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Enhancing Transcription by Release of RNAP II from the Promoter-Proximal Pause

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M.S., Georgia State University, 2001
B.S., Bowling Green State University, 1998

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Abstract

Enhancing Transcription by Release of RNAP II from the Promoter-Proximal Pause

By Wendy A. Kellner

Transcription regulation by promoter-proximal pausing is the rate limiting step for the expression of a large number of genes. The steps necessary for release of RNA polymerase II from this pause are not well characterized. Although covalent histone modifications have been implicated in this process, their role in this regulatory step is not well understood. This study elucidates some of the histone modifying enzymes and the modifications they perform in order to characterize the link between histone phosphorylation and acetylation. 14-3-3 is identified as a critical protein necessary for mediating the connection between histone phosphorylation and acetylation that we show is necessary for release of RNA polymerase into transcriptional elongation. Transcription factors bind through sequence specific interactions that ultimately mediate transcription activation to the promoter region of the genes they regulate. In the large percentage of genes that have paused RNA polymerase II at the 5' end of the gene, the activating signal should mediate the release of the polymerase from a paused state into transcriptional elongation. Histone modifications also play a role in regulation of transcription at enhancers and we identify histone phosphorylation by JIL-1, 14-3-3 recruitment and acetylation of histones by CBP at enhancers and the promoters they regulate suggesting an interaction between these two sequences. This interaction is confirmed by chromatin conformation capture assays and shown to be dependent on JIL-1, 14-3-3 and CBP. Lastly, in order to investigate these steps as a genome-wide phenomenon during transcription activation across we demonstrate that histone phosphoacetylation of both H3K9acS10ph and H3K27acS28ph occur at both enhancers and promoters.

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Chapter 1

Introduction

Regulation of Transcription by Promoter-Proximal Pausing

Cell differentiation leading to the establishment of unique cell types that perform specific functions is regulated by a transcriptional program that is a signature of a given cell type. Whereas the nucleus of every cell of a multicellular organism contains the same DNA sequence, a specific pattern of gene expression characterizes a cell type and defines its function. The differentiation process into distinct terminal cell types is driven by cell signaling from both neighboring and distant cells to modulate transcription levels. Additionally, any given cell type needs to integrate external and internal signals and respond to environmental cues even after achieving a terminally differentiated state. Signal transduction pathways allow cells to receive information from their environment and transform it into a transcriptional response by changing expression levels of specific genes. Ultimately, it is the set of genes expressed and transcriptional responses to stimuli that determine the cell type.

Most studies of gene expression mechanisms have concentrated on the pre-initiation and initiation steps of the transcription cycle. However, work from John Lis and collaborators on the *Drosophila Hsp70* genes first demonstrated that these genes have RNA Polymerase II (RNAP II) initiated at the locus but do not continue to the transcription elongation step until the cells are subjected to elevated temperatures (Rougvie and Lis, 1990). It has become apparent that a large proportion of eukaryotic genes contain a significant amount of initiated RNAPII downstream of the transcription start site regardless of transcription levels. Moreover, developmental genes and gene targets at the end of signal transduction pathways are preferentially loaded with a paused RNAP II. This phenomenon, termed promoter-proximal pausing, is characterized by the presence of disproportionately more initiated RNAP II located at the promoter than elongating polymerase inside the gene and has been shown to be an important

regulatory step for these genes (Krumm et al., 1995; Zeitlinger et al., 2007; Muse et al., 2007). Although RNAP II is paused immediately downstream from the transcription start site (TSS) at these genes, it is competent in producing short transcripts of 25-50 bp (Nechaev et al., 2010). This study addresses some of the steps involved to release RNAPII from promoter-proximal pausing and into productive transcription elongation.

Histone code regulation of transcription

Transcription activation in metazoans is associated with the establishment of a complex histone code in which the transcription status of a gene correlates with post-translational modifications. The histone code hypothesis proposed by Strahl and Allis predicts that certain proteins (writers) carry out post-translational modifications to provide binding platforms for certain domains of other proteins designed to recognize these modifications (readers) to recruit the proper proteins (Strahl and Allis, 2000). Interconnected phosphorylation, acetylation, and methylation of histones at promoters that is necessary for proper transcription of genes is well documented (Guenther et al., 2007; Heintzman et al., 2007; Berger, 2007). For example, trimethylation of H3K27 (H3K27me3) and H3K9 (H3K9me3) is associated with regions of the chromosomes that are not transcribed, and conversely, acetylation of the same residues is associated with active transcription. Heterochromatin protein 1 (HP1), a chromatin condensing protein, binds H3K9me3 using a chromodomain that recognizes methylated lysine residues to achieve maximum chromatin compaction (Jacobs et al., 2002). The SWI/SNF complex required for displacement of nucleosomes during transcription activation binds histone H3 acetylated on lysine 9 (H3K9ac) using bromodomains that recognize acetylated lysine residues (Hassan et al., 2002). These are some examples of how histone modifications provide information for other proteins to facilitate or prevent access to the DNA for transcription activation or silencing.

JIL-1 phosphorylates H3S10 upon transcriptional activation during interphase

Phosphorylation of histone H3 at serine 10 (H3S10ph) is a modification that occurs at the promoter-proximal pause and is required for the release of polymerase into transcription elongation during interphase (Ivaldi et al., 2007). The first goal of this study is to investigate the function of H3S10ph and identify proteins that recognize this modification and might also be necessary for release of RNAPII. In *Drosophila*, H3S10 phosphorylation is performed by the JIL-1 kinase upon transcriptional activation (Jin et al., 1999). During mitosis, the aurora kinase is responsible for phosphorylation of the same residue and thought to play a role in displacement of heterochromatin binding protein HP1 on highly condensed chromatin regions (Fischle et al., 2005). Therefore, it is likely that phosphorylation of H3 during transcriptional activation in interphase serves a different purpose than phosphorylation during mitosis, when there is no transcription. More specifically, phosphorylation of H3S10 by JIL-1 during interphase has been demonstrated to occur after RNAP II initiation and to be necessary for elongation, placing the activity at the promoter-proximal pause (Ivaldi et al., 2007). Interestingly, the mammalian orthologues of JIL-1, Msk1/2, have multiple phosphorylation sites from several kinase cascades such as the Erk, MAPK, and p38 pathways, integrating signals from both internal and external stimuli (McCoy et al., 2005). Msk1/2 activity is regulated by phosphorylation at a number of residues, such that the more highly phosphorylated the higher the activity. This feature would make Msk1/2 or JIL-1 kinases ideal for regulation of transcription targets of cell signaling (Arthur, 2008). Supporting these data is the fact that signal transduction gene targets often have poised RNAPII at the 5' end of the genes, and JIL-1 activity has been suggested to be necessary for release from the promoter-proximal pause (Figure 1-2) (Ivaldi et al., 2007).

14-3-3 is able to bind phosphorylated histones

14-3-3, a phospho-binding protein, is well characterized as playing a critical role in signal transduction pathways. One function of 14-3-3 is mediating interactions by binding two or more phosphorylated residues on either the same protein or different proteins to form dimmers and multimers between the 14-3-3 molecules (Bridges and Moorehead, 2004). There are two serine residues on the histone H3 tail that could be possibly be phosphorylated, serine 10 (S10ph) and serine 28 (S28ph). *In vitro* peptide binding assays show 14-3-3 has the ability to bind both serine residues, with stronger affinity for H3S28ph (Winter et al., 2008). Moreover, 14-3-3 binding to substrates with two 14-3-3 binding motifs demonstrates a 30-fold binding affinity over substrates with one 14-3-3 binding motif suggesting synergysic binding (Yaffe et al., 1997). While some studies have investigated a possible role for H3S10ph during transcription, H3S28ph has largely been ignored (Zippo et al., 2007; Karrasch et al., 2006; Nowak and Corces, 2000). If 14-3-3 is involved during transcriptional activation, it is likely mediated through interactions of both H3S10ph and H3S28ph. Because H3S10ph has already been demonstrated to play a role at the promoter-proximal pause a role for 14-3-3 in regulation at this step will be investigated during this study as well as a role for H3S28ph for helping to recruit 14-3-3.

***Drosophila* as a model for transcription regulation**

Regulation of transcription by histone modifications and promoter-proximal pausing is conserved from mammals to *D. melanogaster*, providing an excellent model system for investigation of the steps involved in this regulation. During larval stages, salivary glands go through many rounds of replication without cell division, resulting in chromosomes containing thousands of copies of the DNA, termed polytene chromosomes. These copies align due to homology and allow for visualization of the

DNA by immunostaining of salivary glands at the end of the third instar larval stage. Staining polytene chromosomes with DAPI, a fluorescent dye that stains DNA, reveals a banding pattern with regions of high DNA compaction and low transcriptional activity indicated by dark staining, termed bands. Regions with low compaction and little staining are termed interbands and are characterized by higher transcriptional activity. Therefore proteins that localize to interband regions by immunostaining are associated with highly transcribed genes.

In addition to being present during interphase, H3S10ph as well as 14-3-3 binding occurs during mitosis at levels much greater than during interphase. This situation creates a problem for looking at *in vivo* levels of H3S10ph and 14-3-3 binding on the chromosomes in any cell population (Figure 1-1). Since the polytene chromosomes of the salivary glands do not enter mitosis, this system affords a way to visualize protein components associated with transcription without contaminating signal from mitotic cells.

