THE MOLECULAR MECHANISM OF ADDITIONAL SEX COMBS (ASX) IN *HSP70* TRANSCRIPTIONAL REPRESSION

by

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Abstract

Additional sex combs (*Asx*) is an Enhancer of trithorax and Polycomb (ETP) group gene in *Drosophila*. Mutations in *Asx* enhance both trithorax group (trxG) and Polycomb group (PcG) homeotic mutations, suggesting that *Asx* maintains both activation and repression of homeotic gene transcription. Asx associates with Trithorax (Trx) *in vivo* and is required for promoter clearance at the homeotic gene *Ultrabithorax* (*Ubx*). Asx is necessary for the heat shock response and is recruited to the heat shock inducible gene *hsp70* promoter region following heat stress. Mutations in *Asx* resulted in a 2-fold increase of the *hsp70* mRNA level upon heat shock induction compared to wild-type, indicating that Asx is a transcriptional repressor at *hsp70* during heat shock induction and recovery. To gain further insights into the molecular mechanism of Asx as a transcriptional repressor at the *hsp70* locus, the effect of Asx on the transcriptional activation marker, histone H3K4 trimethylation, and the repression marker, histone H3K27 trimethylation, were investigated. Asx prevented the deposition of histone H3K4 trimethylation and promoted histone H3K27 trimethylation deposition at *hsp70* during heat shock induction and recovery. I propose that Asx prevents Trx-mediated trimethylation of H3K4 and prevents transcriptional elongation, which indirectly promotes the activity of PRC2-mediated transcriptional repression. At the early stage of *hsp70* heat shock induction, Asx-mediated repression likely plays a role in blocking the recruitment of Heat shock factor (HSF), Mediator or elongation factors rather than altering histone H3K4 or H3K27 trimethylation levels. Combined with other studies in the lab on *Ubx*, we can for the first time propose testable models for Asx function as an ETP.

Preface

Figures 1-1, 1-2, 1-3, 1-4 and 2-1A are used with permission from applicable sources. I (T. Li) performed all additional experiments. Hugh Brock and I conceived the experiments. The other parts of this dissertation are original, unpublished, independent work by the author T. Li.

Table of Contents

Abstract ……..……ii

Preface ……..…iii

Table of Contents ……...…….…….iv

List of Tables ……...…..…….....vi

List of Figures …….……....vii

Acknowledgements …….…....….viii

1. Introduction …….…….…..….1

1.1 Maintenance proteins …….……...1

1.2 Enhancers of trithorax and Polycomb (ETP) …….……...8

1.3 *Additional sex comb* (*Asx*) and mammalian homologues are ETP …….……..9

1.4 Asx is required for the promoter clearance of *Ubx* ……...……..17

1.5 Molecular mechanism of heat shock inducible *hsp 70* locus …….…….22

1.6 Thesis aims …….…….……23

2. Results …….…….....25

2.1 Asx binds to the *hsp70* promoter region upon heat shock induction ……..…...…25

2.2 *hsp70* locus is the alternative target to study Asx-mediated

transcriptional regulation …….……..27

2.3 *hsp70* mRNA level in wild-type embryo with heat shock and recovery ……..…...30

2.4 Asx is required for *hsp70* repression during heat shock induction

and recovery ……31

2.5 Histone H3K4 trimethylation heat shock induction and recovery profile at *hsp70* in wild-type embryos ……..34

2.6 Asx prevents H3K4me3 deposition at *hsp70* during heat shock induction ……....36

2.7 Asx promotes histone H3K27 trimethylation at *hsp70* during heat shock induction and recovery ……...38

3. Discussion ……42

3.1 Asx is required for *hsp70* repression ……..42

3.2 Asx prevents Trx-mediated H3K4 trimethylation and transcription elongation at *hsp70* locus during heat shock induction and recovery ……..44

3.3 Asx promotes PRC2-mediated histone H3K27 trimethylation at *hsp70* locus during heat shock induction and recovery ……....46

3.4 The role of Asx, trxG and PcG interactions at the promoter ……48

3.5 Role of Asx at developmentally regulated genes in relation to studies of *hsp70* ……..51

3.6 *Ubx* as a target to study molecular role of Asx in development ……..53

3.7 Summary ……....54

4. Material and Methods ……...56

References ……..62

List of Tables

1-1 List of PcG and trxG complexes and their subunits …….5

List of Figures

1-1 Domain structure of *Drosophila* Asx ……..…..……11

1-2 Asx is required for both transcriptional repression and activation of *Ubx* ……..…16

1-3 Asx binds to the *Ubx* promoter and is required for Trx recruitment

to the *Ubx* promoter ……....18

1-4 Asx is needed for promoter clearance at *Ubx* …….....21

2-1 Asx binds to *hsp70* promoter region upon heat shock induction ……..……...…..26

2-2 Temporal profile of hsp70 mRNA levels during heat shock and recovery …….....28

2-3 Asx is required for *hsp70* repression during heat shock induction and recovery …...33

2-4 Kinetic analyses of histone H3K4me3 level at the *hsp70* locus with

heat shock induction and recovery in wild-type embryos …….……35

2-5 H3K4me3 level changes at *hsp70* in *Asx*^{3} mutants ……..……...37

2-6 H3K27me3 level changes at *hsp70* in *Asx*^{3} mutants ……..…….39

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1. Introduction

1.1 Maintenance proteins

During development in eukaryotes, the maintenance of gene expression patterns from parental to daughter cells is epigenetically regulated. This is because both parental and daughter cells have the same DNA sequence, but they can have different spatio-temporal gene expression patterns. The restricted gene expression pattern must be inherited when a cell undergoes cell division and replication. Inheritance of gene expression patterns is termed "maintenance". This form of gene expression maintenance is mainly achieved by epigenetic mechanisms, which neither requires the continuous presence of initiation signal for cell fate and identity determination, nor involves alterations in the DNA sequence (Schuettengruber et al., 2011).

Two groups of proteins are required for maintenance. These proteins are termed Maintenance Proteins (MPs, Brock and Fisher, 2005). Polycomb group (PcG) proteins are required to maintain silencing of gene expression (Paro, 1990). The trithorax group (trxG) proteins maintain activation of gene expression (Kennison, 1993). In *Drosophila melanogaster*, maintenance requires complex DNA elements called Maintenance Elements (ME, Brock and van Lohuizen, 2001), often located many kilobases from the promoter (Simon et al., 1990). ME bind PcG and trxG proteins, and the binding sites are called PcG Response Elements (PRE) and trxG Response Elements (TRE) respectively (Tillib et al., 1999). Mutations that alter the DNA sequence of ME cause loss of MP

binding to the ME, which leads to loss of maintenance (Brock and van Lohuizen, 2001).

Maintenance of gene expression patterns cannot be easily distinguished from transcriptional regulation, because any change in transcription will lead to a change in maintenance and vice versa. The situation is particularly complicated because PcG and TrxG proteins act at promoters as transcriptional repressors and activators respectively, in addition to acting at ME (Papp and Muller, 2006; Petruk et al., 2008). Most of what we know about the molecular mechanisms of PcG and trxG proteins has been obtained by studying their roles in transcriptional regulation (Brock and Fisher, 2005). Therefore this thesis will concentrate on transcription.

The well-characterized targets of PcG and trxG proteins include *Homeotic* (*Hox*) genes, which are required for axial pattern of the organism during development (Jurgens, 1985; Kennison and Tamkun, 1988; Lewis, 1978). *Hox* genes are conserved throughout evolution from Metazoans to mammals (Veraksa et al., 2000). In *Drosophila melanogaster*, 8 *Hox* genes are arranged in two gene complexes. The Antennapedia complex (ANT-C) determines the development of anterior segments and the bithorax complex (BX-C) determines the development of posterior segments (Bender et al., 1983; Lewis, 1978; McGinnis and Krumlauf 1992). The developing *Drosophila* embryo is divided into parasegments, which are precursors to segments. Each *Hox* gene has a unique embryonic expression pattern restricted to a specific set of parasegments. Alteration of the expression pattern of *Hox* genes causes morphological transformations

of one segment to another. (Bender et al., 1983; Lewis, 1978; McGinnis and Krumlauf, 1992). The order of *Hox* genes in *Hox* gene complexes correlates with their spatial expression pattern in the developing fly (McGinnis and Krumlauf, 1992) and mammalian embryos (Duboule and Dolle, 1989; Graham et al., 1989; Krumlauf, 1994).

PcG mutations cause posterior transformations, where a more anterior structure is transformed towards a more posterior one (Ingham, 1984; Jurgens, 1985). This transformation occurs because *Hox* genes are expressed outside their normal parasegments. Thus PcG proteins are negative regulators that required for repression of *Hox* genes outside of their normal expression domains. Flies with mutations in two different PcG genes exhibit more severe posterior transformation than single mutants (Cheng et al., 1994, Jurgens, 1985). On the other hand, trxG mutations cause anterior transformations resulting from failure of *Hox* gene activation in their spatial expression domains (Kennison, 1993; McGinnis and Krumlauf 1992). Flies doubly mutant for trxG genes exhibit more severe anterior transformations than single mutants (Shearn, 1989). These observations suggest PcG proteins maintain repression and trxG proteins maintain the activation state of *Hox* genes during development, and that the members within each group have additive or synergistic functions.

Many trxG genes were discovered as suppressors of PcG-mediated posterior transformation (Kennison and Tamkun, 1988; Shearn, 1989). Flies simultaneously mutant for a PcG and trxG gene exhibit suppression of homeotic transformations seen in either

mutant alone and thus produce a fly with nearly normal phenotype (Ingham, 1983; Kennison and Tamkun, 1988; Klymenko and Muller, 2004). These observations suggest that the two groups of proteins either have opposite functions (Ingham, 1983), or act antagonistically to one another (Jones and Gelbart, 1993).

In *Drosophila*, the initial boundary of *Hox* gene expression is determined by the pattern of gap and pair-rule gene expression during the early stage of embryonic development, and then the activation of *Hox* genes is regulated by transiently expressed late segmentation genes that establish the parasegment borders (Harding and Levine, 1988; Irish et al., 1989; White and Lehmann, 1986). Mutants of PcG and trxG genes do not exhibit abnormal *Hox* expression patterns until about 2.5-3 hours after the establishment of normal expression pattern (Simon, 1995; Soto et al., 1995; Struhl and Akam, 1985). Thus MPs are required for maintenance but not initiation of homeotic expression patterns.

To date, about 20 PcG genes and 15 trxG genes have been characterized in *Drosophila* (Brock and Fisher, 2005; Schuettengruber et al., 2011). There may be up to 40 PcG genes in total (Jurgens, 1985; Landecker et al., 1994). Most characterized PcG and trxG proteins have homologs with similar functions in mammals (Brock and Fisher, 2005). Biochemically most MPs are members of multi-protein complexes with distinctive functions (Table 1-1). In *Drosophila*, the Polycomb Repressive Complex 1 (PRC1) contains Polycomb (Pc), Polyhomeotic (Ph), Posterior sex combs (Psc),

Table 1-1. List of PcG and trxG complexes and their subunits. All references are listed in Introduction section 1-1. Complexes labeled (*) indicate only core subunits are listed.

Complex name	Protein	Abbreviation	Function
PcG complex			
PRC1	Polycomb	Pc	Binds H3K27me3
	Posterior sex combs	Psc	Compacts chromatin
	dRing	dRing	H2A ubiquitylase
	Polyhomeotic	Ph	Unknown
	Sexcomb on midleg	Scm	Corepressor
PRC1 variant	dRing	dRing	H2A ubiquitylase
	Posterior sex combs	Psc	Compacts chromatin
	dKDM2	dKDM2	H3K36 demethylase
PRC2	Enhancer of zeste	E (z)	H3K27 methyltransferase
	Suppressor 12 of zeste	Su (z) 12	E (z) cofactor
	Extra sex combs	Esc	E (z) cofactor
	Polycomblike	Pcl	Needed for E (z) binding
	Nurf55/Caf1	Nurf55/Caf1	Histone binding
trxG complex			
BRM	Brahma	Brm	Nucleosome remodeling
	Moira	Moira	
	Osa	Osa	
TAC1	Trithroax	Trx	H3K4 methyltransferase
	dCBP	dCBP	H3K36 acetyltransferase
	Sbf1	Sbf1	cofactor for the complex
COMPASS *	dSET1	dSET1	Global H3K4 methyltransferase
	Ash2	Ash2	Coactivator
COMPASS-like *	Ash2	Ash2	Coactivator
	trithroax-related	trr	H3K4 methyltransferase
	dUTX	dUTX	H3K27 demethylase
COMPASS-like *	Trithroax	Trx	H3K4 methyltransferase
	Asx2	Asx2	Coactivator
	Menin	Menin	Sress response regulator
Bre1/Rad6	Bre1	Bre1	H2B ubiquitylase
	Rad6	Rad6	Coactivator

dRing/Sex comb extra (Sce), Sexcomb on midleg (Scm) and other non-PcG subunits (Levine et al., 2002; Lavigne et al., 2004; Shao et al., 1999). PRC1 inhibits chromatin remodeling through the SWI/SNF complex *in vitro* (Francis et al., 2001). The PRC1 variant complex contains dRing, Psc and dKDM2, which has been termed dKDM2 complex (Lagarou et al., 2008). Polycomb Repressive Complex 2 (PRC2) contains Enhancer of Zeste (E(z)), Extra sex combs (ESC), suppressor 12 of Zeste (Su(z)12), Polycomblike (Pcl) and histone binding protein Nurf55/Caf1 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002).

trxG proteins also form multi-protein complexes. Brahma, Moira and Osa are subunits of Brahma-associated nucleosome remodeling complexes (Kal et al., 2000). Trx, dCBP and Sbf1 are subunits of the TAC1 complex (Petruk et al., 2001). Ash1 forms a complex with dCBP, which has histone methyl transferase (HMT) activity on histone H3K36 and histone acetyl transferase (HAT) activity for histone H3K27 (Tanaka et al., 2007; Bantignies et al., 2000). Recently, the trxG protein Ash2 has been found in the *Drosophila* COMPASS complex with dSET1 and other proteins (Ardehali et al., 2011; Hallson et al., 2012; Mohan et al., 2011), with a role in global gene activation and mediation of the bulk H3K4 trimethylation on chromatin (Hallson et al., 2012; Mohan et al., 2011). Ash2, Trithorax-related (trr), dUTX and other proteins form the COMPASS-like complex, that monomethylates histone H3K4 (trr), and demethylates histone H3K27 (dUTX) (Herz et al., 2012; Smith et al., 2008). Trx is associated in a

COMPASS-like complex with Ash2 and Menin (Mohan et al., 2011). Menin is a regulator of stress response and also a tumor suppressor (Papaconstantinou et al., 2005; Yang and Hua, 2007). In mammals, Trx homolog Mixed Lineage Leukemia 1 (MLL1) is required for H3K4 trimethylation at homeotic genes and transcriptional elongation (Guenther et al., 2005). The association between MLL1, Menin and the Ash2 homolog, ASH2L, within the mammalian COMPASS-like complex is conserved, which is consistent with their functional similarity in *Drosophila* and mammals (Yokoyama and Cleary, 2008). Similarly, the mammalian trr homologs, MLL3 and MLL4, form a complex with UTX and ASH2L, with functional similarity to *Drosophila* trr COMPASS-like complex (Herz et al., 2012). Bre1 is an E3 ubiquitin ligase (Bray et al., 2005). Bre1 forms a complex with Rad6, which is required for histone H2B monoubiquitination and the complex is also required for Notch and Wnt signaling pathways (Bray et al., 2005; Buszczak et al., 2009; Mohan et al., 2010).

