essential for HLB assembly and function. (A) S2 cells were treated with *PTB* (negative control) and *mxc* dsRNAs for 5 days, fixed, and stained with MPM-2 (magenta in merge), α-Spt6 (green in top panel merge), α-FLASH (red in top panel merge), α-Lsm11 (green in middle panel merge), α-Coilin (red in middle panel merge), α-Mute-LS (green in bottom panel merge), α-YY1 (red in bottom panel merge), and DAPI (blue in merge). Note that only Coilin foci are detected in cells treated with *mxc*

dsRNAs. (B) GFP histone mis-processing assay. *PTB* and *Coilin* dsRNAs are negative controls, and *Lsm11* and *SLBP* dsRNAs are positive controls. (C) Syncytial blastoderm (cycle 12) embryos expressing GFP-Mxc were fixed and stained with α-GFP (green in merge), MPM-2 (red in merge), and DAPI (blue in merge). Bar: 10 μm. (D) Graph of mxc^{G48} rescue crosses. Three different GFP-Mxc insertions are shown.

Secondary screen exploring inter-dependencies of HLB components

To validate our primary screen and extend our understanding of the interrelationships between HLB components, we performed secondary screens on our top 95 candidate genes (see Materials and Methods for selection criteria). S2 cells were plated and arrested in S phase in the same manner as in the primary screen, except that cells were stained with anti-Mxc, and anti-FLASH antibodies, as well as MPM-2 and DAPI. We also plated Dmel-2 cells stably expressing the histone pre-mRNA processing GFP reporter (Wagner et al., 2007; Yang et al., 2009) to determine which of these candidate genes are required for correct histone pre-mRNA processing.

The secondary screens validated 15 genes when reduced in expression cause a severe to moderate reduction in the MPM-2 index as compared to control no dsRNA and *SK* treatments (Table 3.2). Fifteen other genes when knocked down caused a slight decrease in the MPM-2 index. We also identified genes required for the correct localization of Mxc and FLASH (Table 3.2). Loss of *mxc* expression severely disrupted the localization of FLASH and the MPM-2 epitope. In contrast, *FLASH* knockdown caused only a slight reduction in the Mxc index. These results suggest that FLASH may have a greater dependency on Mxc for HLB localization than Mxc on FLASH. Interestingly, *MBD-R2* knockdown caused a slightly lowered MPM-2 index, but a moderately reduced FLASH index, perhaps having a greater effect on FLASH because of

its function in histone pre-mRNA processing. *Pointed* (*pnt*) knockdown caused a slight reduction of MPM-2 and Mxc indices, and a moderate reduction in the FLASH index.

In the histone pre-mRNA processing GFP reporter assay, we found that loss of *FLASH* expression caused a very strong histone misprocessing phenotype (Table 3.3 and Figure 3.8B, and (Yang et al., 2009). *Mcrs1* and *MBD-R2* scored strongly as previously reported (Wagner et al., 2007), and so did *mxc* (Table 3.3 and Figure 3.8B). Curiously, reduced expression of *Dim γ-tubulin 1* (*Dgt1*), a factor required for proper recruitment of γ-tubulin to centrosomes and assembly of the mitotic spindle (Goshima et al., 2007), also scored strongly with the histone pre-mRNA processing GFP reporter. Interestingly, Dgt1 occasionally co-localizes with MBD-R2 (Rcd5) (N. Rusan, personal communication). We also obtained 6 new genes that scored moderately and 6 new genes that score weakly with the histone pre-mRNA processing GFP reporter (Table 3.3).

Table 3.2: Gene products required for HLB localization in order of necessity.

Marker	Severe	Moderate	Slight
MPM-2	*Int6* *CG9769* *Mxc* *CSN4* *FLASH*	*CSN5* *CG18591* *CSN3* *CG4849* *RpII18* *CG7597* *CG11985* *Nnp-1* *eIF-3p66* *alien*	*CycK* *pnt* *Tbp-1* *mge* *CG11092* *MBD-R2* *CG13716* *CG5844* *raptor* *Arf102F* *l(1)G0095* *emb* *CG9121* *CG5859* *Peb*
Mxc			*pnt* *FLASH* *Tbp-1*
FLASH	*mxc*	*MBD-R2* *pnt*	*CG4849*

		CG18591	

Table 3.3: Classification of histone pre-mRNA processing GFP reporter hits based on qualitative strength.