In addition to providing a way to visualize proteins associated with the DNA, *Drosophila* provides a simpler system to analyze proteins necessary for transcription. For example, seven kinases have been reported to be capable of carrying out H3S10 phosphorylation in mammals. *Drosophila* has only one kinase responsible for carrying out this modification during interphase, simplifying studies. The same situation is true for 14-3-3 proteins, known to bind the phosphorylated H3S10 residue. Mammals have nine isoforms of 14-3-3 while *Drosophila* has just two, 14-3-3ζ and 14-3-3ε, providing a simple model to study 14-3-3 function. Moreover, six of the nine mammalian isoforms have been demonstrated to

P-TEFb mediates the release of RNAPII from the promoter-proximal pause

It has been propsed that RNAP II is initiated at high levels but does not continue to transcribe past the promoter-proximal pause in order to prevent encroaching repressive marks into the promoter region. This maintains a histone free region with a RNAP II ready to transcribe allowing for a rapid transcriptional response to activating signals (Gilchrist et al., 2010). As a result, genes that have a paused polymerase must have a mechanism to prevent RNAPII from elongating until increased transcript levels of these genes are needed. Negative Elongation Factor (NELF) and DRB-Sensitivity Inducing Factor (DSIF) have been demonstrated to have a role in preventing elongation and stabilizing RNAP II at the promoter-proximal pause (Andrulis et al, 2000; Yamaguchi et al., 1999). Positive transcription elongation factor b (P-TEFb), a complex containing Cyclin T and Cdk 9, is recruited to the poised RNAP II upon transcriptional activation and phosphorylates DSIF and the RNAPII c-terminal domain (CTD) on serine 2, to release RNAP II from the pause (Peterlin and Price, 2006; Lis et al, 2000). The phosphorylation of DSIF results in release of NELF from the complex, eliminating its repressive activity. Additionally, phosphorylation of Spt5, a subunit of DSIF, changes its conformation to that of a clamp for RNAPII needed for processivity into elongation (Cheng and Price, 2007; Martinez-Rucobo et al., 2011).

In mammals, Brd4 recruits P-TEFb to the 5’ end of genes and is thought to release paused polymerase into productive elongation (Jang et al., 2005). Female sterile (1) homeotic [Fs(1)h] is the only BET family member in *D. melanogaster* and is the closest homologue to Brd4 in mammals. Both Brd4 and Fs(1)h have two double bromodomains, making one molecule capable of binding a total of four acetylated lysine residues at a time. The best characterized binding partners of Brd4 via the bromodomains are histones, known to be hyperacetylated upon transcription activation (Dey et al., 2003). Peptide binding assays have shown that one of the bromodomains has high affinity for histone H3 acetylated on both lysines 9 and 14 whereas the second

bromodomain has high affinity for histone H4 acetylated at various combinations of lysines 5, 8, 12, and 16 (Vollmuth et al., 2009). Brd4 appears to be the link between histone acetylation and release of RNAPII by P-TEFb. It also provides an explanation of the necessity for hyperacetylation of the histone tails at the 5' end of genes.

Enhancers regulate transcription

Enhancers are regulatory sequences capable of binding combinations of transcription factors and regulating a specific gene or set of genes in response to either extracellular or intracellular signaling. Enhancers are able to integrate signaling pathways from the cell and translate them into changes in gene expression in order to produce the proteins necessary to orchestrate a response to signaling. Recent genome-wide studies have identified histone modifications unique to enhancers, suggesting that the function of these regulatory elements may depend on combinations of specific covalent histone modifications in these regions. In mammals, enhancer regions are marked prior to activation by monomethylated H3K4 (H3K4me1) and acquire histone H3 acetylated on lysine 27 (H3K27ac) carried out by either CBP (CREB Binding Protein) or p300 (Heintzman et al., 2009) upon activation. In *Drosophila* there is only one acetyltransferase, CBP, reported to carry out acetylation of H3K27. Binding profiles from genome-wide chromatin immunoprecipitation (ChIP) data of 38 different transcription factors from *Drosophila* demonstrate that CBP bound regions contain high numbers of transcription factors binding along with H3K27ac and H3K4me1 (Negre et al., 2011). This suggests the regulation of enhancers at the histone level is a conserved mechanism between *Drosophila* and mammals.

Many enhancers are located at long distances from the promoters they regulate and mechanisms must exist to transfer signals from the enhancer to the promoter in order to activate transcription. ChIP experiments of RNAP II subunits demonstrate

enrichment for both enhancer and promoter sequences, but not the intervening sequences (Wang et al., 2005). This suggests enhancer-promoter contacts that have been confirmed by Chromatin Conformation Capture (3C) experiments, where DNA fragments that are far away in the linear DNA sequence can be detected if they are spatially adjacent *in vivo* (Spilianakis and Flavell, 2004; Vakoc et al., 2005). Additionally, other studies demonstrate that the contacts between enhancer and promoter are necessary for activation of transcription by the enhancer. Given that specific transcription factors vary from one enhancer to another, a ubiquitous mechanism, perhaps using histone modifications and the proteins that bind these modified histones, might be a more likely candidate to facilitate enhancer-promoter interactions.

Scope of the Dissertation

In this study I analyze events that take place at the promoter-proximal pause and are necessary to proceed into productive elongation upon gene activation. I propose that these events, needed to release polymerase from the promoter-pause, serve as a major rate enhancing step of the transcription process. Given that H3S10 phosphorylation occurs at the promoter-proximal pause and may be necessary to proceed into elongation, this event provides a reference point for investigating other steps involved in release of paused RNAP II. There is no evidence suggesting that H3S10ph is directly involved in recruiting P-TEFb to promoters to facilitate the release of RNAP II. Therefore, the first aim of this study is to characterize proteins and events between the phosphorylation of H3S10 and release of RNAP II by P-TEFb.

Transcriptional activation of genes has long been known to be regulated at least in part by enhancers. Therefore, to consider the steps necessary for release of RNAPII

from the promoter-proximal pause, it would be remiss not to investigate the role of enhancers. The second aim of this study is to consider the function of the enhancer in the case where polymerase is already recruited and ready to transcribe at the promoter. Given that there is RNAP II paused at the promoter-proximal site at a large percentage of genes characterized in the genome, I predict that enhancers play a role in the release of the RNAP II from the promoter-proximal pause rather than initiation.

Figure 1-1. Levels of H3S10ph and 14-3-3 in interphase cells are low compared to those present in mitotic chromosomes.

Kc167 cells immunostained for H3S10ph (red), 14-3-3 (green), and DAPI (blue). The mitotic cells are indicated by arrows where both H3S10ph and 14-3-3 have much higher levels of staining during mitosis than during interphase.

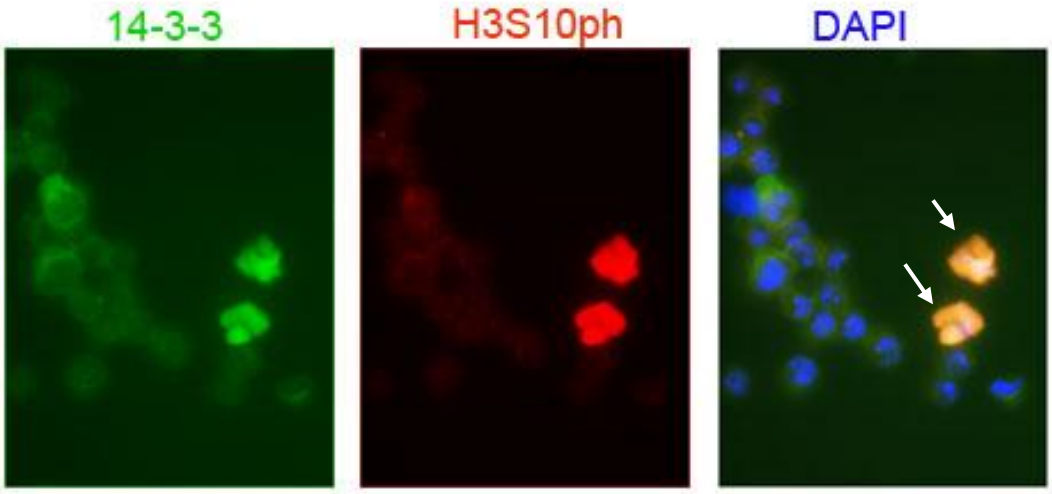
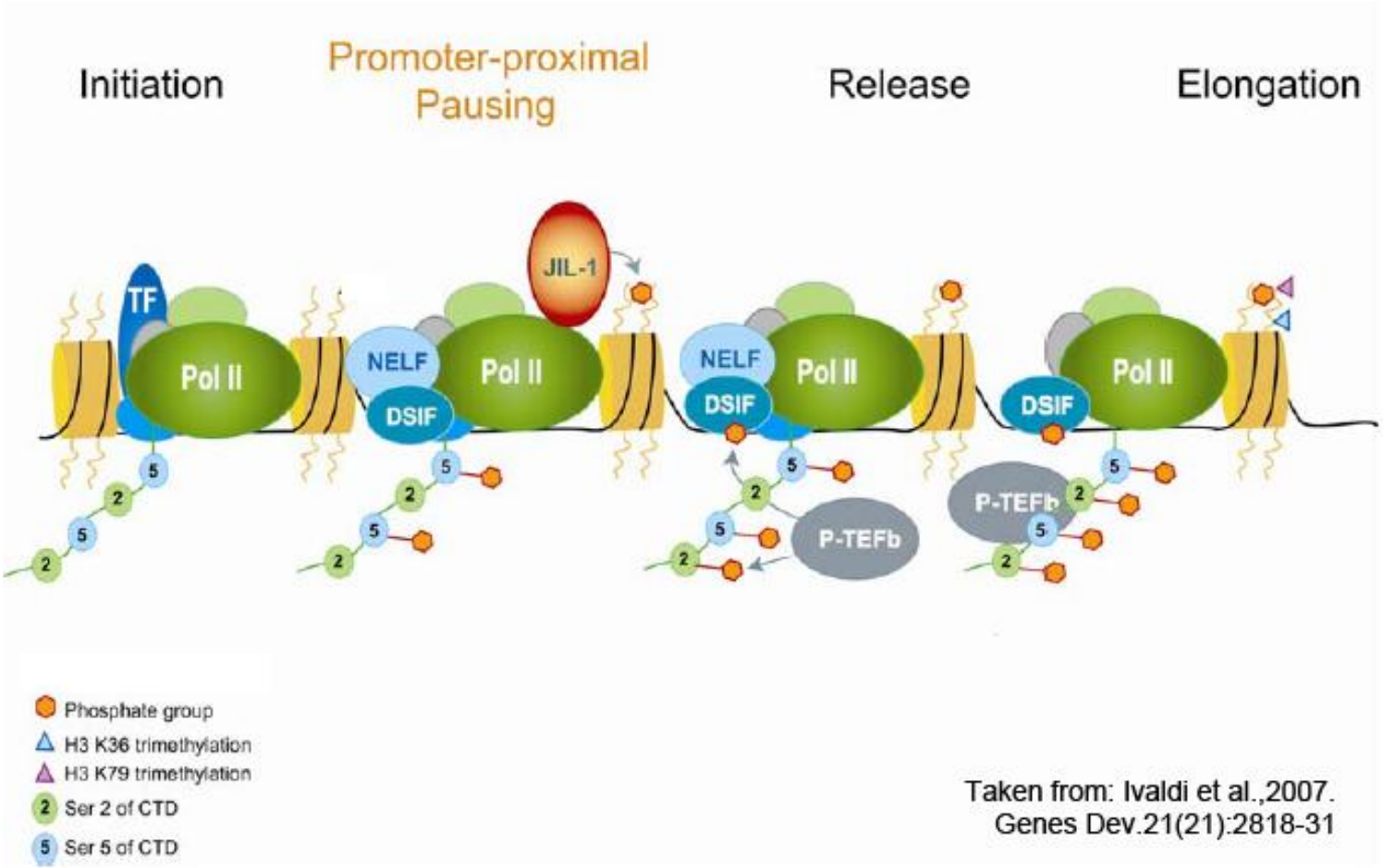