Histones have post-translational modifications (PTM) necessary for activation and repression of gene expression (Li et al., 2007). In *Drosophila*, PcG-mediated gene repression requires histone H2A ubiquitination at gene promoters and downstream of the promoter by the PcG protein dRing/Sce of the PRC1 complex (Wang et al., 2004a). Ubiquitination of H2A is required for histone H3 lysine 27 trimethylation (H3K27me3) by the PcG protein E(z), a member of PRC2 complex (Cao et al., 2002). The PRC2 complex is also responsible for monomethylation and dimethylation of H3K27 (Ebert et

al., 2004). Some studies proposed that H3K27me3 recruits PRC1 via the chromodomain of Pc (Wang et al., 2004b), although other studies argue against this model (Poux et al., 2001). Similarly, trxG-mediated gene activation requires histone H2B ubiquitination by the trxG protein Bre1 (Bray et al., 2005). Ubiquitination of H2B precedes and is required for histone H3 lysine 4 trimethylation (H3K4me3) by the trxG protein Trithorax (Beisel et al., 2007; Smith et al., 2004). These histone modifications are necessary for promoter clearance and transcription elongation following the recruitment of DNA Polymerase II upstream of the promoter (Dellino et al., 2004; Smith et al., 2004).

1.2 Enhancers of trithorax and Polycomb (ETP)

In *Drosophila*, a third group of proteins that is required for maintenance and for transcriptional regulation have been called Enhancers of *trithorax* and *Polycomb* (ETP) because mutations in these genes enhance homeotic phenotypes of both trxG and PcG mutants (Brock and van Lohuizen, 2001; Gildea et al., 2000). This suggests that ETP proteins maintain both transcriptional activation and silencing. Mutations in two genes originally classified as PcG genes, *E(z)* and *Additional sex combs* (*Asx*), results in simultaneous anterior and posterior transformations, which is consistent with a role in activation and repression of homeotic loci (LaJeunesse and Shearn, 1996; Milne et al., 1999). Another candidate is *Trithoraxlike* (*Trl*), which encodes the GAGA factor that synergizes with the ISWI nucleosome remodeling complexes during gene activation

(Tsukiyama et al., 1995). Mutations in *Trl* enhance the homeotic phenotype of PcG gene *ph* mutants, indicating that *Trl* is an ETP (Hodgson et al., 2001). ETP proteins could be members of both trxG and PcG complexes, be required to recruit both trxG and PcG complexes, be required for enzymatic or structural activity of both trxG and PcG proteins, or have a role in nuclear architecture (Brock and van Lohuizen, 2001). However, the identification of the H3K27 histone methyltransferase E(z) as an ETP suggests that the indirect effects of trxG and PcG mutations may make it difficult to predict molecular functions of ETP based on genetic analysis alone.

1.3 *Additional sex comb* (*Asx*) and mammalian homologues are ETP

Additional sex combs (*Asx*) encodes Asx (Jurgens, 1985), which genetically is an ETP (Gildea et al., 2000; Milne et al., 1999; Sinclair et al., 1992). Mutants in *Asx* exhibit both anterior and posterior transformations, because *Hox* genes are derepressed and improperly activated respectively (Milne et al., 1999; Sinclair et al., 1992). Consistent with this model, *Asx* mutants enhance the homeotic transformation of PcG mutations (Campbell et al., 1995) and trxG mutations (Milne et al., 1999).

The mammalian homologous of *Asx* are *Additional sex combs-like 1* (*Asxl1*), *Asxl2* and *Asxl3* (Fisher et al., 2003; Fisher et al., 2006; Katoh and Katoh, 2003; Katoh and Katoh, 2004). These mammalian Asx homologues belong to the ETP group because *Asxl1* mutants exhibit simultaneous derepression and failure to activate *Hoxc8*, a

mammalian homeotic gene in mice (Fisher et al., 2010). *Asxl2* mutations also exhibit simultaneous posterior and anterior transformation in the axial skeleton (Baskind et al., 2009). Murine *Asxl1* genetically interacts with the mammalian PcG gene *Pc* homolog, *Cbx2* (Fisher et al., 2010). ASXL1 functions as a transcriptional co-activator of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) in human retinoic acid-sensitive cancer cell lines, and also functions as a co-repressor of RAR activity in different mammalian cell lines (Cho et al., 2006). The repressive function of ASXL1 on RAR and RXR receptors is achieved through the interaction of ASXL1 with the histone H3K4 demethylase LSD1 (Lee et al., 2010). These results support the model that Asx/ASXL proteins have dual activator and repressor functions in both *Drosophila* and mammals.

The *Drosophila* Asx and mammalian homologs ASXL1, ASXL2 and ASXL3 share a common domain architecture that consist of the ASXH domain in the N-terminal region and a plant homeodomain (PHD) domain in C-terminal region (Figure 1-1, Fisher et al., 2006). The region around the ASXH domain is required for binding the PcG protein Calypso, which has histone deubiquination activity (Scheuermann et al., 2010). This function is conserved in mammals because the ASXH domain of ASXL1 interacts with BAP1, the mammalian homolog of Calypso (Scheuermann et al., 2010). The ASXH domain also promotes binding between ASXL1 and LSD1, which is required for transcriptional repression by the RAR and RXR receptors (Lee et al., 2010). Within the

N-terminal **C-terminal**

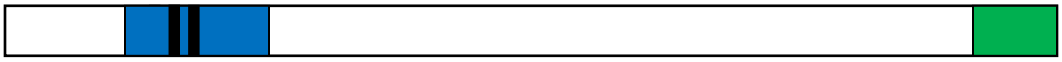


Figure 1-1. Domain structure of *Drosophila* Asx. The boxes represent conserved domains and motifs. ■ Represents ASXH domain, ■ represents ΦXXΦΦ motif (Nuclear receptor), ■ represents PHD domain.

ASXH domain there are two ΦXXΦΦ (Φ is any hydrophobic residue) motifs that are conserved in nuclear receptor (NR) binding motifs. NR binding motifs act together with chromatin-remodeling factors, coactivators and corepressors to regulate gene transcription (Rosenfeld and Glass, 2001). The PHD domain is a histone or DNA binding module for transcription factor and chromatin regulators (Liu et al., 2012; Sancez and Zhou 2011). MPs including Pcl, Trx, Ash1, Ash2 and their mammalian homologs also have PHD domains (Brock and van Lohuizen, 2001). The PHD domain of Pcl interacts with E(z) (O'Connel et al., 2001). The PHD domain in the mammalian Pcl homolog, PCL2, is needed for targeting PRC2 to chromatin (Casanova et al., 2011). Although the target of the Asx and ASXL protein family PHD domain remains to be identified, it is predicted to be histones or DNA.

In humans, mutations in *Asxl1* have been identified in myeloid malignancies such as myelodysplastic syndrome (MDS) (Bejar et al., 2011; Boultwood et al., 2010; Gelsi-Boyer et al., 2009; Thol et al., 2011), acute myeloid leukemia (AML) (Metzeler et al., 2011; Patel et al., 2012), chronic myelomonocytic leukemia (CMML) (Abdel-Wahab et al., 2011; Gelsi-Boyer et al., 2009), chronic myeloproliferative neoplasms (MPN) (Abdel-Wahab et al., 2011; Carbuccia et al., 2009; Guglielmelli et al., 2011) and chronic lymphocytic leukemia (CLL) (Quesada et al., 2011). Among these, *Asxl1* is mutated in 45.3 % of CMML, 34.5% of MPN, 36.5% of AML and 16.2% of MDS cases, and thus there is strong correlation between *Asxl1* mutation and myeloid malignancies

(Gelsi-Boyer et al., 2012). Most of these mutations are nonsense point mutations or frame-shift mutations at the 5'-end of the last exon region, suggesting these mutations could cause a truncated protein product lacking the C-terminal PHD domain (Abdel-Wahab et al., 2011; Gelsi-Boyer et al., 2009). Recent studies suggest that the reduced stability of mutant ASXL1 rather than protein truncation per se is likely the cause of myeloid malignancies (Abdel-Wahab et al., 2012). These studies suggest that ASXL1 act as a tumor suppressor in myeloid malignancies that affects stem cell renewal and proliferation (Gelsi-Boyer et al., 2009).

Mutations in *Asxl2* and *Asxl3* are relatively infrequently associated with myeloid malignancies. *Asxl2* is mutated in 6.0% of castration-resistant prostate cancers (Grasso et al., 2012), and *Asxl3* is mutated in 4.0% of melanomas (Berger et al., 2012). This observation suggests the molecular functions of ASXL family members are different and may be tissue specific in humans.

In human embryonic kidney cells, loss of *Asxl1* caused a global loss of H3K27me3 (Abdel-Wahab et al., 2012). The effects of *Asx11* mutation on histone H2Aub was minimal compared with the effects on H3K27me3 at homeotic loci (Abdel-Wahab et al., 2012). This indicates ASXL1 acts as a PcG protein by modulating the HMT activity of *Drosophila* E(z) homolog EZH2 on H3K27. ASXL1 was co-immunoprecipitated with PRC2 components including EZH2, SUZ12 and EED, suggesting that ASXL1 is crucial for recruitment or stabilization of PRC2 at homeotic genes to maintain the repression

state of the gene (Abdel-Wahab et al., 2012). It remains unclear whether ASXL1 recruits PRC2 directly or indirectly, but results from two independent studies suggest divergence in the function of ASXL1 and ASXL1-BAP1 complexes. If Asx-Calypso and ASXL1-BAP1 are required for histone H2A deubiquitination and favour gene activation, then H3K27me3 level at target genes should decrease. In turn, these observations suggest Asx and ASXL protein family could have different molecular functions in both transcriptional activation and repression in a context-dependent manner.

In *Drosophila,* Asx forms a complex with the chromatin deubiquitin enzyme Calypso to form the Polycomb-repressive deubiquitinase (PR-DUB) complex with the function of removing monoubiquitin from histone H2AK118 and possibly from H2BK117 in bulk histones (Scheuermann et al., 2010). This interaction is critical for the enzymatic activity of Calypso. The mammalian homolog of Calypso, BAP1, shows association with ASXL1, and the ASXL1-BAP1 complex has conserved deubiquitinase activity *in vitro* (Scheuermann et al., 2010). If Asx-Calypso is required for deubiquitination of both H2Aub and H2Bub, then Asx may be regulating transcriptional activation and repression by removing the ubiquitination that is required for H3K4 and H3K27 trimethylation respectively. Asx-mediated deubiquitination of H2A should favour gene activation because it prevents the establishment of the H3K27me3 mark, and deubiquitination of H2B should favour repression because it prevents the establishment of the H3K4me3 mark. Thus a single enzymatic activity associated with Asx could explain the molecular

function of Asx as an ETP.

Surprisingly, no one has demonstrated an unequivocal role for Asx in transcriptional activation in *Drosophila*. Some researchers deny that *Asx* is an ETP (Scheuermann et al., 2010). Early studies on the effects of homeotic gene expression in *Asx* mutants show derepression of homeotic genes *Scr, Antp* and *Ubx* (McKeon and Brock, 1991), or *abd-A* and *Abd-B* in zygotic (Simon et al., 1992), or in maternal effect mutants (Soto et al., 1995). Consistent with these genetic observations, *Asx* mutations decrease the transcription level of a PRC1 member, *Posterior sex combs* (*Psc*) transcripts (Ali and Bender, 2004). Therefore it is crucial to determine if *Asx* does have a role in transcriptional activation, and determine the molecular mechanism of Asx on transcriptional activation.