Category	Gene
5 (very strong)	*FLASH*
4 (strong)	*Mcrs1* *MBD-R2* *mxc* *Dgt1*
3 (medium)	*CG9772* *CG31111* *CG8142* *DMAP1* *CSN5* *CG18591*
2 (weak)	*RpII18* *Trf2* *CG7597* *CG18787/18789* *CG42249* *CSN4*

Discussion

We performed genome-wide RNAi and biochemical screens in S2 cells aimed at identifying Cyclin E/Cdk2-regulated MPM-2 antigen(s) part of HLBs. Both screens identified factors that regulate HLB dynamics and histone mRNA biosynthesis, including at least one MPM-2 antigen, Mxc. Mxc is necessary for HLB assembly and histone pre-mRNA processing in S2 cells. We propose that Mxc provides the first direct connection between Cyclin E/Cdk2 and histone gene expression in *Drosophila.*

HLB assembly during early Drosophila embryogenesis

Our analysis of HLB markers during early embryogenesis favors a model of ordered assembly rather than random aggregation for HLBs. In interphase 10, we first observe co-localizing foci of Mxc and the histone pre-mRNA processing factor FLASH

that are smaller than in later cycles. During nuclear cycle 11, foci of Mxc and FLASH become more prominent and Mute is detected in HLBs for the first time. This is coincident with the appearance of MPM-2 and Lsm11 foci (White et al., 2007) and the appearance of zygotic histone transcripts (Edgar and Schubiger, 1986). Foci of all of these HLB components become increasingly robust from nuclear cycle 11 to 12, which is when transcriptional activation of the histone genes reaches maximal levels (Edgar and Schubiger, 1986).

Competence for transcriptional activation for all genes is first acquired during nuclear cycle 10 (Edgar and Schubiger, 1986). Remarkably, foci of Mxc and FLASH are first detected in nuclear cycle 10. It is tempting to speculate that these two HLB components might be necessary and perhaps sufficient for histone gene transcriptional competence. Because Mxc is required for FLASH localization and Mxc can localize to foci independently of FLASH, we favor a model whereby Mxc recruits FLASH to form the early HLB in nuclear cycle 10, which in turn recruits additional components (including Mute and U7snRNP) forming a fully assembled and functional HLB by nuclear cycle 11. An attractive possible signal for the transition from an early HLB to a mature HLB is phosphorylation of Mxc by Cyclin E/Cdk2, because we first detect MPM-2 foci in nuclear cycle 11.

HLB dynamics during mitosis

Our analysis of HLB composition during mitosis provided surprising results. Both nucleoli and human HLBs have been shown to completely disassemble at the metaphase-anaphase transition (Hernandez-Verdun, 2006; Ma et al., 2000; Zhao et al.,

2000). Therefore, we expected the same to be true for *Drosophila* HLBs, especially because this behavior was observed for Lsm11 and MPM-2 (White et al., 2007). Whereas Mute and Spt6 disassembled by anaphase, both Mxc and FLASH remained in chromatin-associated foci all throughout mitosis. Interestingly, we observed anaphase cells containing 3 or 4 small foci of Mxc and FLASH. Typically only 1 or 2 HLBs are observed per diploid nucleus in a manner that is consistent with homologous chromosome pairing. Three to four foci were occasionally observed when performing FISH to the histone genes (unpublished results). Thus, we speculate that 3 or 4 foci of Mxc and FLASH represent separate sister chromatids and that these factors may directly associate with the histone genes as part of their role as a core HLB. By remaining tightly associated with the histone genes, Mxc and FLASH could facilitate the rapid assembly of a mature HLB in the subsequent S phase. This mechanism would be especially important during post-blastoderm divisions during which there is no G1 phase.