Figure 1-2. Promoter-proximal pausing of RNAPII is a rate limiting step for a large percentage of genes.

ChIP-chip and ChIP-seq experiments have demonstrated that a large number of genes that have low levels of transcription or even no transcription have high levels of initiated RNAP II at the 5' end of the gene. The RNAP II CTD becomes phosphorylated at serine 5 residues upon initiation at the transcription start site and begins to transcribe the first 25-50 bp of the gene. DSIF and NELF as well as the first nucleosome, inhibit progress of the polymerase, resulting in promoter-proximal pausing. Events not well characterized recruit P-TEFb to the paused polymerase where it phosphorylates the RNAP II CTD as Well as components of DSIF. Phosphorylation of DSIF causes it to release NELF and its repressive activity and induces a conformational change in Spt5 subunit in order to form a clamp for the polymerase that increases its processivity for progression of RNAPII. Upon elongation, histone marks such as H3K36me3 and H3K79me3 are deposited inside the body of the gene (Ivaldi et al., 2007).



Taken from: Ivaldi et al.,2007. Genes Dev.21(21):2818-31

Chapter 2

14-3-3 Binding to H3S10ph Mediates Histone Crosstalk in *Drosophila*

These experiments were performed by W. Kellner as my contribution to the manuscript published in PLoS Genetics: Karam CS, Kellner WA, Takenaka N, Clemmons AW, Corces VG. (2010) 14-3-3 mediates histone cross-talk during transcription elongation in Drosophila. PLoS Genet. 6(6):e1000975.

ABSTRACT

Post-translational modifications of histone proteins modulate the binding of transcription regulators to chromatin. Studies in Drosophila have shown that the phosphorylation of histone H3 at Ser10 (H3S10ph) by JIL-1 is required specifically during early transcription elongation. 14-3-3 proteins bind H3 only when phosphorylated, providing mechanistic insights into the role of H3S10ph in transcription. Findings presented here show that 14-3-3 functions downstream of H3S10ph during transcription elongation. 14-3-3 proteins localize to active genes in a JIL-1-dependent manner. In the absence of 14-3-3, levels of actively elongating RNA polymerase II are severely diminished. 14-3-3 proteins interact with Elongator protein 3 (Elp3), an acetyltransferase that functions during transcription elongation. JIL-1 and 14-3-3 are required for Elp3 binding to chromatin, and in the absence of either protein, levels of H3K9 acetylation are significantly reduced. These results suggest that 14-3-3 proteins mediate cross-talk between histone phosphorylation and acetylation at a critical step in transcription elongation.

INTRODUCTION

Promoter-proximal pausing, characterized by the presence of disproportionately more initiated RNAPII located at the promoter of the gene than elongating polymerase inside the gene, has been shown to be a major regulatory step for the transcription of a large number of genes (Rougvie and Lis, 1990; Krumm et al., 1995; Zeitlinger et al., 2007; Muse et al., 2007). Not only is RNAPII at the start of the gene, but it has been demonstrated to be competent in producing short transcripts of 25-50 base pairs inside the TSS (Nechaev et al., 2010). If RNAPII is already transcriptionally competent but not able to proceed into elongation, it begs the question of what are the events necessary to release the polymerase from the pause and achieve active transcription elongation.

Histone phosphorylation as a result of transcriptional activation has been characterized for more than 40 years (Gutierrez and Hnilica, 1967; Cross and Ord, 1971). The same correlation has been made for acetylation of histones and the same histone fractions that are acetylated are also phosphorylated (Jungmann et al., 1970). Later studies identified H3S10 as one of the residues phosphorylated upon transcriptional activation (Chadee et al., 1999) and the JIL-1 kinase as the enzyme responsible for this modification in *Drosophila* (Jin et al., 1999). *JIL-1*^{z2} null mutants show an almost complete loss of H3S10ph by western blots, as well as alterations of other histone modifications and overall chromatin structure (Wang et al., 2001; Zhang et al., 2006). H3S10 phosphorylation during transcription activation in *Drosophila* by JIL-1 takes place at the promoter proximal pause and *JIL-1*^{z2} mutants do not affect the recruitment of transcription factors or initiation of transcription by RNAPII (Ivaldi et al., 2007).

14-3-3, a well characterized phospho-binding protein, has been demonstrated to bind H3S10ph upon transcriptional activation (Macdonald et al., 2005). In addition, 14-3-

3 is recruited more efficiently if the neighboring lysine residues K9 and K14 are acetylated (Winter et al., 2008). These results are confusing, because structural analysis as well as a large amount of literature has characterized 14-3-3 as protein that recognizes phosphorylated residues and not acetylated residues. Due to the connection between acetylation and phosphorylation of H3 during transcriptional activation it is likely the two modifications are regulated in the same pathway. 14-3-3 may play a role in this process by being linked to both the phosphorylation of H3S10 and the acetylation of the neighboring lysines K9 and K14.

Here I explore the role of H3S10ph mediated recruitment of 14-3-3 to chromatin during transcription activation. Results show that 14-3-3 proteins are recruited to active genes in a JIL-1-dependent manner and are required for phosphorylation of RNAPII at Ser2 (RNAP IIo^{ser2}), an event that takes place upon release of RNAP II into elongation. I also examine Elongation protein 3 (Elp3) as a possible candidate for acetylation of H3K9 due to its characterization as a histone H3 acetyltransferase necessary for transcription elongation (Winkler et al., 2002; Kristjuhan and Svejstrup, 2004). Experiments show that Elp3 interacts with 14-3-3 and carries out H3K9 acetylation. The recruitment of Elp3 to chromatin and the subsequent acetylation of H3K9 are dependent on JIL-1 and 14-3-3, demonstrating that 14-3-3 proteins mediate crosstalk between H3 phosphorylation and acetylation during early transcription elongation. Furthermore, loss of 14-3-3 or Elp3 result in normal initiation of trasncription but lack of elongation, suggesting a role in the release of RNAPII from promoter-proximal pausing.

RESULTS

Jll-1 is recruited to the *hsp70* locus after heat activation

A large body of evidence has indicated a role for H3S10ph in transcription activation. The mammalian homologues of JIL-1, Msk1 and Msk2, have been shown to be responsible for H3S10ph during transcription using ChIP (Vicent et al., 2006; Bruck et al., 2008; Drobic et al., 2010). Studies in *Drosophila* indicate that this modification is involved in the release of Pol II from promoter-proximal pausing during early transcription elongation. Interestingly, mutations in *JIL-1* not only result in a genome-wide decrease in transcription but also cause dramatic changes in the structure of polytene chromosomes (Wang et al., 2001; Ivaldi et al., 2007). Although the two effects are probably related, it has been recently questioned whether JIL-1 and H3S10 phosphorylation play a role in transcription and whether the observed recruitment of JIL-1 to heat-shock genes upon induction, and the ensuing H3S10 phosphorylation, are artifacts resulting from the fixation procedures utilized in the immunofluorescence microscopy analyses used to derive these conclusions (Cai et al., 2010). To address these concerns, JIL-1 antibodies were used in standard chromatin immunoprecipitation (ChIP) experiments to examine whether JIL-1 is recruited to the *hsp70* promoter when the gene is induced in *Drosophila* Kc cells. The results confirm our previous observations showing that JIL-1 binds to the promoter region of the *hsp70* gene only after the cells are subjected to heat-shock. Recruitment of JIL-1 to the *hsp70* promoter explains the phosphorylation of H3S10 at heat-shock puffs, which has been observed consistently by various investigators using antibodies from different sources and varying fixation protocols (Nowak and Corces, 2000; Ivaldi et al., 2007; Labrador and Corces, 2003; Buszczak and Spradling, 2006; Schwartz and Ahmad, 2005; Grau et al., 2008; Ciurciu et al., 2009).