However, as with all genetic experiments, we do not know if the effects of Asx mutations on transcriptional regulation are direct or indirect. This problem is acute in the PcG proteins because PcG proteins cross-regulate each other's transcription (Ali and Bender, 2004). *Asx* mutations cause simultaneous derepression and failure of activation of BX-C homeotic gene *Ultrabithorax* (*Ubx*) expression, and this indicates the molecular function of Asx at *Ubx* locus involves both transcriptional activation and repression (Figure 1-2A. Hodgson, unpublished). In wild-type embryos, *Ubx* expression is observed in parasegments 5-13 (White and Lehmann, 1986). At the anterior end of the embryo, *Ubx* expression is suppressed by the early expression of gap gene

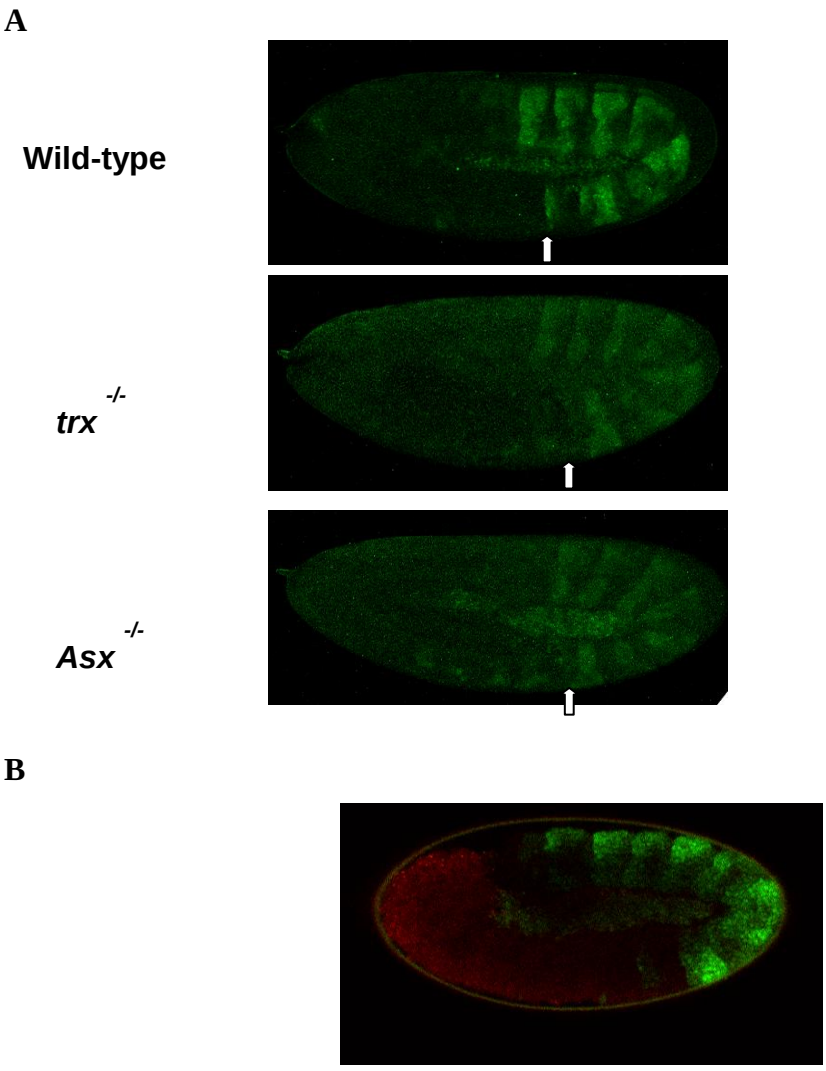


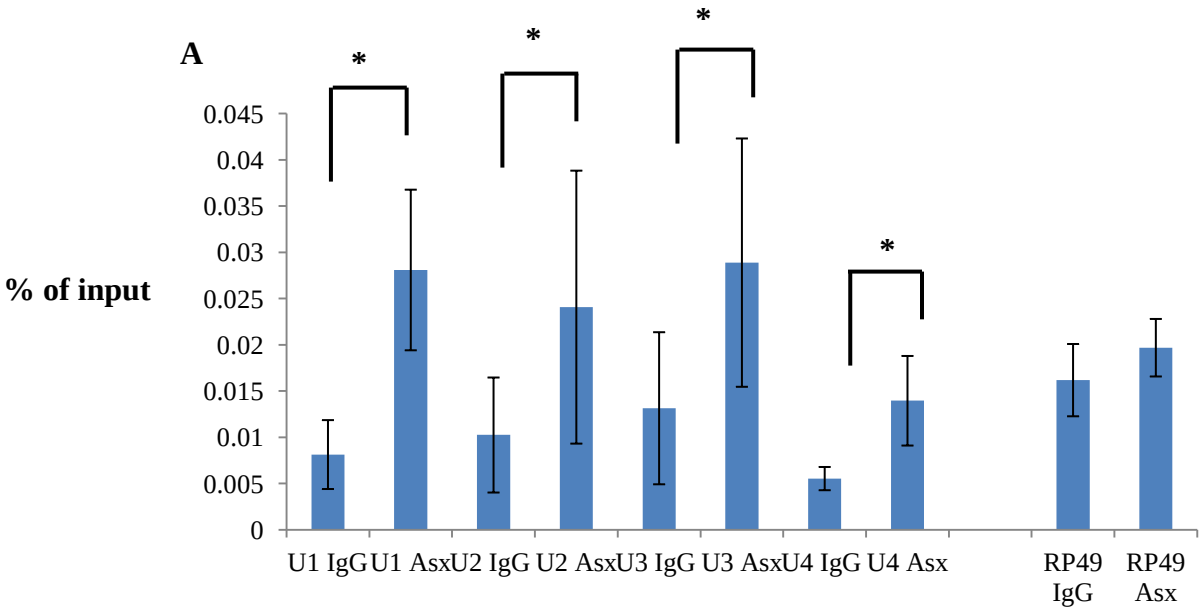
Figure 1-2. Asx is required for both transcriptional repression and activation of *Ubx*. **(A)** *Ubx-eGFP* labeled embryos are mounted with anterior to the left and dorsal up. The position of the border between parasegment 4 and 5 is marked with the arrow. Embryos are homozygous for *trx* and *Asx* mutant as labeled. Images have the same exposure time and same contrast level to ensure the expression level is proportional to the pixel intensity. *trx* mutant embryos showed reduced intensity of transgene expression in posterior segments, showing that Trx has a function in *Ubx* activation. Asx mutant embryos showed simultaneous failure of activation of *Ubx* in posterior segments with derepression of *Ubx* in anterior segments, which indicates Asx is required for both *Ubx* transcriptional activation and repression (Hodgson, unpublished). **(B)** *Ubx-eGFP* (Green) and *hb-mCherry* (Red) dual-labeled embryos is mounted with anterior to the left and dorsal up. The *Ubx* expression at the posterior of the embryo is repressed by gap gene *hb* expression at the anterior end (Hodgson, unpublished).

hunchback (Figure 1-2B; White and Lehmann, 1986). The *trithorax* (*trx*) homozygous mutant embryos exhibit reduced expression of *Ubx* (Figure 1-2A), indicating Trx is required for transcriptional activation of *Ubx* (Mazo et al., 1990). In *Asx* homozygous mutant embryos derepression of *Ubx* expression is observed in parasegments 1-4, with reduced *Ubx* expression levels in posterior parasegments 5-13 similar to those seen in *trx* homozygous mutations (Figure 1-2A). This indicates Asx is required for both *Ubx* transcriptional activation and repression. *Ubx* is one candidate target gene to examine the direct molecular role of Asx in transcriptional regulation, because preliminary data from a chromatin immunoprecipitation (ChIP) assay showed that Asx binds to the *Ubx* promoter in wild-type embryo (Figure 1-3A).

1.4 Asx is required for the promoter clearance of *Ubx*

The C-terminal domain (CTD) of RNA polymerase II (Pol II) contains multiple heptad repeats with a consensus sequence YSPTSPS, which can be multi-phosphorylated during eukaryotic gene transcription (Komarnitsky et al., 2000). During transcription initiation, the assembly of a large protein complex with Pol II and general transcription factors (GTFs) begins at promoter region. RNA Pol II with no CTD phosphorylation is recruited to the promoter region with a set of GTFs includes TATA-binding protein TBP (Lu et al., 1991). Phosphorylation of Ser-5 residues on the CTD by the kinase, Cdk-7, is required for promoter clearance (Schwartz et al., 2003; Yamamoto et al., 2001), in which

A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RP49 IgG
RP49 Asx

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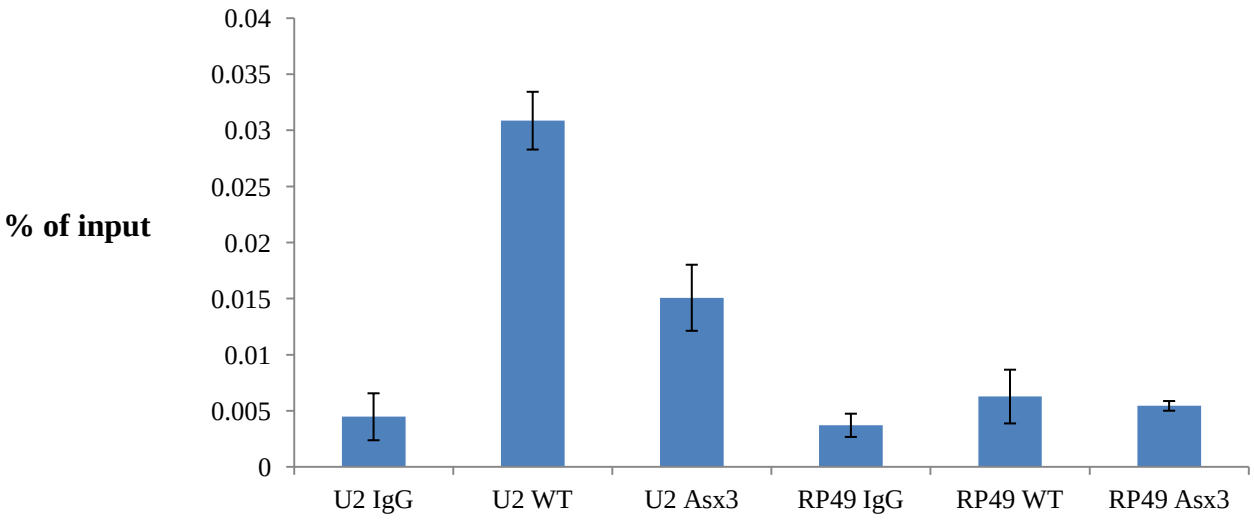
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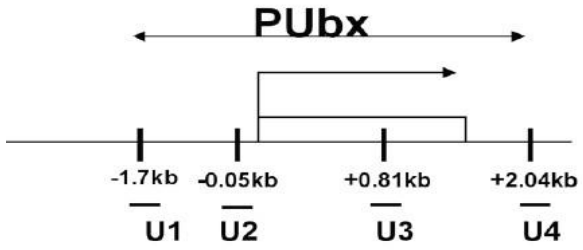
RP49 Asx3

C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+0.81kb
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U1
U2
U3
U4

Figure 1-3. Asx binds to the *Ubx* promoter and is required for Trx recruitment to the *Ubx* promoter. (A) Chromatin immunoprecipitation from wild-type embryos with an anti-Asx antibody and rabbit anti-IgG antibody for comparison. Primers for the *rp49* gene were used as a negative control for Asx binding. *Ubx* primer locations are labeled as in Figure 1-3C. The amount of DNA recovered from the ChIP samples were analyzed by real-time PCR. The signals are represented as mean ±SEM with n=3. (*) $P<0.05$. Data indicates Asx binds to *Ubx* promoter region (Komljenovic unpublished**). (B)** Chromatin immunoprecipitation from wild-type and Asx^3 mutant embryos with an anti-Trx antibody and rabbit anti-IgG antibody for comparison. Primers for the *rp49* gene were used as a negative control for Asx binding. The amount of DNA recovered from the ChIP samples were analyzed by real-time PCR. The signals are represented as mean ±SEM with n=3. Trx is recruited to *Ubx* primer in wild-type embryo but the level of Trx binding shows 2-fold decrease in Asx^3 mutant embryos. The result indicates Asx is required for Trx recruitment at *Ubx* promoter (Komljenovic unpublished**). (C)** *Ubx* primer maps showing primers used in this study. U1-U4 primers are upstream or within the promoter region of *Ubx*.

Pol II leaves the promoter region where GTFs binds, transcribes 25-50 bp and then pauses (Rougvie and Lis, 1988). Phosphorylation of Ser-2 residues by the kinase, Cdk-9, is essential for the transition from polymerase pausing to elongation by making possible elongation factor recruitment and 3'-end processing of the transcript (Eissenberg et al., 2007). The absence of Cdk-7 and Cdk-9 kinases that phosphorylate the Pol II CTD will stall the Pol II at the initiation stage or the promoter pausing stage respectively (Schwartz et al., 2003; Eissenberg et al., 2007). Mutations in the genes encoding the kinases Cdk7 and Cdk9 interrupt transcription, which allows us to study the recruitment of specific protein before initiation stage or promoter pausing stage. The analysis of the phosphorylation status of the Pol II CTD in a specific mutant background allows us to determine what step of transcription requires a given protein. Asx and Trx colocalize on polytene chromosomes at many binding sites. Mutations in *Asx* prevent recruitment of Trx, and conversely mutations in *trx* prevent recruitment of Asx to polytene chromosomes (Petruk et al., 2008). Preliminary data from our lab shows that Asx recruits Trx at the *Ubx* promoter region (Figure 1-3B, Komljenovic unpublished), consistent with a role for Asx in *Ubx* activation (Figure 1-2A). In late embryos, *Asx* is required for promoter clearance at the *Ubx* locus because in *Asx* mutants, Pol II with an unphosphorylated CTD is recruited to the promoter, but Ser-5 and Ser-2 phosphorylated Pol II is not observed in chromatin immunoprecipitation experiments (Figure 1-4. Komljenovic, unpublished). However, the expression of *Ubx* is spatially regulated

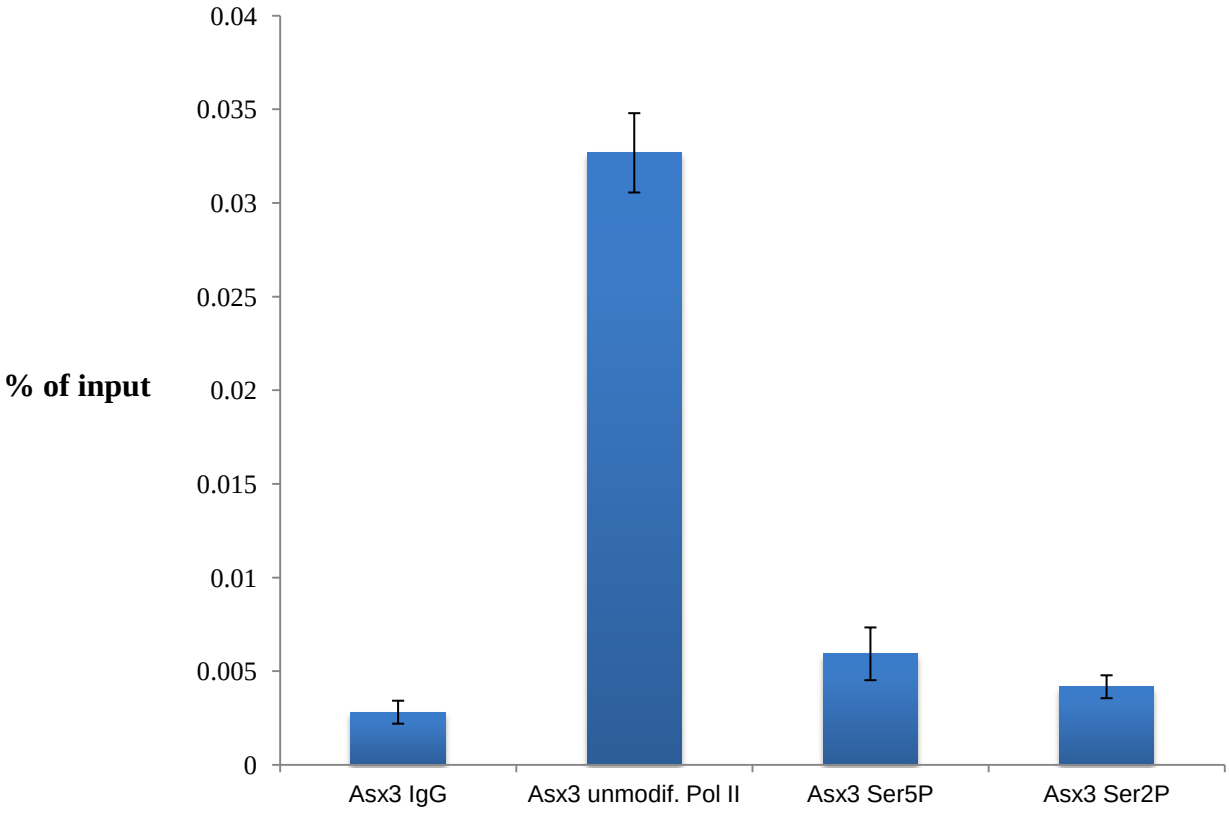

Figure 1-4. Asx is needed for promoter clearance at *Ubx*. *Asx*^{3} mutant embryos 14-16 hr after egg-lay are probed with unmodified Pol II, CTD Serine 5 phosphorylated and Serine 2 phosphorylated Pol II antibodies by chromatin immunoprecipitation, using IgG antibody as a control for non-specific binding. CTD Serine 5 phosphorylation occurs at promoter clearance, and CTD Serine 2 phosphorylation occurs during the transition to transcriptional elongation. The amount of DNA recovered from ChIP samples were analyzed with real-time PCR. U2 Primers specific for the *Ubx* promoter (Figure 1-3C) are used to measure unmodified Pol II, Serine 5 phosphorylated and Serine 2 phosphorylated PolII recruitment to *Ubx*. The percentage of input DNA recovered in the immunoprecipitate is shown along the *y* axis. The signals are represented as mean ±SEM with n=3. The observation of unmodified Pol II but not CTD modified Pol II recruitment indicates in *Asx* mutation the *Ubx* transcription paused before promoter clearance. This indicates Asx is required for promoter clearance at *Ubx*. (Komljenovic, unpublished)