The transcription elongation factor Spt6 is the earliest known component to leave HLBs during mitosis. We hypothesize that the departure of Spt6, Mute, Lsm11, and MPM-2 epitope(s) from HLBs during mitosis coincides with the timing of abortion of nascent histone transcripts (Shermoen and O'Farrell, 1991). Spt6 is also absent from HLBs in cells in G1 that have down-regulated Cyclin E/Cdk2 activity and histone gene transcription, whereas Mute and Lsm11 are present. In post-blastoderm embryos, we observe prominent foci of Spt6 in cells in late G2 that have been shown to be actively transcribing histone genes (Lanzotti et al., 2004). Late G2 cells contain MPM-2 foci and Cyclin E/Cdk2 activity; therefore, we conclude that Spt6 localization does not only depend on Cyclin E/Cdk2 activity, but also on histone gene transcription.

Identification of a Drosophila S phase MPM-2 antigen

MPM-2 has been used as a marker of Cyclin E/Cdk2 activity in *Drosophila* cells for over a decade (Calvi et al., 1998; Narbonne-Reveau and Lilly, 2009; Read et al., 2009; Sun et al., 2008; Zielke et al., 2008). Nevertheless, the identity of the MPM-2 antigens forming nuclear foci in S phase cells has remained elusive. We report that one of these antigens is Mxc, which thus represents a potential novel Cyclin E/Cdk2 substrate. The fact that Mxc contains similar sequences to human NPAT makes it a very attractive candidate for an NPAT homolog. Both Mxc and NPAT are large proteins and contain a LisH domain in their N-terminus, which in NPAT is necessary for H4 transcription (Wei et al., 2003). *NPAT* expression is regulated by the transcription factor E2F (Gao et al., 2003). There is emerging evidence suggesting that the *mxc* gene may also be an E2F target, because in a ChIP assay using two different anti-E2F antibodies,

sequences located just upstream of *mxc* were pulled down (M. Korenjak and N. Dyson, personal communication).

MPM-2 co-immunoprecipitates Spt6, both long and short forms of Mute, and Mxc. Western blot analysis revealed that Mxc is MPM-2 reactive. These results along with immunofluorescence data suggest that Mxc, Mute, and Spt6 could form a complex, possibly held together by Mxc. Alternately, Mute and Spt6 may independently interact with MPM-2 reactive protein(s).

Mxc is a novel regulator of histone mRNA biosynthesis

We propose that Mxc is a core component of HLBs, because it is one of the first markers detected at HLBs in early embryogenesis and remains assembled during mitosis and in G1 cells having low Cyclin E/Cdk2 activity. In S2 cells, Mxc is necessary for the correct localization of all HLB components we tested. Moreover, Mxc is required for correct histone pre-mRNA processing *in vivo*, such that reduced *mxc* expression causes the accumulation of inappropriately polyadenylated histone GFP reporter transcripts.

Mxc regulates histone mRNA biosynthesis by mediating both HLB assembly and histone pre-mRNA processing. It is also possible that Mxc stimulates histone gene transcription, at least as part of its role in promoting HLB assembly. A dual role in histone transcription and pre-mRNA processing has been recently proposed for NPAT (Pirngruber and Johnsen, 2010). DNA damage induces the disassembly of NPAT and FLASH positive HLBs that are associated with a down-regulation of histone expression (Bongiorno-Borbone et al., 2010). This type of regulation is consistent with a model in which NPAT and FLASH coordinate HLB assembly, and Cyclin E then stimulates the initiation of histone mRNA biosynthesis.

Summary

Through genome-wide RNAi and biochemical screens in S2 cells, we identified factors regulating HLB dynamics and histone mRNA biosynthesis. This includes one MPM-2 antigen, Mxc. We showed that Mxc is necessary for both HLB assembly and histone pre-mRNA processing in S2 cells. We propose that Mxc provides the first direct connection between Cyclin E/Cdk2 and histone pre-mRNA processing in *Drosophila.* Understanding how Mxc regulates histone mRNA biosynthesis and polycomb gene function will likely reveal interesting clues about how proliferation is coordinated during development.

Acknowledgements

We are grateful to Sarada Bulchand for her generous sharing of reagents, especially anti-Mute antibodies, and for unpublished information. We are also grateful to Nasser Rusan and Greg Rogers who made the genome-wide screen possible with their gift of S2 cells plated to the dsRNA library. We thank Xiao-cui Yang for making and generously sharing anti-Mxc and anti-FLASH antibodies, and Brandon Burch for assistance with the histone pre-mRNA processing GFP reporter assays. This work was supported by CIHR grant MDR-85476 to A.E.W. and NIH grant 5-52141 to R.J.D.