14-3-3 binding is dependent on H3S10ph and plays a role in transcription elongation genome-wide

In order to investigate the role of H3S10 phosphorylation in the recruitment of 14-3-3 during transcription activation I first investigated mutants of 14-3-3. Previous studies have shown that two human 14-3-3 proteins (14-3-3ζ and 14-3-3ε) associate with active genes in an H3S10ph-dependent manner (Macdonald et al., 2005; Winter et al., 2008; Zippo et al., 2009). However, these studies are limited to the analysis of specific genes and it is yet unclear whether 14-3-3 plays a more general role in transcription. To address this question, I analyzed mutations in the two *Drosophila* 14-3-3 isoforms. Existing 14-3-3ζ mutant alleles are lethal at the embryonic stage whereas 14-3-3ε (*leo*) mutant alleles have been isolated that display lethality at the first instar larval stage; larvae homozygous mutant for this allele were thus selected for western analysis. These mutants compensate for the loss of the 14-3-3ε isoform by upregulating the 14-3-3ζ isoform detected with an antibody that recognizes both isoforms of 14-3-3. The two isoforms appear to have overlapping functions described in the literature and, as a result, no loss of elongating RNAPII activity was seen in these mutants (Figure 2-2A). In order to obtain a fly model that is depleted of both isoforms to analyze a possible role for 14-3-3 in transcription elongation, I turned to the conditional Gal4-UAS RNAi system available for most genes in *Drosophila*. An *hsp70-Gal4* strain that expresses Gal4 in the salivary glands in the absence of any heat-shock treatment (Armstrong et al., 2002) was used to express UAS-RNAi against *14-3-3ζ, 14-3-3ε,* or both simultaneously in order to investigate the role of these proteins in transcription. When 14-3-3ζ is knocked down by RNAi, the lower band is severely diminished, with significant increase in the signal corresponding to the higher band. When RNAi against 14-3-3ε is used, a severe reduction of the higher band is observed, accompanied by partial reduction of the lower band. RNAi against both isoforms leads to a reduction in both bands, the higher band corresponding to 14-3-3ε while the lower one corresponding to 14-3-3ζ. Additionally, knockdown of both isoforms results in a depletion of elongating RNAPII (Figure 2-2B).

Immunofluorescence microscopy was carried out to determine the localization of 14-3-3 proteins on polytene chromosomes from salivary glands of *Drosophila* third instar larvae. These results reveal a broad distribution of 14-3-3 proteins, with almost precise overlap and equal intensity with H3S10ph, at interband regions, where higher levels of transcription take place. 14-3-3 immunostaining is dramatically decreased in larvae expressing RNAi against both isoforms of 14-3-3, verifying the specificity of the antibody used in these analyses. Co-staining with H3S10ph in polytene chromosomes from 14-3-3 RNAi knockdown larvae demonstrates that H3S10 phosphorylation still takes place at normal levels in the absence of 14-3-3, placing phosphorylation upstream of 14-3-3 recruitment genome-wide (Figure 2-3A).

The heat-shock paradigm was carried out to understand the dynamics of 14-3-3 distribution during transcription. When larvae are subjected to temperature elevation, the heat-shock (*hsp*) genes are turned on while all genes that were previously active are turned off. Immunostaining analysis of polytene chromosomes using antibodies against H3S10ph show that heat-shock treatment leads to disappearance of the modification from previously active genes and its redistribution to the induced *hsp* genes (Nowak and Corces, 2000). If 14-3-3 is recruited by phosphorylation of H3S10, the distribution of 14-3-3 should exhibit similar behavior to that of H3S10ph upon heat-shock. Third instar larvae were incubated at 37°C for 20 min and their salivary glands were immediately dissected and fixed for immunostaining analysis using anti-14-3-3 and anti-H3S10ph antibodies. Results show that the pattern of 14-3-3 binding, like that of H3S10 phosphorylation, changes from a broad distribution throughout the genome to one that is restricted to the heat shock genes (Figure 2-3B). This suggests that 14-3-3 proteins are recruited to actively transcribed genes and that their binding to chromatin correlates with H3S10ph.

The experiments described above were then repeated in *JIL-1*^{z2} mutants to determine whether the recruitment of 14-3-3 is dependent on H3S10 phosphorylation. A previous study reported that despite the disrupted structure of the chromosomes of these mutants, RNAP IIo^{ser5}, initiated polymerase remains on chromatin at wild-type levels (Ivaldi et al., 2007). Polytene chromosomes from *JIL-1*^{z2} mutants are stained with antibodies to 14-3-3 and Su(Hw), a DNA binding protein not involved in transcription included to show equal exposures between *JIL-1*^{z2} mutant and wild type chromosomes. The results indicate genome-wide loss of 14-3-3 binding to the chromosomes in the *JIL-1*^{z2} mutant. Using the heat shock paradigm, antibodies against RNAP IIo^{ser5} mark the heat shock loci and no 14-3-3 protein can be detected on the chromosomes of *JIL-1*^{z2} mutants after heat-shock. Additionally, levels of RNAP IIo^{ser5} recruited to the heat shock loci are unaffected suggesting normal initiation can still take place in the absence of 14-3-3 (Figure 2-4). From these results it can be concluded that 14-3-3 binding to the chromosomes during transcription is dependent on JIL-1 kinase activity.

Acetylation of H3K9 by Elp3 during transcription activation depends on 14-3-3 and JIL-1

14-3-3 proteins function as versatile dimeric structures that can modulate various forms of protein-protein interaction in response to signaling cues. In many instances 14-3-3 moelcules serve as scaffolds, bridging proteins that cannot directly interact (Tzivion et al., 2001). I therefore hypothesized that the binding of 14-3-3 to H3S10ph may function to regulate the interaction between H3S10ph and other chromatin-binding proteins. Such proteins would have to (1) interact with 14-3-3, (2) be chromatin-related, and (3) function during transcription elongation. Various reported biochemical screens, aimed at isolating 14-3-3-binding proteins, provide a vast database to search for candidates that fit these criteria. An exhaustive review of 14-3-3 interactors identified the

histone acetyltransferase Elp3 (Winkler et al., 2002; Pozuelo et al., 2004), a subunit of the Elongator complex that co-purifies with RNAP II (Wittschieben et al., 1999) and is required for H3 acetylation specifically during transcription elongation (Winkler et al., 2002; Han et al., 2008; Wittschieben et al., 2000). In order to confirm an interaction between Elp3 and 14-3-3 in *Drosophila*, anti-14-3-3 antibodies were used to immunoprecipitate proteins from Kc167 cell extracts followed by western analysis using anti-Elp3 antibodies. Both Elp3 and H3S10ph co-precipitate with 14-3-3, suggesting these proteins interact directly or indirectly *in vivo* (Figure 2-5A). A mutant $Elp3^{EX1}$ allele has been generated by imprecise excision of a P-element inserted 65 bp upstream of the transcription start site, creating a deletion from the P-insertion site to the triplet encoding K277 (Walker et al., 2009). Using lysates from this $Elp3^{EX1}$mutant , western analysis demonstrates severely reduced levels of elongating RNAP II levels as well as H3K36me3, a histone modification correlated with transcription elongation. These data suggest a conserved role for Elp3 in *Drosophila* (Figure 2-5B&C).

To examine binding of Elp3 to chromatin, immunostaining experiments were performed on polytene chromosomes using antibodies recognizing Elp3. Chromosomes were co-stained with antibodies to RNAP IIo^{ser5} in order to mark transcriptionally active regions. Co-localization of the two proteins suggests a global role for Elp3 in transcription of most *Drosophila* genes (Figure 2-6A). In addition, Elp3 is present at heat-shock puffs upon elevated temperates (Figure 2-6B), suggesting it is recruited to genes upon induction. If Elp3 is recruited to chromatin via 14-3-3, a loss of Elp3 on the chromosomes of 14-3-3 RNAi flies is expected. This is demonstrated genome-wide as well as during active recruitment under heat shock conditions, confirming that Elp3 recruitment during transcription is dependent on 14-3-3 (Figure 2-6 A&B). Additionally, these experiments demonstrate normal initiation of the RNAP II in 14-3-3 RNAi flies

genome-wide as well as at the heat shock loci consistent with a role at the promoter-proximal pause rather than RNAP II initiation.

Using *Elp3*^{EX1} mutant larvae as a comparison, immunostaining looking at Elp3 levels in both *JIL-1*^{z2} mutants and 14-3-3 RNAi salivary glands with Su(Hw) co-staining included as a control for intensity, show that Elp3 binding to the chromosomes is lost across the genome in *JIL-1*^{z2} mutants and 14-3-3 RNAi flies (Figure 2-7). These data suggest Elp3 recruitment to the chromosomes is dependent on JIL-1 and 14-3-3.To identify the role of Elp3 in acetylation of histones, western blot analyses of salivary gland lysates were performed in wild type, *Elp3*^{EX1} and *JIL-1*^{z2} mutants along with 14-3-3 RNAi flies. Comparison of levels of different histone modifications present in these mutants compared with wild type shows a large reduction in H3K9ac but not H3K14ac, another modification associated with histone phosphorylation. This suggests Elp3 acetyltransferase activity is specific for H3K9. In addition, *JIL-1*^{z2} mutant along with 14-3-3 RNAi salivary gland lysates also show a marked reduction in H3K9ac, confirming the dependence of Elp3 activity on the function of these two proteins. Antibodies to the dual modification phosphorylated H3S10 and acetylated H3K9 (H3K9acS10ph) show the same trend of severely reduced levels of H3K9acS10ph in both the *Elp3*^{EX1} and 14-3-3 RNAi salivary glands (Figure 2-8A). However, the antibody to the dual modification phosphorylated H3S10 and acetylated H3K14 (H3S10phK14ac) shows normal levels in these mutants, suggesting that proteins other than 14-3-3 and Elp3 are necessary for H3K14 acetylation. *Elp3*^{EX1}mutant *a*nd 14-3-3 RNAi salivary glands still have phosphorylated H3S10 consistent with the hypothesis that H3S10ph is upstream of 14-3-3 recruitment and Elp3 activity (Figure 2-8A). Elp3 acetyltransferase activity was investigated by immunoprecipitation of both 14-3-3 and Elp3 followed by an *in vitro*

acetylation assay. This shows 14-3-3 associates with a histone acetyltransferase activity that is specific for H3K9 and not H3K14 (Figure 2-8B).