(Akam and Martinez-Arias, 1985), so to study the molecular mechanism of Asx in *Ubx* repression, it is necessary to isolate cells that do or do not express green fluorescent protein (GFP) under the control of *Ubx* enhancers (Petruk et al., 2006) using fluorescence-activated cell sorting (FACS). So far, this has presented technical problems, so I was not able to further investigate Asx function at *Ubx.*

1.5 Molecular mechanism of heat shock inducible *hsp 70* locus

Immunohistochemical staining of *Drosophila* polytene chromosomes with antibodies to Asx shows Asx is recruited to the heat shock inducible gene *hsp70* region (87AB) upon 20 min of heat shock (Figure 2-1A. Brock, unpublished). Therefore Asx could have a role in regulating heat shock induction at *hsp70*. The molecular mechanism of transcriptional activation of the *hsp70* gene is well characterized. Before heat shock induction, the *hsp70* promoter region is maintained in a nucleosome-free conformation by the GAGA factor encoded by *Trl* (Tsukiyama et al., 1994). This uninduced state also maintains a paused Pol II approximately 25 nucleotides downstream of the transcription starting site (Rasmussen and Lis, 1993; Rougvie and Lis, 1988). The paused Pol II shows phosphorylated Ser-5 but not phosphorylated Ser-2 (Boehm et al., 2003). The induction of transcription of *hsp70* is achieved through recruitment of series of protein factors including heat shock factor (HSF), which trimerizes and binds to heat shock elements (HSE) (Westwood and Wu, 1993). This activation process also includes the recruitment of Positive Transcription Elongation Factor b (P-TEFb), Mediator, and various elongation

factors for synthesis of full-length transcripts (Andrulis et al., 2000; Kaplan et al., 2000; Saunders et al., 2003). P-TEFb contains Cdk9 that is required for Pol II CTD Ser-2 phosphorylation and transcription elongation (Boehm et al., 2003).

After heat shock induction, recruitment of the TAC1 complex, including trxG protein Trx, is required to maintain high levels of transcription elongation and H3K4me3 at the promoter region (Smith et al., 2004). In *trx* mutants, the level of *hsp70* transcription decreases significantly compared to wild-type, accompanied by a decrease of the H3K4me3 level at the *hsp70* promoter (Smith et al., 2004). The PcG protein Pleiohomeotic (Pho) is required to repress *hsp70* transcription after heat shock during the recovery phase (Beisel et al., 2007). Our lab showed that PcG-mediated repression acts on *hsp70* during heat shock activation to prevent runaway transcription, as well as acting during recovery after heat shock (Kovermann, unpublished). Therefore, both trxG-mediated activation and PcG-mediated repression control *hsp70* gene activity, which suggests that the *hsp70* gene is an excellent target for studying the role of Asx in regulating transcription. The *hsp70* promoter is activated by heat shock in each cell of the fly embryo, making it possible to investigate the role of Asx in heat shock repression at whole embryo level.

1.6 Thesis aims

The molecular mechanism of Asx function in transcriptional repression is still

unknown. Preliminary data showed that after heat shock induction, *Asx* mutants exhibited an increase in *hsp70* mRNA level and less effective recovery, suggesting that Asx acts as a repressor in both activation and repression phases of heat shock response at *hsp70* locus (Kovermann, unpublished).

In this thesis, I will investigate the role of Asx in *hsp70* transcriptional repression during heat shock and recovery after heat shock. Chapter 1 will address whether Asx binds to the *hsp70* promoter region during heat shock and recovery, and whether Asx is required for *hsp70* transcriptional repression. Chapter 2 will address the molecular mechanism of Asx in transcriptional repression, and whether Asx acts as an *hsp70* transcriptional repressor by preventing the activity of other activators, by acting as a conventional PcG protein or if Asx has dual role on these two mechanisms. Combined with data from previous studies, the results from this thesis will provide a possible molecular function of Asx as an ETP protein. I will connect the results with previous genetic experiments showing that *Asx* is an ETP gene.

2. Results

2.1 Asx binds to the *hsp70* promoter region upon heat shock induction

Experiments in which salivary glands were subjected to 20 minutes of heat shock, stained with antibodies to Asx, and compared to preparations from glands that were not heat shocked show that Asx was recruited to *hsp70* region (87AB) upon 20 min of heat shock (Figure 2-1A; Brock, unpublished). This result suggests that Asx acts directly on heat shock loci, and that it is recruited after heat-shock.

Polytene staining experiments are of insufficient resolution to confirm that Asx binds specific genes at a specific location, and are not quantifiable. To improve the resolution I decided to use chromatin immunoprecipitation at the *hsp70* locus in embryos. If Asx regulates *hsp70* transcription in heat shock, then binding of Asx at *hsp70* promoter region should be observed after heat shock. In order to do these experiments an N-terminal region of Asx was expressed in bacteria as a GST-fusion, and purified on a column containing glutathione, and used to immunize rabbits. IgG was prepared from crude serum by precipitation with ammonium sulphate. I used Western blotting on wild-type *Drosophila* embryo nuclear extract with pre-immune serum and purified IgG to confirm the specificity of the Asx antibody. A band of expected size of 180 kDa (Sinclair et al., 1998) was present in the lane with purified IgG but not in the lane probed with pre-immune serum, suggesting that the antibody is Asx-specific (data not shown).

The IgG purified antibody was used in a chromatin immunoprecipitation (ChIP)

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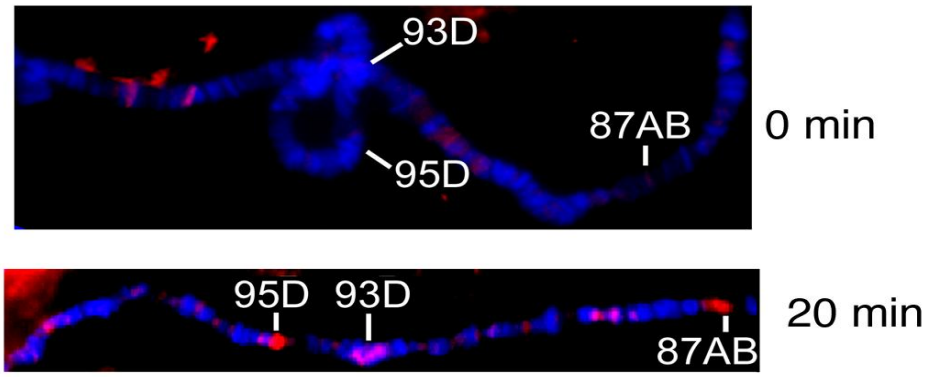

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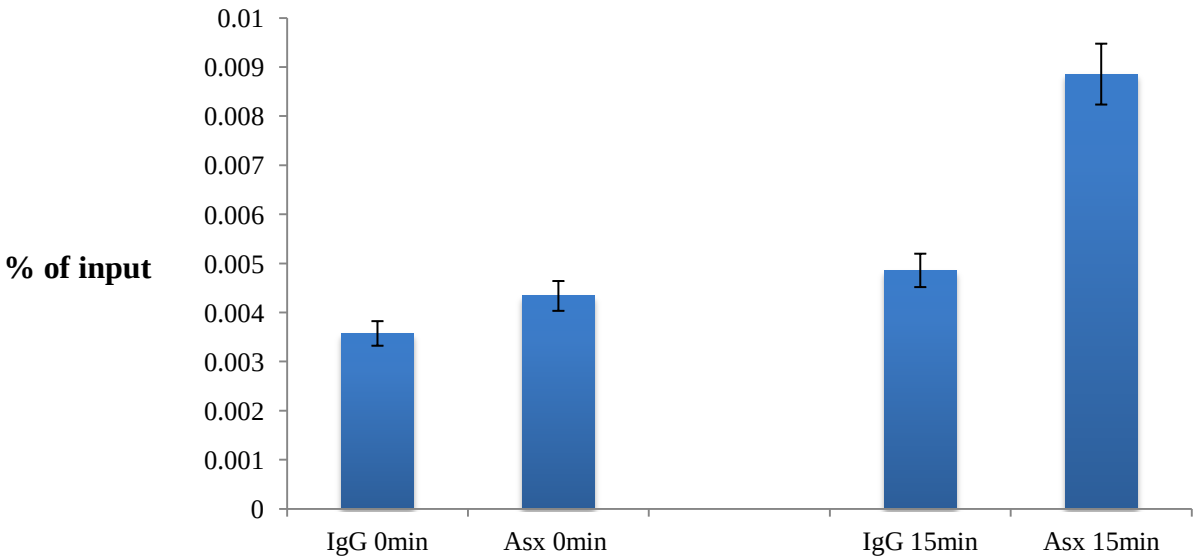

Figure 2-1. Asx binds to the *hsp70* promoter region upon heat shock induction. (A) Asx antibody staining of the proximal portion of wild-type 3R polytene chromosomes before and after 20 min heat shock at 37°C. Major heat shock loci 87A, 93D and 95D are labeled. After 20 min heat shock, Asx has been recruited to major heat shock loci as indicated (Brock, unpublished data). **(B)** Chromatin immunoprecipitation from 14-16 hr wild-type embryos before and after 15 min heat shock at 37 °C with the anti-Asx antibody and rabbit anti-IgG antibody for comparison. The amounts of DNA recovered from ChIP samples were analyzed by real-time PCR. Primers specific to 218 and 392 bp down stream of *hsp70* transcription start site were used to measure Asx recruitment to the heat shock gene before and after heat shock induction. The percentage of input DNA recovered in immunoprecipitate is shown along the *y* axis. After 15 min heat shock induction, Asx has been recruited to *hsp70* promoter region. The signals are represented as mean ±SEM with n=3.

experiment to verify the binding of Asx to the *hsp70* promoter region with rabbit IgG antibody as a negative control. At 0 min of heat shock, the difference in levels of DNA recovered at the *hsp70* promoter region between IgG antibody control and Asx antibody was not significant. This indicates without heat shock, Asx does not significantly bind at the *hsp70* promoter region. After 15 min of heat shock, the level of DNA recovered with the Asx antibody was 2-fold higher than with the IgG antibody control (Figure 2-1B), indicating that Asx is recruited to *hsp70* promoter upon heat shock induction *in vivo*, which is consistent with the immunohistochemistry on polytene experiments. Together these experiments confirm that the *hsp70* locus is a direct target of Asx.

2.2 The *hsp70* locus is the alternative target to study Asx-mediated transcriptional regulation

The ChIP results indicates Asx was directly associated with *hsp70* locus after heat shock induction, which suggests the *hsp70* locus is a candidate to study the effect of Asx on transcriptional regulation (Figure 2-1B). I used a sensitive quantitative PCR assay to determine the steady-state levels of *hsp70* transcripts at various times after heat shock in *Drosophila* embryos. Embryos were collected, heat-shocked for the times indicated, with or without recovery time at room temperature as described in the Material and Methods. I prepared total RNA, used the Superscript III Reverse Transcriptase kit to prepare cDNA,