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CHAPTER IV

DISCUSSION AND FUTURE DIRECTIONS

Insight into the mechanisms controlling gene expression will increase our understanding of how cellular identity is specified. Genome function is regulated by the organization of the nucleus into nuclear bodies, which mediate interactions between proteins regulating gene expression and chromatin (Matera et al., 2009; Misteli, 2007). Work described in this dissertation explored the relationship between histone locus bodies (HLBs) and histone gene expression. Specifically, it focused on how Cyclin E/Cdk2 stimulates replication-dependent histone mRNA biosynthesis during S phase. Using MPM-2 as a tool in *Drosophila*, we have shown that a component of HLBs contains a phospho-epitope that is dependent on Cyclin E/Cdk2 activity (Chapter II). MPM-2 epitopes first appear precisely when zygotic histone gene transcription begins during nuclear cycle 11. Intriguingly, MPM-2 antigens along with U7 snRNP particles can assemble HLBs independently of the histone genes. Based on this work, we hypothesize that MPM-2 detects a Cyclin E/Cdk2 substrate and HLB component regulating histone mRNA biosynthesis. We next sought to identify the MPM-2 antigen in order to better understand the mechanisms controlling histone mRNA biosynthesis. Performing genome-wide RNAi and biochemical screens, we identified 4 HLB factors as well as 30 putative regulators of HLB dynamics (Chapter III). Notably, we identified Mxc as a novel MPM-2 target and regulator of histone pre-mRNA processing and

HLB assembly. Based on our study, we conclude that the cell cycle regulation of nuclear organization is a critical mechanism modulating histone mRNA biosynthesis.

Cell cycle regulation of HLB dynamics

Cyclin E/Cdk2 activity was previously shown to be required for histone mRNA biosynthesis in *Drosophila*, because histone mRNAs are greatly depleted in late stage *cyclin E* mutant embryos (Lanzotti et al., 2004). We show that Cyclin E/Cdk2 activity is necessary for the appearance of nuclear MPM-2 foci co-localizing with HLBs. Therefore, we hypothesized that Cyclin E/Cdk2 directly regulates histone mRNA biosynthesis. Further analysis of the localization of newly found HLB components in G1 cells revealed two classes of HLB components: cell cycle-dependent and cell cycle-independent. Whereas Mxc, Mute, FLASH, and U7 snRNP particles remain assembled in HLBs in G1 arrested cells of the embryo, Spt6 cannot be detected at HLBs in cells lacking Cyclin E/Cdk2 activity. This result raised the question of whether Spt6 co-localization with HLBs depends on Cyclin E/Cdk2 activity or is a secondary consequence of low histone gene transcription. We favor the interpretation that Spt6 co-localization with HLBs is a consequence of histone gene transcription. However, based on our analysis it is impossible to distinguish between whether Spt6 is actively or passively recruited. This question might be better addressed by utilizing histone deletion mutant embryos, which lack histone gene transcription and have unperturbed Cyclin E/Cdk2 activity. If Spt6 localizes to HLBs in mutant embryos lacking the histone genes, then perhaps Cyclin E/Cdk2 promotes the assembly of a complex containing transcription initiation and elongation factors even in the absence of a DNA template.

The cell cycle regulation of nuclear body dynamics is a general feature of mammalian nuclear organization (Matera et al., 2009). In human cells, HLBs and nucleoli disassemble during mitosis and reform in the subsequent interphase (Hernandez-Verdun, 2006; Ma et al., 2000; Zhao et al., 2000). We therefore expected *Drosophila* HLBs to behave similarly during mitosis. Instead, we observed that not all HLB components disassemble in mitosis. Spt6 disassembles first during metaphase, followed by the MPM-2 epitope(s), Mute, and U7 snRNP at the metaphase-anaphase transition. In contrast, foci of FLASH and Mxc are present throughout mitosis in anaphase and telophase cells. We speculate that the foci of FLASH and Mxc remaining in late mitosis might accelerate HLB assembly during S phase in the post-blastoderm cell cycles lacking a G1 phase. Whether HLB dynamics are the same in cells undergoing the G1-S-G2-M cell division cycle and that only express Cyclin E/Cdk2 activity in S phase is not known. This question could be addressed with the constitutively expressing GFP-Mxc (*ubiGFPmxc*) fly line that we generated. Mxc localization could be imaged in live *ubiGFPmxc* larval imaginal tissues and brains, which undergo G1-S-G2-M cell divisions. Wild-type and *ubiGFPmxc* larval imaginal tissues and brains could also be fixed and stained with the HLB markers recognizing FLASH, Mxc, Mute, and Spt6. Filming GFP-Mxc throughout embryogenesis would give a more complete picture of HLB dynamics, because only fixed tissues have been examined and provide static images of development. Another method to explore HLB dynamics during the canonical cell cycle would be to image S2 cells expressing the *mxc::Venus* transgene. Other HLB factors could be similarly tagged and their localization imaged in live cells. For example, we