To take this a step further, acetylation of H3K9, the modification Elp3 performs in the previous assays, was investigated by immunostaining. *JIL-1*^{z2} mutant and 14-3-3 RNAi polytene chromosomes show a genome-wide depletion of H3K9ac when compared with the wild type, consistent with the proposed role of Elp3 in performing H3K9 acetylation. Furthermore, the lack of its recruitment to the chromosomes in *JIL-1*^{z2} mutant and 14-3-3 RNAi flies confirms the dependence of Elp3 on JIL-1 and 14-3-3 and its function of acetylating H3K9 (Figure 2-9).

H4K16ac, a modification associated with transcriptional elongation, is dependent on JIL-1 kinase but not 14-3-3

The results discussed above suggest that 14-3-3 proteins recruit the histone H3 acetyltransferase Elp3, which in turn acetylates H3 in the Lys9 residue. This observation is interesting in the context of recent findings suggesting that 14-3-3 can recruit the histone acetyltransferase MOF to acetylate H4K16 in the mammalian FOSL1 gene (Zippo et al., 2009). To test whether this is also the case in *Drosophila*, I determined whether levels of H4K16 acetylation are affected by downregulation of 14-3-3 using RNAi against the two genes encoding this protein in *Drosophila*. Results show that the presence of H4K16ac in protein extracts from salivary glands (Figure 2-9A) or immunostaining of polytene chromosomes (Figure 2-10) is not affected in flies lacking 14-3-3 proteins. To further confirm the absence of association between 14-3-3 and *Drosophila* MOF, 14-3-3 and associated proteins were isolated by immunoprecipitation

with 14-3-3 antibodies and used for an *in vitro* acetylation assay using recombinant histones as a substrate. The results of these experiments show absence of H4K16 acetylation when compared to the no antibody control and suggest that recruitment of MOF does not require 14-3-3 in flies (Figure 2-9B). However, western analysis (Figure 2-9A) as well as immunostaining (Figures 2-10 & 2-11) demonstrate a JIL-1 dependency of MOF function in carrying out H4K16 acetylation on the autosomes in males and across the genome in females, an observation already described during dosage compensation in *Drosophila* males (Jin et al., 2000; Lerach et al., 2005).

DISCUSSION

The phosphorylation of histone H3 at S10 has been shown to be accompanied by the recruitment of members of the 14-3-3 protein family to specific genes upon induction (Macdonald et al., 2005; Winter et al., 2008; Zippo et al., 2009). Here I extend these observations and present evidence that 14-3-3 mediates a novel histone crosstalk that promotes transcription elongation in *Drosophila*. I show that 14-3-3 has a broad distribution across the genome at actively transcribed regions, suggesting this is a global step in transcriptional activation. Moreover, I demonstrate that in a 14-3-3 mutant, Pol II is still able to initiate transcription at active loci, but fails to elongate, suggesting a role in promoter-proximal pausing. This builds on our previous study showing H3S10 phosphorylation by JIL-1 takes place at the promoter-proximal pause by demonstrating 14-3-3 recruitment is dependent on this phosphorylation.

14-3-3 provides a mechanistic connection between histone phosphorylation and acetylation during transcription activation. The dependency on JIL-1 kinase

phosphorylation of H3S10 for acetylation of the neighboring H3K9 provides insight on the connection observed between to two modifications. Here I propose a mechanism like that described by the “histone code” hypothesis by Struhl and Allis, where in response to cellular signaling, JIL-1 phosphorylates H3S10 proving a binding platform for 14-3-3 binding, and 14-3-3 subsequently recruits Elp3 for acetylation of H3K9. This pathway results in a combinatorial code that likely has subsequent modifications, such as H4K16ac, to cause the hyperacetylation seen on the majority of histone lysine residues reported at promoters during transcription activation.

Interestingly, it was recently shown in mammals that 14-3-3 proteins bind phosphorylated H3S10 at the *FOSL1* gene and serves to recruit the H4K16 acetyltransferase MOF. MOF then acetylates histone H4 at the Lys16 residue and is required for recruitment of Brd4 and P-TEFb. Although the specific combinations of lysine residues required for recruitment of Brd4 in mammals have not been explored in detail, studies have suggested that acetylation of H3K9 might also play a role (Zippo et al., 2009; Vollmuth et al., 2009). Consistent with this observation, it has been shown that phosphorylation of H3S10 is required for recruitment of P-TEFb to heat-shock genes in *Drosophila* (Ivaldi et al., 2007). Nevertheless, it appears that in *Drosophila* 14-3-3 does not play a major role in the recruitment of MOF; instead, 14-3-3 recruits Elp3 and this protein is then required for MOF recruitment, based on the observation that H4K16Ac is dramatically reduced in flies carrying a mutation in the *Elp3* gene. Taken together, the data strongly support a role for crosstalk between histone phosphorylation and acetylation during the release of RNAP II from promoter-proximal pausing.

The relationship between histone H3 phosphorylation and acetylation has been the subject of some debate. These two modifications are known to occur in response to the same stimuli, in the same tissue and on the same histone tails. Two possible models

have been consequently put forward to explain these observations (Mahadevan et al., 2004). The first proposes that the two modifications are synergistic and coupled such that one is dependent on the other. This is supported by the fact that, *in vitro*, HATs preferentially acetylate histone tails that are phosphorylated, suggesting that histone phosphorylation provides a stronger binding site for HATs than unphosphorylated ones (Cheung et al., 2000). The second model envisions the two modifications being performed by regulatory machineries that are recruited simultaneously yet independently to active genes (Mahadevan et al., 2004). Studies that support this model have utilized specific antibodies that recognize the modifications either individually or together. Two populations of histones were detected at active genes using these antibodies, a larger highly acetylated population that is not phosphorylated and a smaller phosphoacetylated population, suggesting that phosphorylation is not a prerequisite to acetylation (Mahadevan et al., 2004). Our results support the 'synergistic and coupled' model, but under the premise that 14-3-3 acts as a bridge, rather than one modification acting as a binding site for the next enzyme. At the same time, the data do not rule out the other scenario. In fact, while different groups have in the past advocated one model over the other, the two are by no means mutually exclusive. It is becoming increasingly evident that multiple layers of regulation come into play during transcription activation.

MATERIALS AND METHODS

Drosophila stocks

Stocks were maintained in standard medium at 18°C or 25°C. Oregon R larvae were used for wild type (wt) controls in all experiments. The *JIL-1*^{z2} stock was a gift from Dr. K. Johansen (Iowa State University). The *Elp3*^{EX1} mutant was a gift from Dr. J. Svejstrup (Cancer Research UK London Research Institute). UAS-14-3-3ζ RNAi flies were

obtained from VDRC (Stock #48724) and UAS-14-3-3ε RNAi flies were obtained from NIG (Stock #31196R-4). To express siRNA in salivary glands these stocks were crossed to +/+; hsp70-Gal4/hsp70-Gal4 (Bloomington, 1799). To express RNAi against both 14-3-3 isotypes simultaneously, 48724/48724; 31196R-4/31196R-4 flies were crossed to +/+; hsp70-Gal4/hsp70-Gal4. Leo mutants (14-3-3 zeta) obtained from VDRC are homozygous lethal at first instar stage so are maintained as heterozygotes P{PZ}14-3-3ζ07103/cyo-GFP. Homozygotes can be selected by the absence of GFP in the first instar larvae.

Preparation of *Drosophila* protein extracts and western analysis

Approximately 100 pairs of salivary glands from third instar wild type or mutant larvae or glands subject to RNAi were homogenized in 100 µl RIPA buffer with EDTA free protease inhibitors (Roche) and phosphatase inhibitors (Sigma #P2850) and left on ice for 15 min. Laemmli's buffer and beta-mercaptoethanol were added and lysates were incubated at 65°C for 20 min to solubilize proteins and then insoluble fractions were spun down. Samples were run on NuPage 4–12% gradient Bis-Tris gels and transferred to PVDF membranes for immunodetection. Membranes were incubated overnight at 4°C in antibody dilution buffer (PBS/0.05% TIen/5% milk or BSA in case of Pol II antibodies) containing primary antibodies at concentrations of 1:1000 rabbit α-14-3-3 (SCBT), 1:1000 mouse α-Pol IIo^{ser2} (H5, Covance), 1:5000 mouse α-lamin C (Developmental Studies Hybridoma Bank), 1:1000 rabbit α-H3S10P (Millipore), 1:5000 rabbit α-H3K9Ac (Millipore, 07-352), 1:5000 rabbit α-H3K14Ac (Millipore, 07-353), 1:1000 rabbit α-H3S10PK9Ac (Abcam, ab12181), 1:1000 rabbit α-H3S10PK14Ac (Millipore, 07-081), 1:1000 rabbit α-H3K79me (Abcam) and 1:10000 rabbit α-histone H3 (Abcam).The membranes were washed twice with PBS/0.25% Tween, incubated for 1h at room

temperature in the appropriate HRP secondary antibody (Jackson ImmunoResearch Laboratories) and washed twice with PBS/0.25% Tween. Antibody signal was visualized using chemi-luminescence detection methods (SuperSignal West Pico kit, Pierce).

Induction of the heat-shock response and analysis of polytene chromosomes

Salivary gland polytene chromosome squashes were prepared from wandering third instar larvae maintained at 18°C. For heat-shock experiments, third-instar wild type and *JIL-1*^{z2} mutant larvae were subjected to heat-shock treatment as described previously (Nowak et al., 2003). Salivary glands were dissected in 0.7% NaCl and fixed for 2 min in 45% acetic acid/1.85% formaldehyde. Fixed salivary glands were subsequently squashed in 45% acetic acid on subbed slides. The slides were frozen in liquid nitrogen and stored dry at −70°C. For immunostaining of 14-3-3 proteins, salivary glands were fixed for 1 min in 3.7% acetic acid, 2 min in 45% acetic acid/3.7% formaldehyde and 3 min in 45% acetic acid. Slides were incubated overnight at 4°C in antibody dilution buffer (PBS/0.1% Triton X-100/1% BSA) containing primary antibodies at concentrations of 1:50 α-14-3-3 (K19, SCBT), 1:100 rabbit α-JIL-1, 1:20 rabbit α-Elp3 (Winkler et al., 2002), 1:30 mouse α-Pol IIo^{ser2} (H5, Covance), 1:30 mouse α-RNAP IIo^{ser5} (H14, Covance), 1:150 rat α-Su(Hw), 1:50 rabbit α-H3S10phK14ac and 1:50 rabbit α-H3K9ac. Following incubation, slides were washed three times in PBS/0.1% Triton X-100 and incubated for 1 h at 37°C in the appropriate secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:200 in antibody dilution buffer. Slides were washed three times as described above and stained with 0.5 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) and mounted in Vectashield mounting medium (Vector Laboratories) for viewing.