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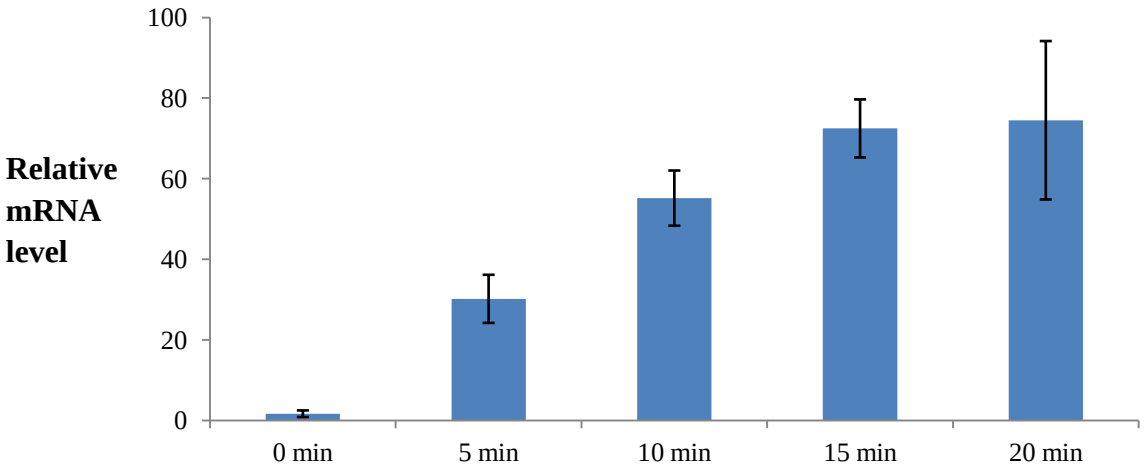
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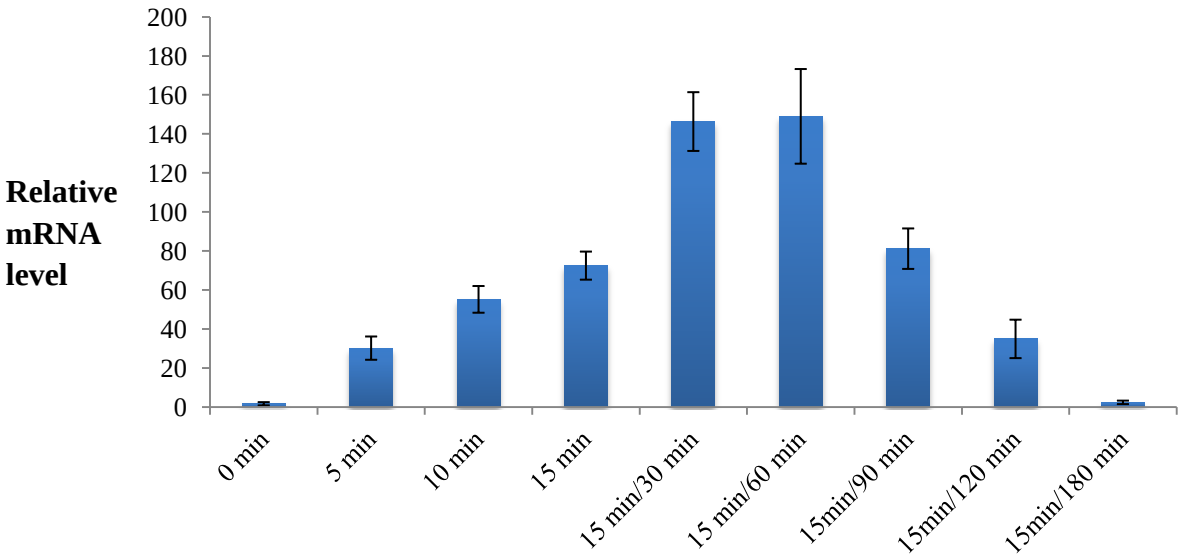
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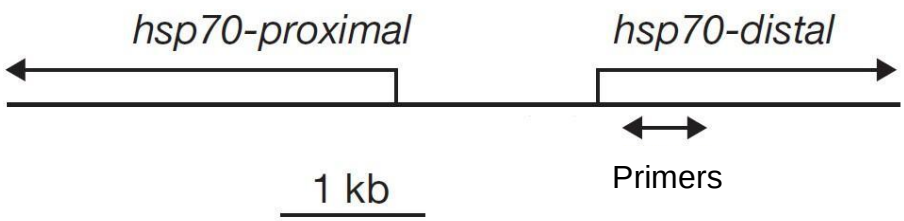
hsp70-proximal
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Primers

Figure 2-2. Temporal profile of *hsp70* mRNA levels during heat shock and recovery. **(A)** The relative amount of cDNA of wild-type 14-16 hr embryos obtained from reverse-transcribed mRNA was measured by real-time PCR with primers downstream of *hsp70* transcription starting site. The *x* axis is the heat shock induction time at 37^{o}C, and the *y* axis is the *hsp70* relative mRNA level. The results were expressed as *hsp70* mRNA level normalized to *Ahcy89E* mRNA level. *Ahcy89E* is a control gene located close to *Ubx* locus and its transcription is not affected by heat shock induction. The signals are represented as mean with SEM as error bar. Input was from three independent experiments with two technical replicates within each experiment. The relative mRNA level reached maximum at 15 min heat shock induction. **(B)** Kinetic analyses of *hsp70* mRNA level with heat shock induction and recovery. Heat shock recovery was under 22 ^{o}C after 15 min of 37^{o}C heat shock induction. The maximum relative mRNA level was observed after 60 min of recovery with 15 min heat shock induction. **(C)** Primer map showing the location of primers used in A and B. Primers are specific to 218 and 392 bp downstream of *hsp70* transcription starting site.

and 100 ng of diluted cDNA was used in each PCR reaction using the primers shown in Figure 2-2C. For each time point, I carried out three independent experiments. For each experiment I carried out two technical replicates. The error bar indicates standard error of the mean (SEM). To determine when the maximum level of mRNA was reached after heat shock, I carried out a time course experiment. To control for variability in RNA yield between experiments, the relative mRNA level of *hsp70* was normalized to the mRNA level of an endogenous control gene, *Ahcy89E,* whose expression was not affected by heat shock (data not shown). As shown in Figure 2-2A, the relative mRNA level of *hsp70* increased 18-fold after 5 min of heat shock induction, and 44-fold after 15 min compared to the mRNA level without heat shock induction. The steady-state of *hsp70* level at 15 min and 20 min heat shock induction did not differ markedly, which indicates the *hsp70* transcription is maximal after 15 min of heat shock induction (Figure 2-2A). Therefore, I decided to use 15 min of heat shock in subsequent experiments, so that I achieved maximum change in mRNA levels with the minimum amount of heat shock. These results show that the transcriptional level change at the *hsp70* locus upon heat shock induction is rapid, and that there is enough dynamic range to detect small effects when comparing control and experimental embryos.

2.3 *hsp70* mRNA level in wild-type embryo with heat shock and recovery

In order to characterize the effect of Asx on *hsp70* transcription during heat shock

induction and recovery in wild-type embryos, I extended the previous results to include the recovery phase. When heat stress is removed, *hsp70* transcription ends, and the steady state level of mRNA decreases (DiDomenico et al., 1982). These data provide a baseline for investigating the effects of Asx mutations. As noted, because in wild-type embryo heat shock induction the *hsp70* relative mRNA level maximized at 15 min heat shock, I characterized *hsp70* recovery phase following 15 min of heat shock.

As shown in Figure 2-2B, the levels of *hsp70* mRNA increased during heat shock. Interestingly, the steady state levels doubled after 30 minutes of recovery. This result suggests that when heat shock stress is removed, elevated transcription continues for some time. There may be a lag between transcription of *hsp70* and accumulation of the transcript. The plateau of *hsp70* mRNA levels between 30 and 60 min of recovery may indicate that the *hsp70* mRNA is stable if transcription has stopped, or that there is a balance between transcription and decay of the mRNA. After 60 minutes of recovery, the level of mRNA drops rapidly until it attains pre-heat shock levels after 3 hr of recovery. These results suggest that there is no more transcription after 60 min of recovery. It is also possible that the half-life of the mRNA changes during this process.

2.4 Asx is required for *hsp70* repression during heat shock induction and recovery

In order to characterize the effect of Asx on *hsp70* transcription during heat shock and recovery, I compared the *hsp70* mRNA levels of Asx^{3} mutants during heat shock and

recovery to those of wild-type embryos. The *Asx*^{3} mutant was chosen because it is a null mutation with 1.3kb deletion in the middle of coding region, which results to a truncated protein product with only approximately 800 N-terminal amino acids (Jurgens, 1985; Milne et al., 1999; Sinclair et al., 1998). As above, I carried out three independent experiments for each time point, and carried out two technical replicates. These experiments are demanding because *Asx* mutations are embryonic lethal. Mutant embryos have to be picked by hand from the heterozygous and homozygous balancer siblings. If embryos stay too long under the dissecting microscope, the mRNA quality declines rapidly, and also increases the likelihood of inadvertently subjecting the embryos to heat stress. It took about 5 days to collect enough embryos for each time point. For ease of comparison, Figure 2-3 compares the data from *Asx*^{3}mutants to the wild-type data already shown in Figure 2-2.

As seen in Figure 2-3, the level of *hsp70* mRNA before heat shock induction between wild-type and *Asx*^{3} mutant embryos did not differ markedly (Figure 2-3), showing that the extra handling required to collect mutants did not subject the embryos to greater stress than wild-type embryos. The *hsp70* mRNA level in the *Asx*^{3} mutant was significantly higher during all time points for heat shock induction and recovery than in wild-type embryos. The mRNA level difference between the *Asx*^{3} mutant and wild-type was at least 2-fold during heat shock induction and recovery, and at 90 min recovery, the difference reached at maximum level at 2.7-fold (Figure 2-3). In *Asx*^{3} mutants, the

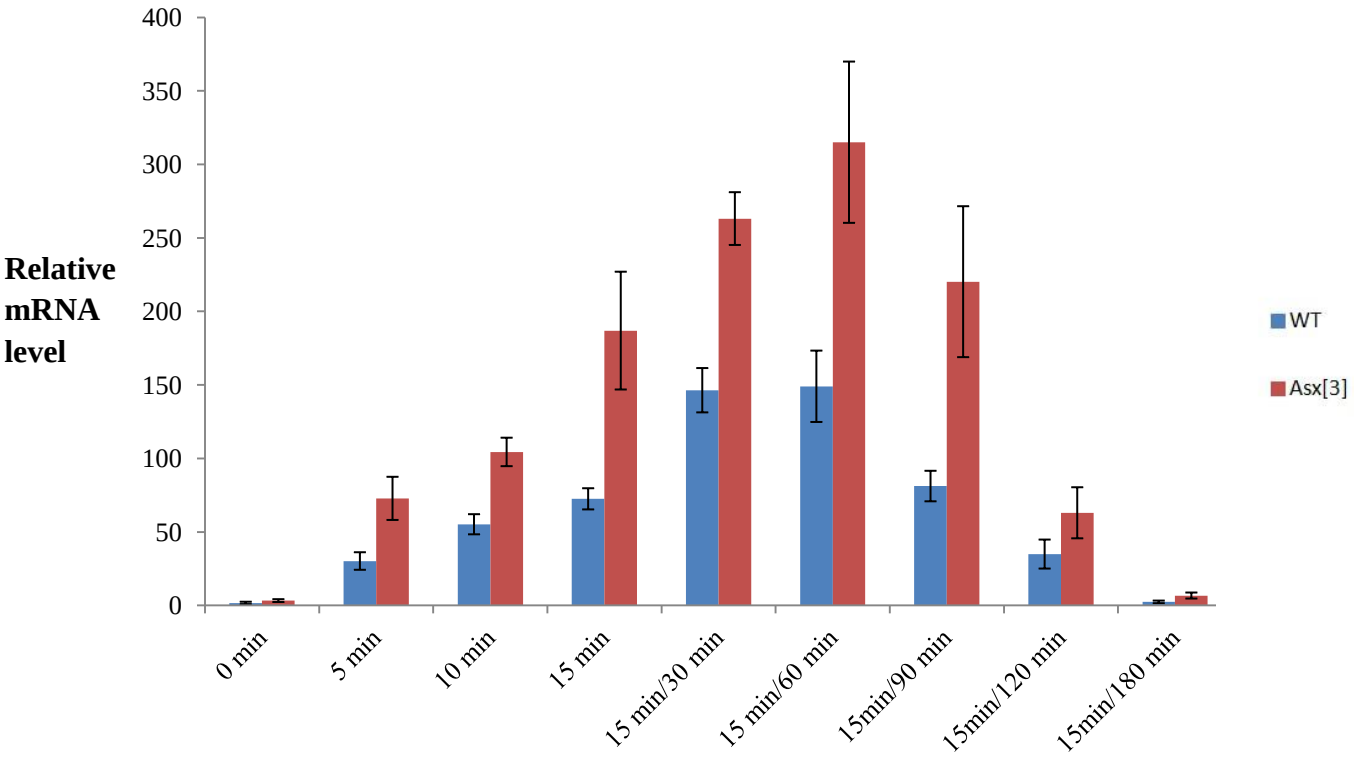

Figure 2-3. Asx is required for *hsp70* repression during heat shock induction and recovery. The relative mRNA levels in wild-type and *Asx*^{3} homozygous mutant embryos were measured by qRT-PCR. The *x* axis is the various heat shock induction times at 37^{o}C, and heat shock recovery times at 22 ^{o}C after 15 min 37^{o}C heat shock induction. The *y* axis is the *hsp70* relative mRNA level. The results were expressed as the *hsp70* mRNA level normalized to the control gene *Ahcy89E* mRNA level. The signals are represented as mean±SEM with n=3. The *Asx* mutation showed at least a 2-fold increase of relative mRNA level compared to wild-type. At 90 min recovery after heat shock induction, the difference reached at maximum level at 2.7-fold. These indicate Asx is required for *hsp70* repression during heat shock induction and recovery.

maximum level of induction (200-fold) was observed at 60 min recovery after heat shock, similar to wild-type (Figure 2-3). Together these data show that *Asx* represses the *hsp70* locus during heat shock induction and recovery. After 180 min recovery, the *hsp70* mRNA level in both wild-type and Asx^3 mutant decreased back to the level before heat shock induction (Figure 2-3), indicating that Asx is not required for repressing the transcription to the level before heat shock induction.