already have made constructs that constitutively express Venus-tagged versions of Mute-Short.

HLB assembly during early *Drosophila* development

Zygotic histone gene transcription begins during nuclear cycle 11, while most other genes start being transcribed in nuclear cycle 14 (Edgar and Schubiger, 1986). Interestingly, all genes first acquire competence for transcriptional activation in nuclear cycle 10 (Edgar and Schubiger, 1986). When we analyzed the localization of HLB components during early embryogenesis, we observed correlations between the appearance of HLB components and these hallmarks of histone gene expression. We first detect small HLBs containing Mxc and FLASH during nuclear cycle 10. Then in nuclear cycle 11, the MPM-2 epitope(s), U7 snRNP particles, and Mute all co-localize with Mxc and FLASH. It is tempting to speculate that Mxc and FLASH might be sufficient for transcriptional competence of the histone genes and that through Cyclin E/Cdk2-mediated phosphorylation of Mxc (appearance of the MPM-2 epitope(s)), the HLB fully assembles precisely when zygotic histone gene transcription begins.

Our analysis raised several unanswered questions. First, is incorporation into HLBs gradual or sudden? Mxc dynamics during the early syncytial cycles could be examined in *ubiGFPmxc* flies and GFP-Mxc fluorescence quantified to determine the mode of incorporation. Second, is Mxc a stable or dynamic component of HLBs? Using fluorescence recovery after photobleaching (FRAP) analysis in *ubiGFPmxc* syncytial embryos, HLBs could be bleached and the kinetics of reincorporation of GFP-Mxc into HLBs studied. Additionally, a single HLB in a nucleus containing two HLBs (unpaired homologs) could be bleached and the fluorescence of each HLB measured to determine

whether components are exchanged in nuclei containing two HLBs. Finally, the nuclear area surrounding the HLB could be bleached and loss of fluorescence in the HLB measured (FLIP).

We reported that *mxc* is necessary for HLB assembly in S2 cells. A significant question remaining is whether *mxc* is also required for HLB assembly in the embryo. The mxc^{G48} allele is late embryonic/early larval lethal (Saget et al., 1998; Santamaria and Randsholt, 1995). Embryos from late developmental stages (12-14) could be fixed and stained with HLB markers to address this question. Another experimental approach would be to generate maternal/zygotic (m/z) *mxc* mutants by homologous recombination in the ovary to examine HLB assembly in the absence of the *mxc* gene product in the early embryo. We hypothesize that m/z *mxc* mutant embryos will lack HLBs and arrest at stage 11, because embryos lacking the histone genes arrest at stage 11 due to a deficiency in histone biosynthesis (Smith et al., 1993).

Foci of Lsm11 and MPM-2 form in histone deletion mutant embryos, however, these foci are smaller and some are aberrant. For example, 8% of cells in histone deletion mutant embryos contained foci of Lsm11 that do not co-localize with MPM-2 (Table 2.1). The localization of other HLB markers, FLASH, Mute, and Mxc, could be examined in mutant embryos lacking the histone genes to determine whether other HLB factors exhibit mislocalization. Another aspect of HLB assembly remaining to be assessed in histone deletion mutants is whether HLBs first form in nuclear cycle 11, and if so whether they are smaller than in wild-type or are they formed at a normal size in nuclear cycle 11 and later become reduced in size. To test this it would be necessary to identify histone deletion mutant embryos earlier in development than what was done in

Chapter II (using Histone H3 *in situ*). Using a DNA FISH probe that recognizes the histone genes, histone deletion mutant embryos could be distinguished from their siblings by the lack of a histone DNA FISH signal. Because nucleolus size is determined by the amount of ribosomal gene transcription (Hernandez-Verdun, 2006), we expect that HLBs formed in histone deletion mutants will first assemble at a normal size and decay from lack of histone gene transcription.