Co-immunoprecipitation experiments

Goat anti-14-3-3ζ serum (SCBT) was covalently crosslinked to Protein G sepharose beads 4 Fast Flow (GE Healthcare) using DMP. *Drosophila* Kc cells grown to 80% density were spun down, washed twice in PBS and crosslinked for 10 min in 1% paraformaldehyde. Cells were washed twice in PBS to stop the reaction and then lysed in RIPA buffer containing phosphatase inhibitors and protease inhibitors for 20 min on ice. The insoluble fraction was spun down and the soluble fraction split in two, half on the beads with antibody and the other half with beads but no antibody as a control; a small fraction was reserved for the input lane. The samples were run on 4–12% NuPage Bis/Tris gel, transferred to nitrocellulose, blocked in 1% BSA and blotted against rabbit α-14-3-3ζ (SCBT) 1:2000, rabbit α-Elp3 1:2000 (Winkler et al., 2002), and mouse α-H3S10P (Millipore).

***In vitro* acetylation assay**

Wild type third instar larvae were repeatedly washed in PBS and then ground and vortexed in RIPA buffer with protease inhibitors. The lysate was diluted 10-fold with 1% Triton X-100/150 mM NaCl/50 mM Tris and spun down to eliminate insoluble fractions. The soluble lysate was incubated with either no antibody, Elp3 polyclonal antibody, or 14-3-3 polyclonal antibody and pulled down with protein G beads. The beads were washed 5 times in 1% Triton X-100 buffer and 3 times in 50 mM Tris pH8/150 mM NaCl containing protease inhibitors. To 25 µl of the beads containing pull-down, 50 µl 50 mM Tris pH8.0/150 mM NaCl, 20 µl of 1 mg/ml histones and 5 µl of 5.69 mM AcetylCoA were added per 75 µl reaction and incubated at 30°C for 45 min with mixing. The histones were then collected for western analysis.

Chromatin immunoprecipitation experiments

Drosophila Kc167 cells were grown at 25°C to 7×10^6 cells/ml in serum-free HyQ-CCM3 medium (HyClone Laboratories, Inc.). Cells were subjected to heat shock by addition of an equivalent volume of medium preheated to 48°C to the growing cells. After holding the cells at 36.5°C for 15 min, the cells were immediately cooled down to 25°C with the addition of 1/3 total volume of 4°C medium immediately prior to cross-linking . Cells were cross-linked with 1% formaldehyde for 10 min, (Boehm et al., 2003) quenched with 0.125 mM glycine and washed with PBS. Nuclear lysates were sonicated to generate 200–1000 bp DNA fragments. Immunoprecipitation was performed with 7 µl α-JIL-1 or with no antibody. Immunoprecipitated DNA was extracted and amplified with primers described in Boehm et al., 2003:

hsp70+4F, 5'-CAATTCAAACAAGCAAAGTGAACAC

hsp70+112R, 5'-TGATTCACTTTAACTTGCACTTTA.

Figure 2-1. JIL-1 is recruited to the promoter of the *hsp70* gene after heat-shock.

(A) Immunolocalization of H3S10ph (red) and initiating polymerase (RNAP IIo^{ser5}) (green) on wild type (wt) and mutant *JIL-1*^{z2} polytene chromosomes after heat shock (hs).

B) Chromatin immunoprecipitation of Kc cell extracts by anti-JIL1 antibodies before and after heat-shock followed by PCR using primers that amplify the promoter of the hsp70 gene.

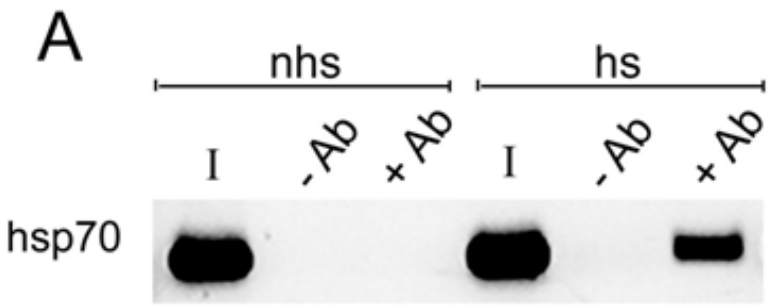
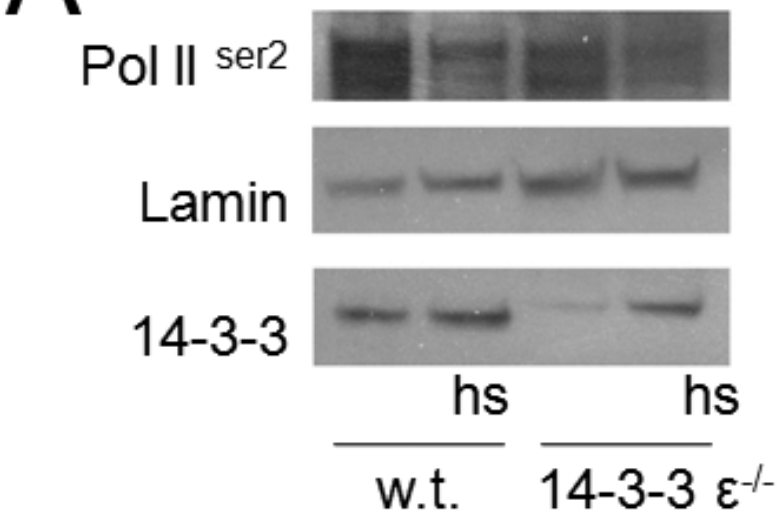

A
nhs
hs
I
-Ab
+Ab
I
-Ab
+Ab
hsp70

Figure 2-2. 14-3-3 proteins are required for transcription elongation of most *Drosophila* genes.

(A) Western analysis of first instar larvae of wt and *leo*$^{-/-}$ (14-3-3ε) mutants show levels of RNAP IIo^{ser2} and both 14-3-3 isoforms. Lamin is used as a loading reference.

(B) Western analysis of 14-3-3 proteins, RNAP IIo^{ser2} and lamin in salivary glands from wild type (wt) larvae, *JIL-1*^{Z2} mutant larvae, or salivary glands expressing RNAi against 14-3-3ζ (ζRNAi), 14-3-3ε (εRNAi), or both simultaneously (ζ+εRNAi).

A


Pol II ser2
Lamin
14-3-3
hs
hs
w.t.
14-3-3 ε-/-

B

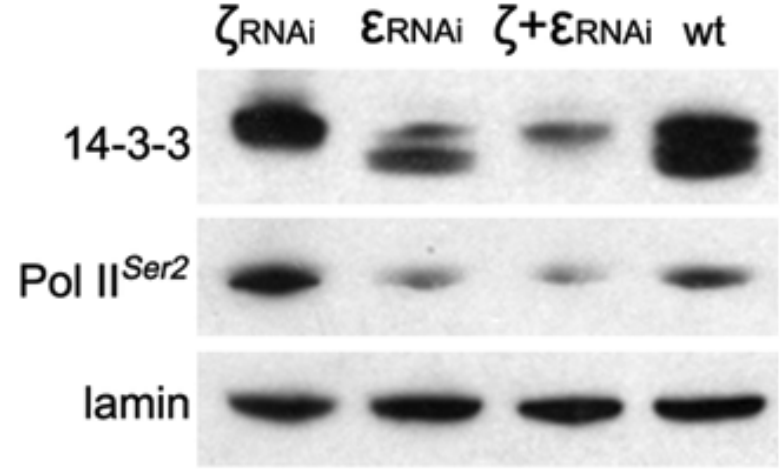

ζRNAi
εRNAi
ζ+εRNAi
wt
14-3-3
Pol IISer2
lamin

Figure 2-3. H3S10ph and 14-3-3 overlap at the majority of sites of active transcription.

(A) Immunolocalization of H3S10ph (green) and 14-3-3 (red) on wild type (wt) and *14-3-3 RNAi* knockdown polytene chromosomes at 25˚C (non heat shock, nhs).

(B) Immunolocalization of H3S10ph (green) and 14-3-3 (red) polytene chromosomes after heat shock (hs) of wild type (wt) or *14-3-3 RNAi* knockdown larvae. DAPI (blue) stains DNA.