2.5 Histone H3K4 trimethylation heat shock induction and recovery profile at *hsp70* in wild-type embryos

Activation by the trxG is correlated with H3K4me3 mediated by Trithorax, whereas repression by the PcG correlates with H3K27me3 mediated by E(z). The data shown in Figure 2-3 show that levels of *hsp70* increase in Asx^3 mutants, but the molecular mechanism is unknown. The absence of Asx could lead to an increase in methylation of H3K4, thus increasing transcription, or to a decrease in methylation of H3K27, which could also increase transcription, or both if Asx is acting as an ETP. Therefore I decided to examine histone H3 trimethylation levels at *hsp70* in wild-type and Asx^3 mutant embryos using ChIP in an attempt to answer this question. The general experimental design is the same as already outlined for the analysis of *hsp70* mRNA, and each time point results from 3 independent experiments, with 2 technical replicates. The embryos

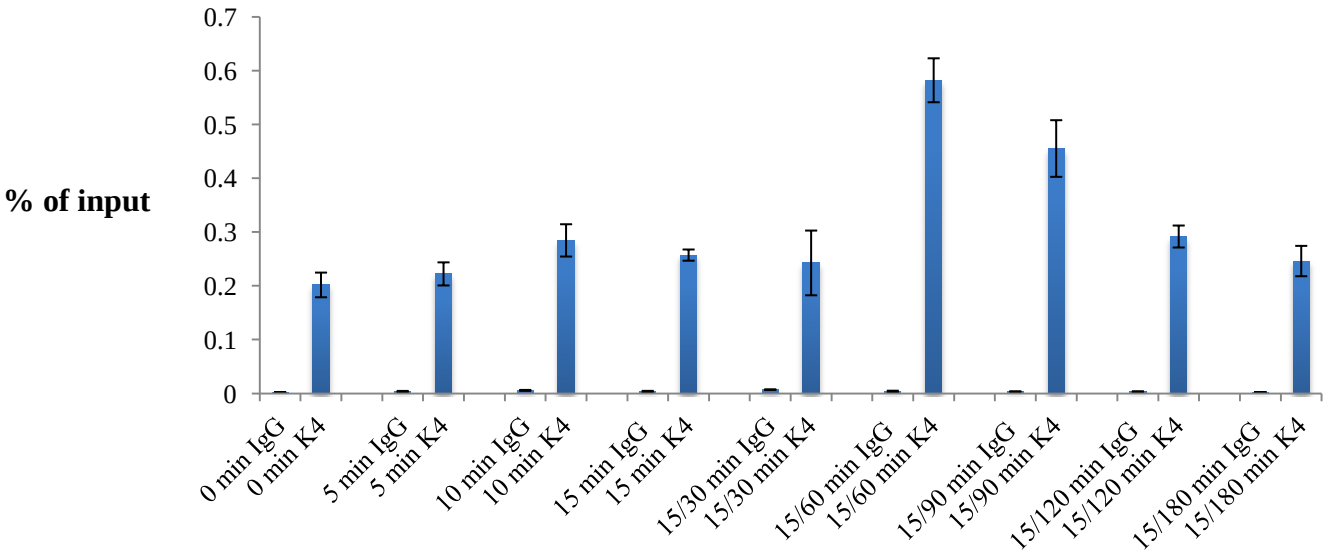

Figure 2-4. Kinetic analyses of histone H3K4 trimethylation level at the *hsp70* locus with heat shock induction and recovery in wild-type embryos. Chromatin immunoprecipitation from wild-type embryos with trimethylated histone H3K4 antibody and rabbit anti-IgG antibody for control. ChIP samples were taken at several heat shock induction and recovery time points as labeled on the *x* axis, and the amount of DNA recovered from ChIP samples was analyzed with real-time PCR. Primers specific to 218 and 392 bp down stream of *hsp70* transcription starting site were used to measure the histone H3K4me3 level at the heat shock promoter. The percentage of input DNA recovered in immunoprecipitate is shown along the *y* axis. The signals are represented as mean±SEM with n=3. The H3K4 trimethylation level peaked at after 60 min of recovery with a 2-fold increase compared to before heat shock induction.

were fixed, sonicated, and subjected to ChIP with control and experimental antibodies as described in the Materials and Methods.

As shown in Figure 2-4, in wild-type embryos, the trimethylated H3K4 level did not show dramatic increase during 0-15 min of heat shock, or during the first 30 min of recovery. The H3K4 trimethylation level peaked at after 60 min of recovery with a 2-fold increase compared to the level before heat shock induction (Figure 2-4). Following this, the H3K4 trimethylation level decreased gradually to the level before heat shock up to 180 min of recovery (Figure 2-4). These results indicate that increased transcriptional level at the *hsp70* locus upon heat shock induction and up to 30 min recovery is not entirely reflected through H3K4 trimethylation level change at the promoter region. These results appear to contradict those obtained previously (Smith et al. 2004) and will be discussed further in the Discussion.

2.6 Asx prevents H3K4 trimethylation deposition at *hsp70* during heat shock induction

If Asx acts as an *hsp70* transcriptional repressor by preventing the activity of other activators, then in Asx^{3} mutant embryos I expected to observe increased levels of H3K4me3 at the *hsp70* promoter region upon heat shock compared to wild-type. As shown in Figure 2-5, the level of H3K4 trimethylation in wild-type and Asx^{3} mutant did not differ markedly in first 10 min of heat shock induction. However at 15 min heat

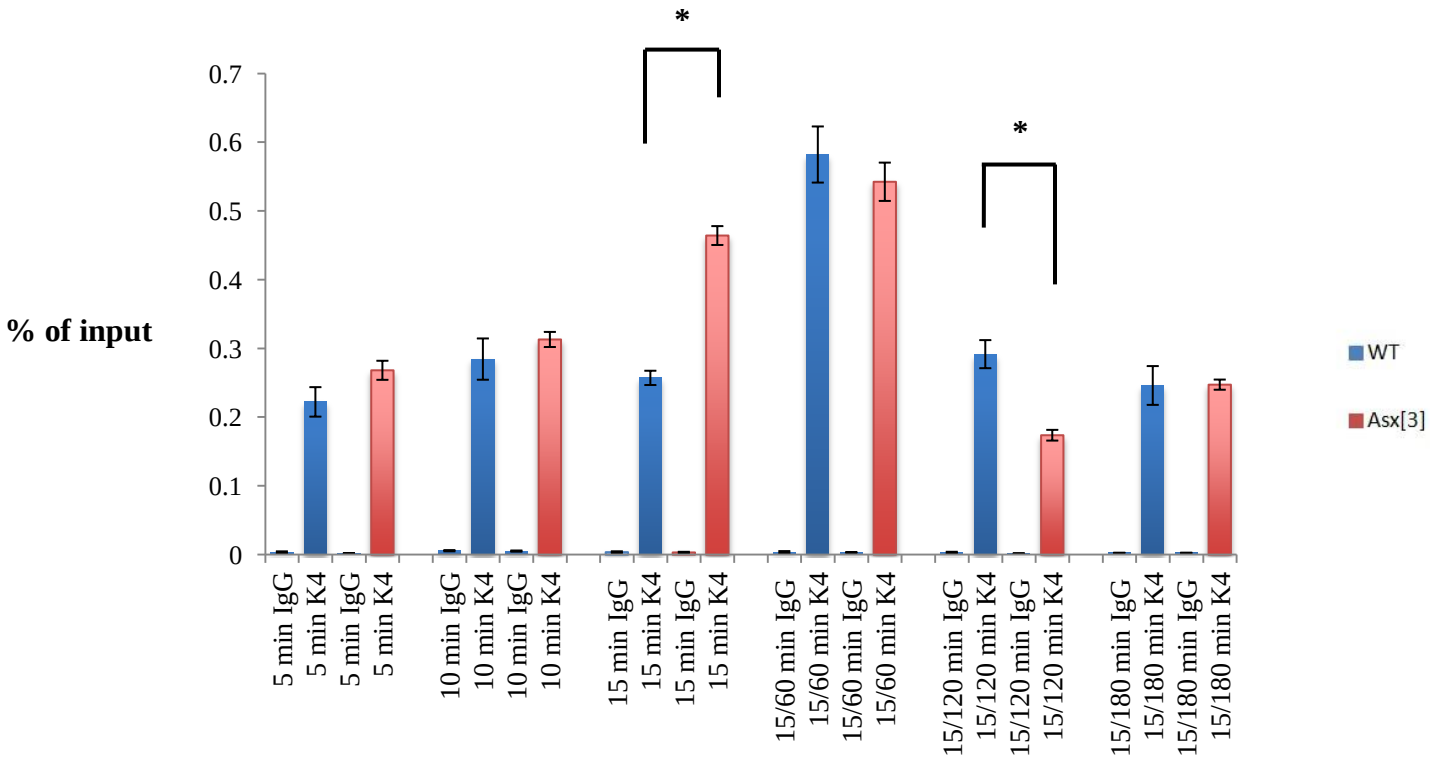

Figure 2-5. H3K4me3 level changes at *hsp70* in *Asx*^{3} mutants. ChIP-qPCR analysis with wild-type and *Asx*^{3} mutant embryos, probed with trimethylated histone H3K4 antibody and rabbit anti-IgG antibody for control. Various heat shock induction time and recovery time after heat shock were labeled on the figure. Primers specific to 218 and 392 bp down stream of *hsp70* transcription starting site were used. The signals are represented as mean ±SEM with n=3. (*) P<0.05. At 15 min heat shock induction the level of H3K4 trimethylation in *Asx*^{3} mutant was 1.8-fold higher than in wild-type. This indicates at 15 min heat shock induction, Asx acts as an *hsp70* transcriptional repressor by preventing the activity of Trx that leads to deposition of H3K4 trimethylation mark.

shock induction the level of H3K4 trimethylation in *Asx*^{3} mutants was 1.8-fold higher than in wild-type (Figure 2-5). This indicates at 15 min heat shock induction, Asx acts as an *hsp70* transcriptional repressor by preventing the activity of Trx, the histone methyltransferase that deposits the H3K4 trimethylation mark. Nevertheless, the observation does not imply the effect of Asx on regulating Trx activity is direct. Afterwards, loss of Asx has no significant effect on H3K4me3 for about 60 min (Figure 2-5). Interestingly, *Asx*^{3} mutants showed less H3K4me3 than wild-type embryos after 120 min of recovery from heat shock. This observation is consistent with the rapid drop of *hsp70* mRNA level during 120 min recovery after heat shock induction (Figure 2-3). The results of the time course analysis argue that Asx's effects on H3K4me3 are transient, which in turn, suggests that Asx activity at *hsp70* is regulated.

2.7 Asx promotes histone H3K27 trimethylation at *hsp70* during heat shock induction and recovery

If Asx acts as an *hsp70* transcriptional repressor by acting as a PcG protein, then I expect to observe decreasing of H3K27 trimethylation level in *Asx*^{3} mutants compared to wild-type during heat shock induction and recovery. Enhancer of Zeste (E(z)), the histone H3K27 methyltransferase is a component of PRC2, and therefore the increased H3K27 trimethylation level upon heat shock induction and recovery is a direct reflection of E(z)/PRC2 activity at *hsp70* (Cao et al., 2002; Czermin et al., 2002). Nevertheless, such

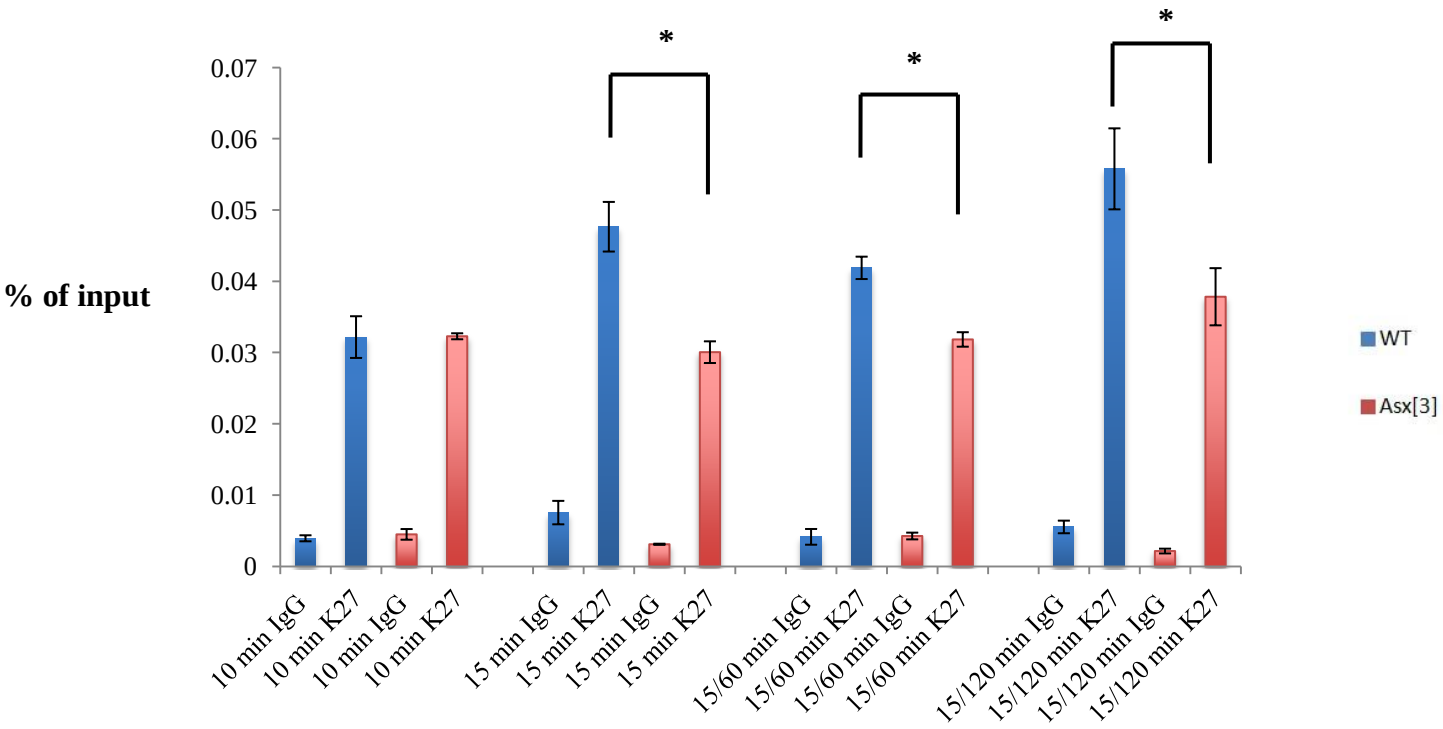

Figure 2-6. H3K27me3 level changes at *hsp70* in *Asx*^{3} mutants. Chromatin immunoprecipitation from wild-type and *Asx*^{3} mutant embryos with trimethylated histone H3K27 antibody and rabbit anti-IgG antibody for control. Various heat shock induction time and recovery time after heat shock were labeled on the figure. Primers specific to 218 and 392 bp down stream of *hsp70* transcription starting site were used. The signals are represented as mean ±SEM with n=3. (*) $P<0.05$. At 15 min heat shock induction to 120 min recovery after heat shock, the level of H3K27 trimethylation in *Asx*^{3} mutant showed continuous 1.5-fold lower level than in wild-type. These indicate Asx acts as an *hsp70* transcriptional repressor by promoting PRC2-mediated repression.

an observation does not necessarily imply that Asx acts directly on E(z) or PRC2.

The level of H3K27 trimethylation in wild-type and *Asx*^{3} mutant did not differ markedly at 10 min of heat shock induction. In subsequent time points from 15 min heat shock induction to 120 min recovery after heat shock, the level of H3K27 trimethylation in *Asx*^{3} mutant was 1.5-fold lower than in wild-type (Figure 2-6). These results show that Asx is a transcriptional repressor at *hsp70* by directly or indirectly increasing PRC2-mediated histone methyltransferase activity, consistent with its PcG activity. These results are consistent with the results from the transcriptional level difference between *Asx*^{3} mutant and wild-type. The increased level of transcription in *Asx*^{3} mutant could be the result of decreased H3K27 trimethylation.