Mxc is a novel regulator of histone mRNA biosynthesis

We provided evidence that Mxc is a novel regulator of HLB dynamics and histone pre-mRNA processing. Whether Mxc directly regulates aspects of histone gene transcription has not yet been shown. In human cells, NPAT is a Cyclin E/Cdk2 substrate that stimulates histone gene transcription (Ma et al., 2000; Wei et al., 2003; Zhao et al., 2000). No ortholog to NPAT has been yet reported in *Drosophila*. A critical structural feature of NPAT is a LisH domain found in its amino terminus, which was shown to be necessary for histone H4 transcription (Wei et al., 2003). The *Drosophila* proteome contains 15 predicted LisH domain-containing proteins, including Mxc. Based on BLAST search comparisons, the LisH domain contained in Mxc is the most similar to NPAT. Thus, we hypothesize that Mxc represents the functional ortholog of NPAT. To test a role in histone gene transcription, *in situs* could be performed on *mxc* mutant embryos with histone H3 probe to determine if histone gene transcription is perturbed. Additionally, *mxc* mutants could be analyzed using a highly sensitive real time PCR assay that has been developed in the Duronio/Marzluff labs to detect changes in histone mRNA levels as well as the presence of inappropriately polyadenylated histone mRNAs.

A requirement for *mxc* in efficient histone pre-mRNA processing can be confirmed *in vivo* by hybridizing *mxc* mutant embryos with the *H3-ds* probe, which detects nascent polyadenylated histone mRNAs (Lanzotti et al., 2004). We expect that the H3-ds probe will detect misprocessed histone transcripts in embryos with declining maternal supplies of Mxc, and histone transcripts will no longer be detected as Mxc becomes sufficiently depleted later in embryogenesis.

We showed that Mxc is MPM-2 reactive and propose that Cyclin E/Cdk2 phosphorylates it. We suggest two methods to assess whether Mxc is a Cyclin E/Cdk2 substrate. An *in vitro* kinase assay would determine whether Mxc could be a Cyclin E/Cdk2 substrate. Additionally, S2 cells containing an inducible hairpin directed at *cyclin E* expression could be made, which would enable the culture of large quantities of *cyclin E* knockdown cells needed for biochemical analysis. Mxc IPs from nuclear extracts made from *cyclin E* knockdown cells could be assayed for loss of MPM-2 reactivity by Western blotting. Mxc contains many putative Cdk phosphorylation sites (S/T-P) and mapping the phosphorylation sites (and the MPM-2 epitope(s)) would increase our understanding of how Mxc activity is regulated by the cell cycle. Phosphorylation sites could be found first by deletion mapping followed by mutating putative sites to Alanines, and then testing for loss of MPM-2 reactivity. The effects a non-phosphorylatable Mxc would have on HLB assembly and histone gene transcription and pre-mRNA processing could then be analyzed.

We showed that Mxc is necessary for HLB assembly in S2 cells, suggesting that it may act as a scaffold, perhaps to couple histone gene transcription and pre-mRNA processing events. Whether Mxc interacts with other known HLB factors could be

evaluated by IP-Western. Mxc (or other HLB components) could be immunoprecipitated and interacting proteins identified by LTQ OrbiTrap liquid chromatography-mass spectroscopy in order to uncover all the components in HLBs. In this manner, perhaps hits from our RNAi screens may be discovered as other novel HLB proteins.

HLBs coordinate histone gene transcription and pre-mRNA processing

Work described in this dissertation explored how histone locus bodies are cell cycle regulated, assemble, and contribute to histone gene expression as a model of nuclear body function. Our data and the work of others support the conclusion that Cyclin E/Cdk2 regulates histone mRNA biosynthesis at the levels of HLB dynamics and histone gene transcription and pre-mRNA processing. These multiple levels of control ensure that histone gene expression and DNA replication are properly coordinated during cell division.

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