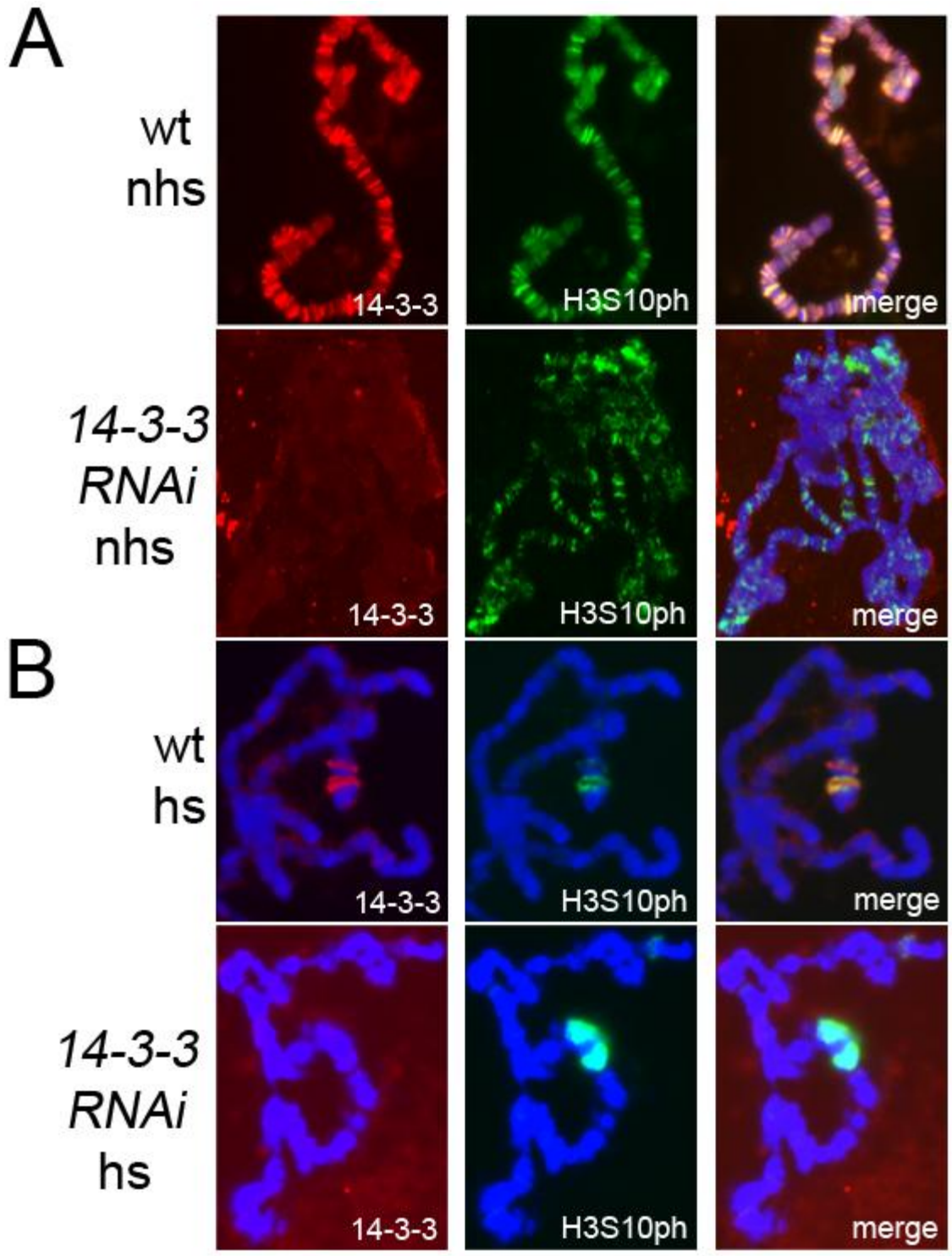

A
wt
nhs
14-3-3
H3S10ph
merge
14-3-3
RNAi
nhs
14-3-3
H3S10ph
merge
B
wt
hs
14-3-3
H3S10ph
merge
14-3-3
RNAi
hs
14-3-3
H3S10ph
merge

Figure 2-4. Binding of 14-3-3 to chromosomes during transcription is dependent on JIL-1 kinase.

(A) Immunolocalization of 14-3-3 (green) on polytene chromosomes from wild type larvae or larvae expressing 14-3-3 RNAi at 25˚C (non heat shock, nhs). Su(Hw) (red) was used to control for signal intensity.

(B) Immunolocalization of 14-3-3 (red) and initiating polymerase, RNAP IIo^{ser5} (green) on polytene chromosomes of wild type (wt) or *JIL-1*^{z2} mutant larvae. DAPI (blue) stains DNA.

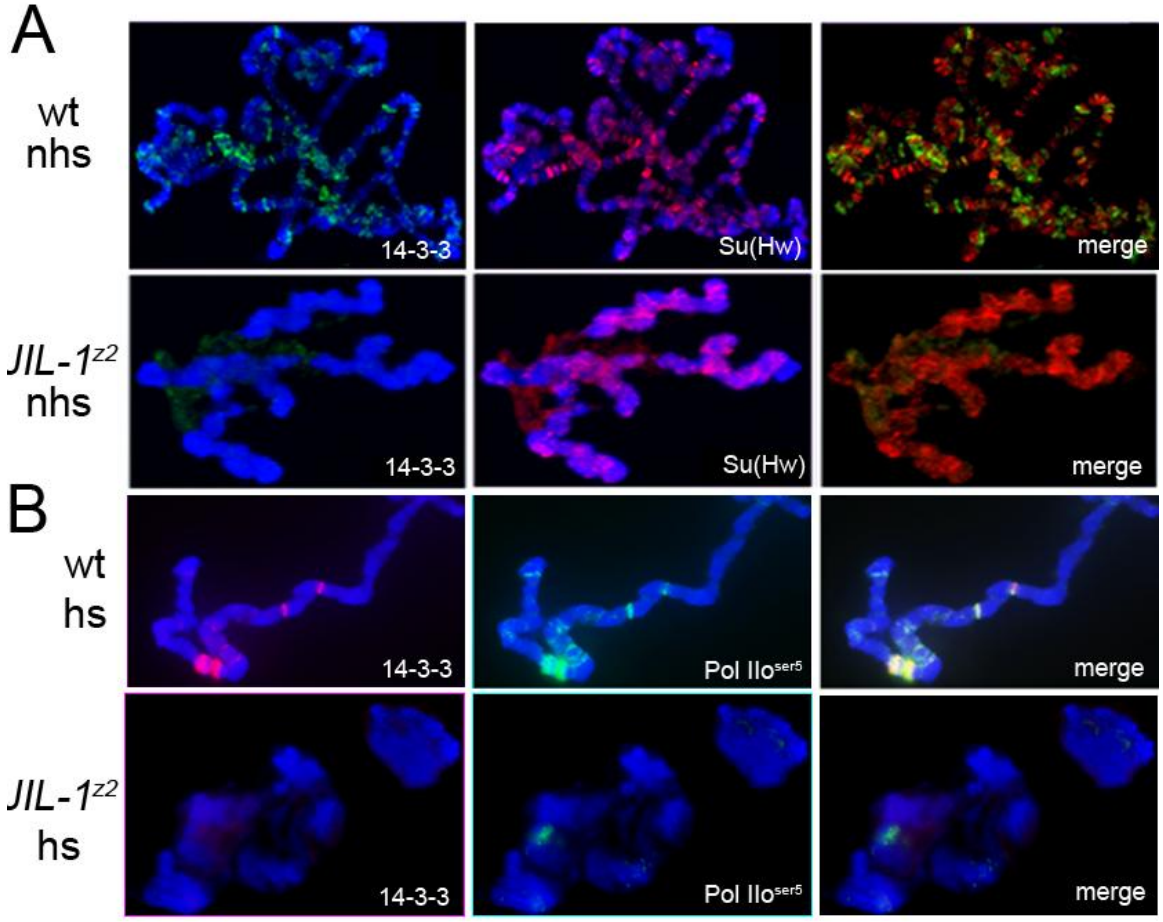

A
wt
nhs
14-3-3
Su(Hw)
merge
JIL-1z2
nhs
14-3-3
Su(Hw)
merge
B
wt
hs
14-3-3
Pol IIoser5
merge
JIL-1z2
hs
14-3-3
Pol IIoser5
merge

Figure 2-5. 14-3-3 proteins interact with the elongation protein Elp3 and are required for transcription elongation of most *Drosophila* genes

(A) Western analysis of Kc cell extract immuno-precipitated with 14-3-3 antibodies (+Ab) or beads alone (-Ab) and probed for Elp3, 14-3-3 and H3S10ph. I = input.

(B) Western analysis of 14-3-3 proteins, RNAP IIo^{ser2} and lamin in salivary glands from wild type (wt) larvae, *JIL-1*^{Z2}, *Elp3*^{EX1} mutant larvae, or salivary glands expressing RNAi against 14-3-3 ζ+ε.

(C) Western analysis of elongation histone mark H3K36me and lamin used as a loading control in salivary glands from wild type (wt) larvae, *JIL-1*^{Z2}, *Elp3*^{EX1} mutant larvae, or salivary glands expressing RNAi against 14-3-3 ζ+ε.