Interestingly, in the *Asx*^{3} mutant the level of H3K27 trimethylation did not show any significant difference at all four time points examined (Figure 2-6). This indicates that the effect of Asx on PRC2-mediated transcriptional repression persist through the entire recovery phase. Combined with results observed with H3K4 trimethylation experiments, these indicate Asx act as a repressor by the combinatorial effect of transiently preventing the molecular function of the transcriptional activator Trx and by promoting PRC2-mediated transcriptional repression throughout the late induction phase and recovery phase.

Prior to 15 min heat shock induction, H3K4 and H3K27 trimethylation levels between *Asx*^{3} mutant and wild-type did not show any significant difference (Figure 2-5,

Figure 2-6), even though effects on transcription are already detected (Figure 2-2). Therefore, at early stages of heat shock induction, the transcriptional repressive role of Asx does not act through H3K4 or H3K27 trimethylation, but must act as a transcriptional repressor by a different molecular mechanism.

3. Discussion

3.1 Asx is required for *hsp70* repression

The comparison of the *hsp70* transcriptional profile of wild-type and Asx^{3} mutant embryos with qRT-PCR indicates that Asx governs *hsp70* transcription by repressing it to certain levels during both heat shock induction and recovery after heat shock (Figure 2-3). These results suggest that Asx functions as a transcriptional repressor. These observations are consistent with the initial identification of *Asx* as a PcG gene because *Hox* genes are derepressed in *Asx* mutant embryos (Jurgens 1985; Sinclair et al., 1992; Sinclair et al., 1998). The results obtained in this thesis are the first demonstration of a role for Asx as a transcriptional repressor on heat stress inducible loci.

Upon heat shock induction, the TAC1 complex that contains the trxG protein Trx is recruited to the promoter of *hsp70*, and is required for maintaining the high level of transcription (Smith et al., 2004). The paused Pol II at *hsp70* shows phosphorylated Ser-5 but not phosphorylated Ser-2 (Boehm et al., 2003), which indicates paused Pol II has been escaped from the general transcription factors at transcription starting site. Therefore, Trx is required for transcriptional elongation because after heat shock induction, the *hsp70* transcriptional level in *trx* mutants was significantly lower than in wild-type (Smith et al., 2004).

Asx also binds to the *hsp70* locus upon heat shock induction (Figure 2-1). Interestingly, Asx and Trx are mutually required to recruit each other at many binding

sites on polytene chromosomes including the *hsp70* locus region 87AB (Petruk et al., 2008; Smith et al., 2004), and a similar observation has been made at the *Ubx* locus (Komljenovic, unpublished). These results could explain why *Asx* mutations enhance the homeotic phenotype of *trx* mutants (Milne et al., 1999). However, these observations suggest a paradox, because collectively they suggest that Asx should be an activator during *hsp70* heat shock induction, rather than a transcriptional repressor as I observed. However, none of the previous studies directly address the molecular function of Asx at *hsp70.* The protein-protein interaction indicates physical association between Trx and Asx, but an interaction does not necessarily imply that they have similar molecular functions.

Therefore, comparing the transcriptional profile of *hsp70* in Asx^{3} mutants with wild-type embryos was an essential first step to begin analysis of Asx function. My studies clearly show that the major molecular function of *Asx* at *hsp70* locus is to repress the transcription to certain level, but *Asx* is not significantly required for transcriptional activation of *hsp70*. However, the data do not rule out a role for Asx in transcriptional activation at *hsp70*. *Asx* is genetically defined as an ETP group gene (Milne et al., 1999). If the primary function of Asx is transcriptional repression at *hsp70* heat shock induction and early recovery phase, then the role of Asx for activation could be masked by its primary repression role at this stage. In the next part I will further discuss the potential role of Asx as *hsp70* transcriptional activator during heat shock recovery phase.

3.2 Asx prevents Trx-mediated H3K4 trimethylation and transcriptional elongation at *hsp70* locus during heat shock induction and recovery

Asx is required for repressing *hsp70* transcription to a certain level upon heat shock induction and recovery after heat shock. However, analysis of the mRNA levels could not reveal whether the absence of Asx leads to increased H3K4 trimethylation, consistent with increased activity of Trx, or decreased H3K27 trimethylation, consistent with less activity of the PcG HMT, E(z), or both.

My results show that the H3K4me3 level in Asx^{3} mutant embryos is elevated at 15 min of heat stress relative to wild-type embryos. Therefore at this time, Asx acts as an *hsp70* repressor by reducing the activity of H3K4 methyltransferase Trx. This observation is consistent with a role for Trx in transcriptional elongation at paused promoters, and with the data showing that mutations in *trx* abolish H3K4 trimethylation after heat shock induction (Smith et al., 2004). Data from our lab shows that Asx coimmunoprecipitates with a small proportion of Trx in embryo nuclear extracts and that the interaction is of moderate affinity but transient (Hodgson, unpublished). The Trx SET domain was recovered in a yeast two-hybrid system screen with Asx as a bait protein (Kyba, unpublished), and this interaction was confirmed *in vitro* with full-length Asx and the Trx SET domain (Hodgson, unpublished). I suggest that Asx and Trx interact directly through the Trx SET domain, and thus that after 15 min of heat stress, Asx reduces Trx HMT

activity.

Strikingly, after 120 min recovery after heat shock induction, the level of H3K4me3 in *Asx*^{3} mutants was lower compared to in wild-type (Figure 2-5). At this time Asx could transiently act as a transcriptional activator to modulate the rate of transcription and prevent the rate of decay from being too fast. I suggest that Asx acts as a governor to regulate the H3K4me3 level at an appropriate level during heat shock and recovery. This conclusion supports the genetic observations that Asx is an ETP. Importantly, this is the first observation known to me of Asx acting as an ETP on the same target in the same cell. This observation raises the interesting question of how the activity of Asx is modulated during heat shock and recovery.

As shown in Figure 2-3 and Figure 2-4, the increased *hsp70* mRNA level in wild-type embryos up to 30 min recovery after heat shock is not entirely reflected by an H3K4me3 level change at the promoter region. My data do not agree with data published previously (Smith et al. 2004). As described in the Introduction, the RNA Polymerase II at *hsp70* pauses downstream of the core promoter before heat shock induction (Rasmussen and Lis, 1993). Upon induction of heat shock stress, the recruitment of HSF and other transcription factors are required for the elongation process (Andrulis et al., 2000; Park et al., 2001; Boehm et al., 2003; Saunders et al., 2003). These data suggest that the promoter region of the *hsp70* locus should contain H3K4 trimethylated histones before heat shock induction because H3K4 trimethylation must be present for promoter escape to occur

(Figure 2-4). If so, the rapid transcriptional activation upon heat shock induction would not require a rapid boost of trimethylated H3K4 level because it relies on recruitment of HSF, Mediator and elongation factors with subsequent modification of Pol II to releasing paused Pol II and initiate elongation (Andrulis et al., 2000; Boehm et al., 2003; Saunders et al., 2003; Park et al., 2001).

I observed an increase of H3K4 trimethylation level 15 min after heat shock induction, which I suggest is required for maintaining the transcription at a stabilized high level. During the first 5-10 min of heat shock induction, the mRNA level of *hsp70* is 2-fold greater in *Asx* mutants versus wild-type, but the trimethylated level of H3K4 did not show significant difference (Figure 2-3, Figure 2-5). At these early stages of heat shock induction, the Asx could block the recruitment of HSF, Mediator or elongation factors at this stage, rather than acting through H3K4 trimethylation or Trx.

3.3 Asx promotes PRC2-mediated histone H3K27 trimethylation at *hsp70* locus during heat shock induction and recovery

The model proposed above suggests that Asx modulates Trx activity, and may act indirectly during early induction on recruitment of other factors. However, these observations do not rule out the possibility that Asx also acts as a PcG protein, perhaps by modulating the HMT activity of E(z) on H3K27. The increased H3K27me3 level at *hsp70* during heat shock induction and recovery is a direct reflection of E(z)/PRC2

activity because E(z) is the only H3K27 trimethylase (Cao et al., 2002; Czermin et al., 2002). My observation that the level of H3K27me3 is lower in Asx^3 mutants during heat shock induction and recovery compared to wild-type is consistent with Asx being required to promote PRC2-mediated transcriptional repression. *Asx* was originally classified as a PcG gene because *Asx* mutations in *Drosophila* exhibit posteriorly directed homeotic transformations and enhance the homeotic phenotype of other PcG gene mutations (Sinclair et al., 1992; Milne et al., 1999). Thus it is not surprising that my results at *hsp70* support a functional association between Asx and PRC2. Nevertheless, the effects of Asx on PRC2 may be indirect. *Asx* mutations decrease levels of *Posterior sex combs* (*Psc*) transcripts (Ali and Bender, 2004). Although Psc is a member of PRC1 complex (Shao et al., 1999), Asx is potentially required for activation of other PcG genes.

As mentioned in the Introduction, in humans the loss of *Asxl1* caused a global loss of H3K27me3 and is also associated with increased expression of homeotic genes (Abdel-Wahab et al., 2012). These observations from a mammalian system are consistent with the results from this thesis that Asx modulates the HMT activity of PRC2 component E(z) on H3K27 trimethylation. ASXL1 was co-immunoprecipitated with the PRC2 components EZH2, SUZ12 and EED, strongly suggesting that ASXL1 interacts with PRC2 and ASXL1 plays a key role in maintain the repression state of genes (Abdel-Wahab et al., 2012). In the future, to detect whether the Asx-PRC2 interaction also exist in *Drosophila*, similar immunoprecipitation experiments can be performed with

nuclear extract from *Drosophila* embryos.

Interestingly, in Asx^{3} mutants the level of trimethylated H3K27 was not significantly different after 10 min heat shock compared to wild-type (Figure 2-6). Therefore during early stage of heat shock induction, Asx-mediated repression is not caused by changing the level of H3K4 or H3K27 trimethylation, lending further support to the hypothesis that Asx-mediated repression blocks the recruitment of HSF, Mediator or elongation factors at early stage of heat shock induction. PcG and trxG genes are required to stably maintain the repression or activation of homeotic loci but not required for establishing the early expression pattern (Jurgens, 1985; Struhl and Akam, 1985; Simon, 1995). The observation from this thesis on Asx-mediated repression is consistent with the observations on homeotic genes.

3.4 The role of Asx, trxG and PcG interactions at the promoter

Data from other systems indicate that it may not be simple to untangle the roles of Asx at *hsp70*. Increased levels of H3K4me3 level at *in vitro* reconstituted mono-nucleosomes disrupt PRC2 binding by blocking the binding site of the Nurf-55-Su(z)12 sub-module of PRC2 (Schmitges et al., 2011). This direct inhibition of PRC2 by H3K4me3 is conserved in *Drosophila* and mammals. Trx functions as an anti-repressor that prevents PcG-mediated transcriptional repression of *Hox* genes at their normal expression domain (Klymenko and Muller, 2004). In wild-type embryos, I

suggest that Trx-mediated transcriptional elongation with its H3K4 methyltransferase activity at the *hsp70* promoter region is the key event that maintains the transcriptional level after heat shock induction. In the absence of Asx, the HMT activity of Trx would decrease about 15 min after heat shock, and this would lead to a secondary increase in trimethylation of H3K27 by PRC2. Consistent with this model, inhibition of Trx activity at *hsp70* indirectly promotes the activity of PRC2-mediated transcriptional repression and accompanied with increased level of H3K27 trimethylation (Poux et al., 2002).

Interestingly, at 5 min of heat shock induction, the promoter region of *hsp70* with paused Pol II has both H3K4me3 and H3K27me3 (Figure 2-5; Figure 2-6). This observation of possibly H3K4me3 and H3K27me3 bivalently marked *hsp70* promoter region prior to transcription is strongly reminiscent of many genes in mammalian embryonic stem (ES) cells and some differentiated cells, where it is proposed that bivalent marks allow developmental plasticity (Bernstein et al., 2006; Mikkelsen et al., 2007; Pan et al., 2007; Zhao et al., 2007). The existence of possible bivalent marks suggests that Asx modulation of *hsp70* could occur through regulation of H3K4me3 or H3K27me3 demethylases.

JMJD3 is the mammalian homolog of *Drosophila* dUTX (Smith et al., 2008). The JMJD3-mediated histone H3K27me3 demethylation at bivalently marked promoter regions with paused Pol II is required for initiation of transcription elongation (Smith et al., 2008; Chen et al., 2012). JMJD3 is also required for recruitment of elongation factors,

which supports the model that JMJD3 is required for activating gene transcription by demethylating H3K27me3 and promoting transcriptional elongation (Chen et al., 2012).

dUTX is recruited to heat shock promoter regions upon heat shock induction in *Drosophila* S2 cells, which supports the model that dUTX-mediated H3K27me3 demethylation is associated with *hsp70* elongation, and the function of H3K27me3 demethylation in transcription elongation of genes with paused Pol II is likely conserved in mammals and flies (Smith et al., 2008). My results do not rule out the possibility that H3K27me3 demethylation is required preceding Trx-mediated trimethylation of H3K4 during *hsp70* transcription elongation. Asx alters both H3K4me3 and H3K27me3 level starting at 15 min after heat shock induction, so it is possible that Asx-mediated *hsp70* repression prevents H3K27me3 demethylation at the bivalently marked promoter region, which prevents transcription elongation. However, the effects of Asx-mediated *hsp70* repression I observed occur after the time of potential demethylation of H3K27me3 that occurs during the transition to transcriptional elongation. Therefore, I prefer the model that Asx directly prevents the Trx activity on *hsp70.*

In *Drosophila,* Asx forms the PR-DUB complex with Calypso and this interaction is critical for the enzymatic activity of Calypso on global H2A deubiquitination (Scheuermann et al., 2010). This interaction is conserved in mammalian homologs ASXL1-BAP1 (Scheuermann et al., 2010). As mentioned in the Introduction, this complex is possibly required for deubiquitination of both H2Aub and H2Bub that favour

gene activation and repression respectively, and this could explain the molecular function of Asx as an ETP. However, the results from this thesis and other findings from mammalian systems are in conflict with this hypothesis. If Asx-Calypso and ASXL1-BAP1 are required for histone H2A deubiquitination that favour gene activation, then loss of H2Aub will lead to a H3K27me3 level decrease at target genes. However, observations at the *hsp70* locus and at the *Hox* loci in human embryonic kidney cells showed that the loss of *Asx*/*Asxl1* causes loss of H3K27me3 (Figure 2-6; Abedl-Wahab et al., 2012), suggesting that Asx/ASXL1 promotes PRC2 activity by modulating the HMT activity of E(z)/EZH2 on H3K27me3. Consistent with these observations, in human embryonic kidney cells, the effects of *Asxl1* mutation on H2Aub was minimal compared with the effects on the level of H3K27me3 decrease at homeotic loci (Abedl-Wahab et al., 2012). My preliminary Western blot data on H2Bub levels in *Asx* or *calypso* mutations showed that a global H2Bub level decrease was not detected (data not shown), suggesting that Asx-Calypso is not required for global deubiquitination of H2B. I cannot exclude the possibility that Asx-Calypso functions as histone H2A and H2B deubiquitinase at specific loci, as only very few loci has been investigated in this thesis and in the mammalian system.

3.5 Role of Asx at developmentally regulated genes in relation to studies of *hsp70*

It is still unknown how Asx could have different molecular roles at different gene

targets during embryonic development, or in different cell types. The mammalian *Asx* homolog ASXL1 is a transcriptional co-activator of the retinoic acid receptor (RAR) and retinoid X receptor (RXR) in human retinoic acid sensitive cancer cell lines (Cho et al., 2006). ASXL1 also functions as a co-repressor of RAR activity in different mammalian cell lines (Cho et al., 2006). These authors proposed that ASXL1 activity was mediated through the functional cooperation with steroid receptor coactivator-1 (SRC-1), which associates with the CBP/p300 transcriptional co-activators. CBP is a histone H3K27 acetyltransferase (Tie et al., 2009). In *Drosophila,* CBP-mediated H3K27 acetylation antagonizes PRC-2 mediated H3K27me3 and this constitutes a critical part of the molecular mechanism of Trx antagonizing PRC-2 mediated silencing (Tie et al., 2009). Combined with the observations that Asx and Trx coordinately recruit each other at *Ubx* (Komljenovic, unpublished) and Trx belongs to the TAC1 protein complex (Smith et al., 2004) which contains CBP, it is possible that Asx and its mammalian homologues act as a transcriptional activators by forming different protein complexes that are required for transcriptional activation.

As noted above, the molecular analysis of H3K4me3 and H3K27me3 levels in an *Asx* mutant background is insufficient to show whether the effect of Asx on *hsp70* is direct or not. In future, to detect the direct molecular mechanism of Asx on *hsp70* repression, ChIP experiments similar to those shown in Figure 1-4 need to be performed at the *hsp70* locus with the same Pol II antibodies as described earlier. If Asx blocks transcriptional

initiation, promoter clearance or elongation, then I would expect to observe increase binding of unmodified, Ser-2P or Ser-5P modified Pol II in *Asx*^{3} mutant embryos compared to wild-type. If Asx is not involved in blocking any transcriptional processes at the early stage of heat shock induction, then at the early induction stages Asx may act on heat shock response repression without affecting steps involved in the generation of a productive elongation as listed above. It would also be possible to study the role of Asx in *hsp70* repression by overexpressing *Asx* with a similar gain-of-function assay as described previously (Dellino et al., 2004). Asx acts as an *hsp70* repressor during heat shock induction and recovery. Overexpressing *Asx* should further repress *hsp70* activity during heat shock and recovery. Initiation of transcription requires recruitment of unmodified Pol II. In later promoter clearance and transcription elongation Ser-5 and Ser-2 residue at CTD of Pol II are sequentially phosphorylated. Therefore a decrease in unmodified or CTD phosphorylated Pol II binding in *Asx* mutant strains compared to wild-type is expected, which is easier to detect compared to an increase of binding.

3.6 *Ubx* as a target to study the molecular role of Asx in development

This thesis provides further molecular evidence that Asx is an ETP. However, the activity of Asx as an activator or repressor depends on the gene target, cell type, and time of development. *Asx* mutations cause simultaneous derepression and failure of activation of *Ubx* expression, suggesting that *Ubx* is an excellent candidate for future analysis. As

described in the Introduction, ChIP analysis confirms that Asx binds to the *Ubx* promoter region, and shows Asx is required for promoter clearance of *Ubx* in bulk prepared late embryos. However, *Ubx* expression is spatially regulated, so future studies should not use whole embryos. *Ubx* is expressed only in posterior segments of the embryo (Akam and Martinez-Arias, 1985), and in the anterior end of the embryo, *Ubx* expression is repressed by the gap gene hunchback (White and Lehmann, 1986). To study the molecular mechanism of Asx in repression of *Ubx* in early embryos, it will be necessary to use fluorescence-activated cell sorting (FACS) to obtain cells that do and do not express *Ubx*. As shown in Figure 1-2B, our lab has obtained embryonic lines that express green fluorescent protein (GFP) under the control of *Ubx* enhancers to mark cells in which *Ubx* is expressed (Petruk et al., 2006). We use mCherry under the control of *hb* enhancers to mark cells in which *Ubx* is repressed. In the future *Ubx* could be studied in both *Asx* mutant and wild-type cells in which *Ubx* is or is not expressed to better understand the molecular role of Asx as an ETP.

3.7 Summary

This thesis is the first study examining the molecular mechanism of Asx at the inducible gene *hsp70*. My data supports a model in which Asx directly prevents Trx activity during *hsp70* heat shock induction and indirectly promotes the activity of PRC2-mediated repression. At the early stages of *hsp70* heat shock induction,

Asx-mediated repression likely plays a role in blocking the recruitment of Heat shock factor (HSF), Mediator or elongation factors rather than altering histone H3K4 or H3K27 trimethylation level. Combined with other studies in the lab on *Ubx*, we can for the first time propose testable models for Asx function as an ETP.

4. Materials and Methods

Culture conditions and *Drosophila* stocks

Fly cultures were grown in vials and bottles with standard media (cornmeal, yeast, molasses, antibiotics and mould inhibitors). All stocks were maintained at 18℃ unless otherwise noted. The description of all stocks and strains can be found on the Flybase website (www.flybase.org).

Embryo collection

Embryos from stocks were collected at 22℃ by placing approximately 300 flies of equal male to female ratio into an embryo collection chamber capped with nylon mesh at the top to ensure sufficient air supply. Eggs were deposited onto an egg lay plate (100 mm Petri dish containing 1% sucrose, 2% agar, 3.5% ethanol and 1.5% apple cider vinegar in water), supplemented with live yeast paste. Flies were acclimatized to the laying chamber for two days before 14-16 hr embryos were collected, with changing of laying plates twice every day to reduce the collection of stored eggs. Embryos on laying plates were immediately washed into a nylon sieve to remove excess yeast, and immersed into 50% bleach for 1.5 min to dechorionate. Embryos were washed twice with cold Embryo Washing Buffer (120 mM NaCl; 0.02% Triton X-100), followed by two times 1X PBT (1X PBS; 0.05% Triton X-100) (1X PBS; Sambrook et al., 1989) wash. *Asx*^{3} mutants were previously balanced with *CyO Twist-GAL4, UAS-eGFP* balancer. *Asx*^{3}

homozygous mutant embryos were identified by the absence of *GFP* expression under a wide-field GFP fluorescence microscope. The wild-type strain Oregon R was used as a control.

Heat shock induction and recovery

Wild-type or homozygous Asx^{3} mutant embryos were collected and transferred into 1.5 mL plastic microcentrifuge tubes with 200 μL 1X PBT, and the tubes were incubated in a 37.0 ℃ water bath to induce the heat shock response for the designed induction time. Only 15 min heat shocked embryos were used to perform the heat shock recovery experiment. These embryos were transferred onto a small piece of moist filter paper placed in a 100 mm Petri Dish-sized moist chamber. After reaching the designed recovery time, embryos were transferred to a new tube with 200 μL 1X PBT for further analysis.

Determining *hsp70* mRNA level in embryos

Following designated heat shock induction and recovery treatment, embryos were rinsed twice with water to wash off excessive salts. Total RNA was extracted based on the manufacturer's protocol (Invitrogen) with 50 embryos for each data point. In brief, embryos were homogenized in 1mL Trizol (Invitrogen) for 1 min, insoluble material was removed by centrifuging for 10 min at 12,000 g, and 200 μL of chloroform was added to the supernatant, which was centrifuged for 15 min at 12,000 g in 4 ℃ to obtain the

aqueous phase with total DNA and RNA. Then 500 μL of isopropanol was added to the extract, the DNA and RNA allowed to precipitate for 10 min at room temperature, and the DNA/RNA recovered by centrifugation for 10 min at 12,000 g in 4 ℃ for 10 min. Pellets were washed twice with 75% ethanol, air dried for 10 min and resuspended in 50 μL nuclease free water. Pellets were treated with a DNAse treatment and removal kit (DNA-*free*; Ambion, Invitrogen) to remove DNA from the mixture. The RNA recovered was quantified using the Quant-iT RNA assay kit (Molecular Probes, Invitrogen). First-strand cDNA synthesis was performed with 0.3 μg total RNA primed with 250 ng of random pentadecamer using 200 U of the Superscript III Reverse Transcriptase kit (Invitrogen) and RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen) at 55 ℃ for 60 min. The reaction was inactivated by heating at 75 ℃ for 15 min. For quantitative PCR (q-PCR) analysis of *hsp70* and *Ahcy89E* transcripts in wild-type and Asx^{3} homozygous mutant embryos, cDNA was diluted to an amount equivalent to 100 ng of total input RNA. Samples were amplified with 1 U Hot Start Taq polymerase (Fermentas) in a 25 μL reaction containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 μM primers and EvaGreen dye (Biotium) with the following cycle paramters: one cycle at 95 ℃ for 5 min; 40 cycles at 95 ℃ for 20 s/58 ℃ for 20s/72 ℃ for 20s. Primers for amplification were: *hsp70*: +218 to +392 (forward 5'-TCA ACA AGT CGT TAC CGA GG-3' and reverse 5'-CGT TCC GAA TCT GTG AAA GC-3'); *Ahcy89E* (forward 5'-TCT GAT GTG TCC ACC TGC TC-3' and reverse 5' –GCC TTC CAA ATG CAC TCT TC-3'). A dilution

series of *Drosophila* genomic DNA was used to generate standard curves for *hsp70* and *Ahcy89E*. All quantitative PCR reactions were performed with StepOnePlus Real Time PCR system (ABI). The results were expressed as *hsp70* expression normalized to *Ahcy89E*. The signals are represented as mean ± standard error of the mean (SEM) with n=3.

Chromatin immunoprecipitation (ChIP)

After heat shock induction or heat shock with recovery treatments, embryos were washed once with room temperature 1X PBT. Formaldehyde was added to a final concentration of 2% and embryos were kept on a rocker for 10 min at room temperature, and then placed at 4℃ for 20 min. The crosslinking was terminated by adding glycine solution to a final concentration of 0.1 M with incubation at 4℃ for 5 min. Samples were washed twice with 1X PBT then twice with sonication buffer (10 mM Tris-HCl at pH 8.0, 10 mM EDTA and 10 mM EGTA) and stored in 200 µL sonication buffer with protease inhibitors (cOmplete, mini, EDTA-free; Roche) at -80℃. Embryos were then divided so that each sample contained 200 embryos and embryos were sonicated for 5 cycles of 10 s with 50 s pulse at 30% power on ice with the ultrasonic processor (CPX 130PB, Cole Parmer). Samples were mixed with 200 µL of 6 M urea with 10 min incubation on ice and insoluble material was removed by centrifuging for 10 min at 12,000 g in 4℃. The supernatant was divided into 4 x 100 µL aliquots, and adjusted to a final volume of 1 mL

with 500 μL IP buffer (10 mM Tris-HCl at pH 8.0, 11 mM EDTA, 0.5 mM EGTA, 167 mM NaCl, 1.1% Trition X-100 and 0.1% deoxycholate) and protease inhibitors, so that each sample contained the equivalent of approximately 50 embryos. Samples were pre-cleared by adding 20 μL of 50% protein G Agarose beads (Sigma) and incubated at 4 ℃ for 1 hr on a nutator. Beads were removed by centrifugation at 6000 g for 2 min at 4℃, and then appropriate amount of antibodies were added and samples were incubated overnight on a nutator at 4℃. An equivalent amount of Rabbit polyclonal IgG antibody was added to a second tube to control for non-specific binding. Next, 20 μL of 50% protein G Agarose beads were added and incubated on a nutator at 4℃ for 2 hr. Beads were spun down at 2500 g for 1 min, the supernatant was removed, and 1 mL of Low Salt washing buffer (0.2 M NaCl, 20 mM Tris-HCl at pH 8.0, 2 mM EDTA, 1% Triton X-100 and 0.1 % SDS) was added and samples were incubated at 4℃ for 30 min. Next, samples were washed with High Salt washing buffer (Low Salt buffer except 0.5M NaCl) for 30 min, and LiCl washing buffer (0.25 M LiCl, 10 mM Tris-HCl at pH 8.0, 1 mM EDTA, 1% IGEPAL CA-630 and 1% deoxycholate) for 10 min, followed with 2X TE buffer wash (10 mM Tris-HCl at pH 8.0 and 1 mM EDTA) for 5 min. Each time, the washing solution was removed after centrifugation at 3000 g for 1 min at 4℃. After the final washing solution was removed, 100 μL of 10% Chelex 100 resin (Biorad) were added to Protein G beads with vortexing, and samples were incubated at 95℃ for 10 min. After cooling down to room temperature, 1 μL of 10 mg/mL Proteinase K (Roche) was added and

mixed, and samples were incubated at 55 ℃ for 30 min, followed with 95 ℃ for 10 min. Samples were cooled down to room temperature, and 90 μL of supernatant were transferred out to new tubes as the immunoprecipitated chromatin sample. Another 100 μL of nuclease free water was added to the original tubes and after mixing then centrifuging, 90 μL of supernatant were transferred out to combine with previous supernatant for a total of 180 μL. Immunoprecipitated material was then assayed by quantitative PCR using primers specific to sequences at the *hsp70* locus as described above. Approximately 3% of the immunoprecipitated material was used for q-PCR analysis.

ChIP experiments were performed with polyclonal rabbit anti-trimethyl histone H3K4 antibody (Active Motif, Cat.# 39159) at 1/1000 dilution and polyclonal rabbit anti-trimethyl histone H3K27 antibody at 1/100 dilution (Millipore, Cat.# 07-449). The rabbit anti-Asx antibody was raised against *Drosophila* Asx amino acid sequence 200-356. The epitope for antibody production was expressed as a GST-fusion protein in *E.coli* and purified under denaturing conditions. Rabbit polyclonal IgG antibody at 1/200 dilution (abcam, Cat.# ab27478) was used as a negative control. The signals are represented as mean ±SEM with n=3. The statistical significance of the data was determined with one-tailed Student's t-test with a significance level of 0.05.

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