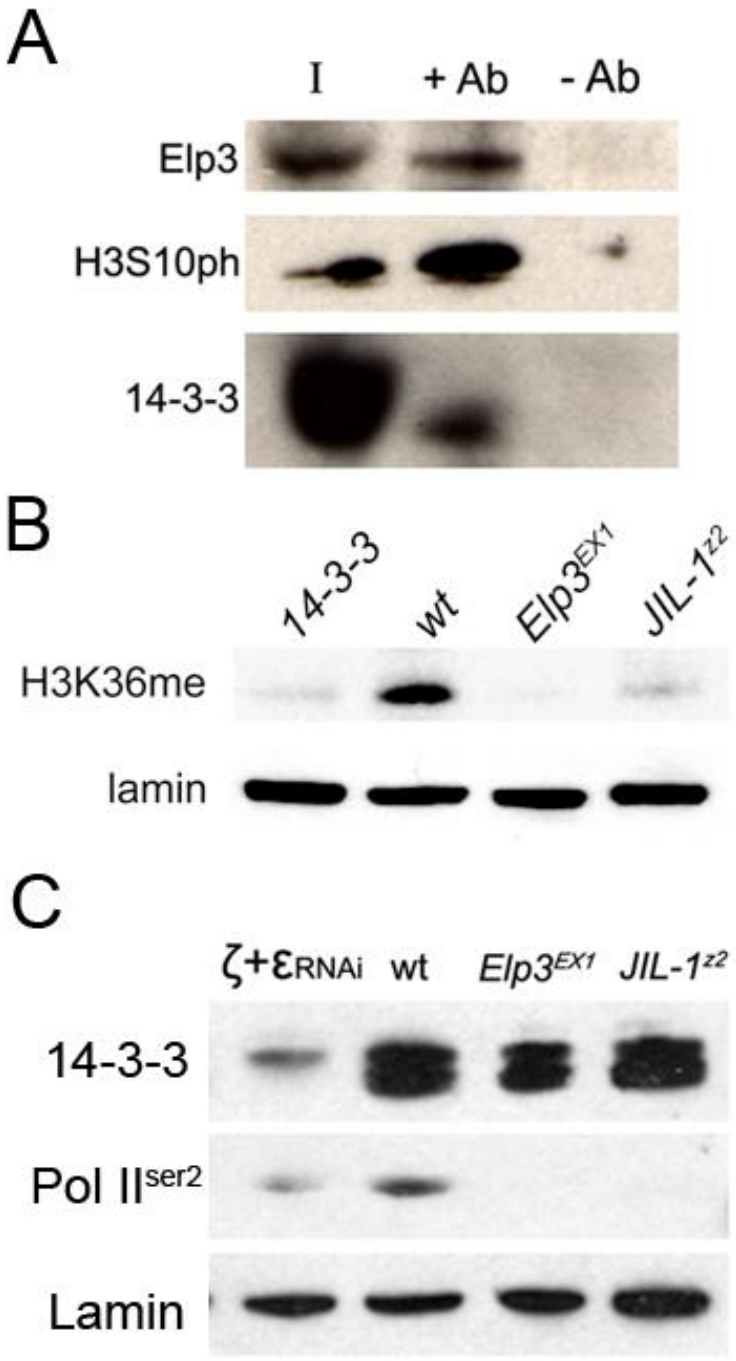

A
I
+ Ab
- Ab
Elp3
H3S10ph
14-3-3
B
14-3-3
wt
Elp3EX1
JIL-1z2
H3K36me
lamin
C
ζ+εRNAi
wt
Elp3EX1
JIL-1z2
14-3-3
Pol IIser2
Lamin

Figure 2-6. Elp3 co-localizes with active chromatin and is dependent on 14-3-3 for recruitment to the chromosomes.

(A) Immunolocalization of Elp3 (red) and RNAP IIo^{ser5} (green) on wild type (wt) or *14-3-3 RNAi* knockdown larvae polytene chromosomes. DAPI (blue) stains DNA.

(B Immunolocalization of Elp3 (red) and RNAP IIo^{ser5} (green) on wild type (wt) or *14-3-3 RNAi* knockdown larvae polytene chromosomes after heat shock (hs). DAPI (blue) stains DNA.

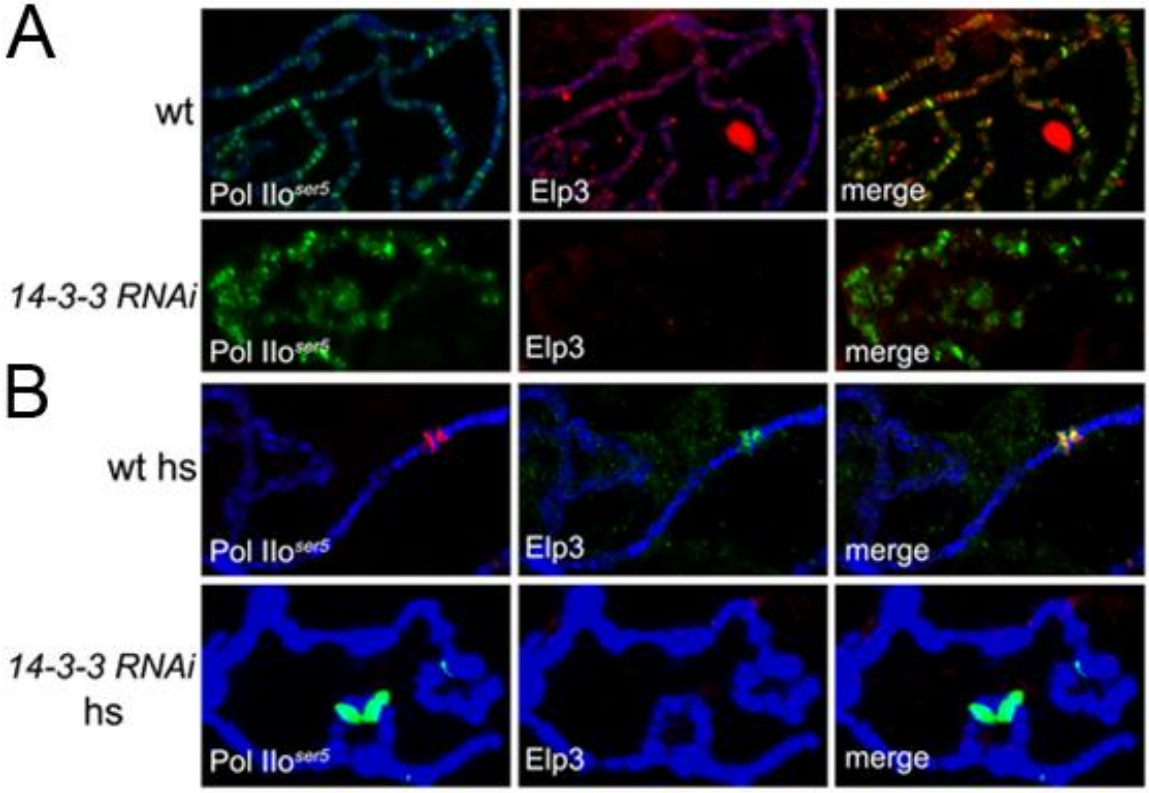

A
wt
Pol IIo ser5
Elp3
merge
14-3-3 RNAi
Pol IIo ser5
Elp3
merge
B
wt hs
Pol IIo ser5
Elp3
merge
14-3-3 RNAi
hs
Pol IIo ser5
Elp3
merge

Figure 2-7. Elp3 recruitment to chromatin is dependent on JIL-1 and 14-3-3.

Immunolocalization of Elp3 (green) in wild type larvae, *JIL-1*^{Z2} or *Elp3*^{EX1} mutant larvae, or larvae expressing 14-3-3 RNAi. Su(Hw) (red) was used as an internal control and DAPI stains DNA (blue).

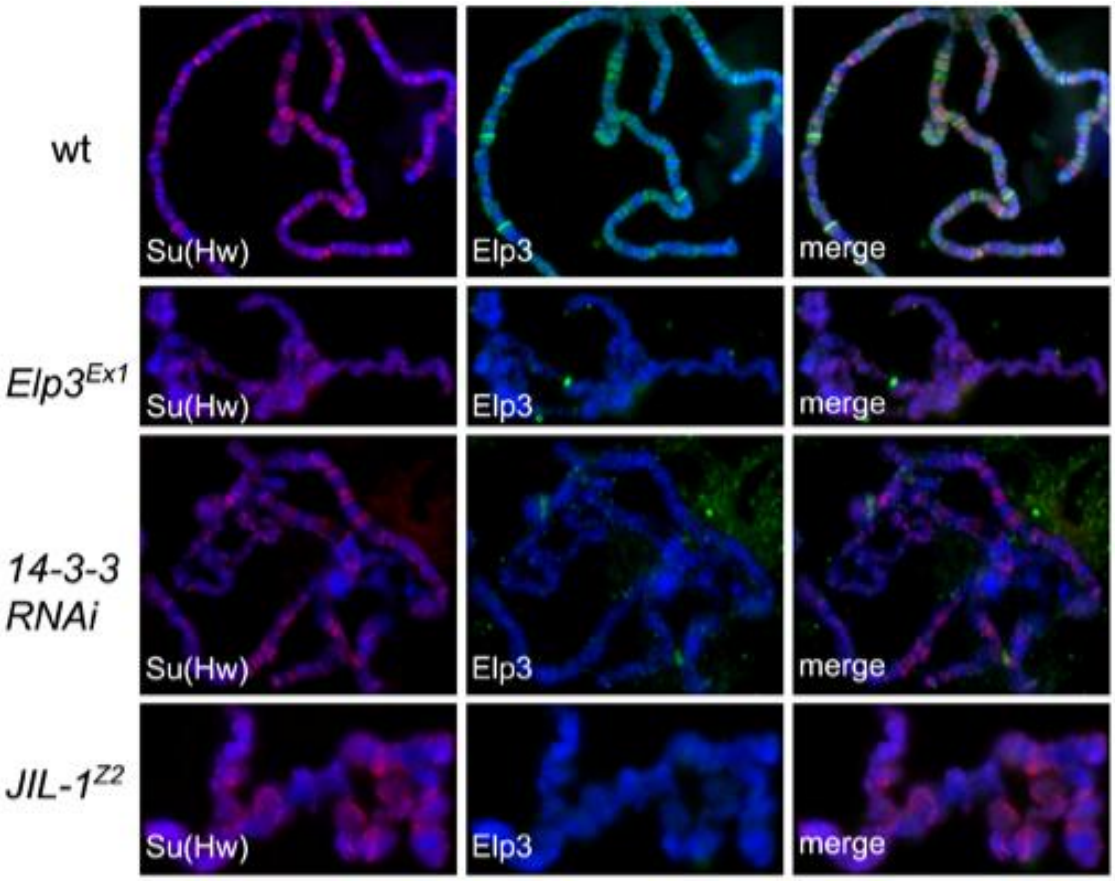

wt
Su(Hw)
Elp3
merge
Elp3Ex1
Su(Hw)
Elp3
merge
14-3-3
RNAi
Su(Hw)
Elp3
merge
JIL-1Z2
Su(Hw)
Elp3
merge

Figure 2-8. JIL-1, 14-3-3, and Elp3 are required for H3 acetylation.

(A) Western analysis of H3S10ph, H3K9ac, H3K9acS10ph, H3K14ac, H3S10phK14ac, H4K16ac, total H3 and lamin in salivary gland cell extracts of wild type (wt), *Elp3*^{EX1}, or *JIL-1*^{Z2} mutant larvae or those expressing RNAi against both 14-3-3 isoforms (14-3-3).

(B) Western analysis of acetylation levels of recombinant histones (H3K9ac, H3K14ac, or H4K16ac) subjected to an *in vitro* histone acetylase assay using larval lysates that were co-immunoprecipitated with either anti-Elp3 or anti-14-3-3 antibodies. Coomassie stain shown as loading control.

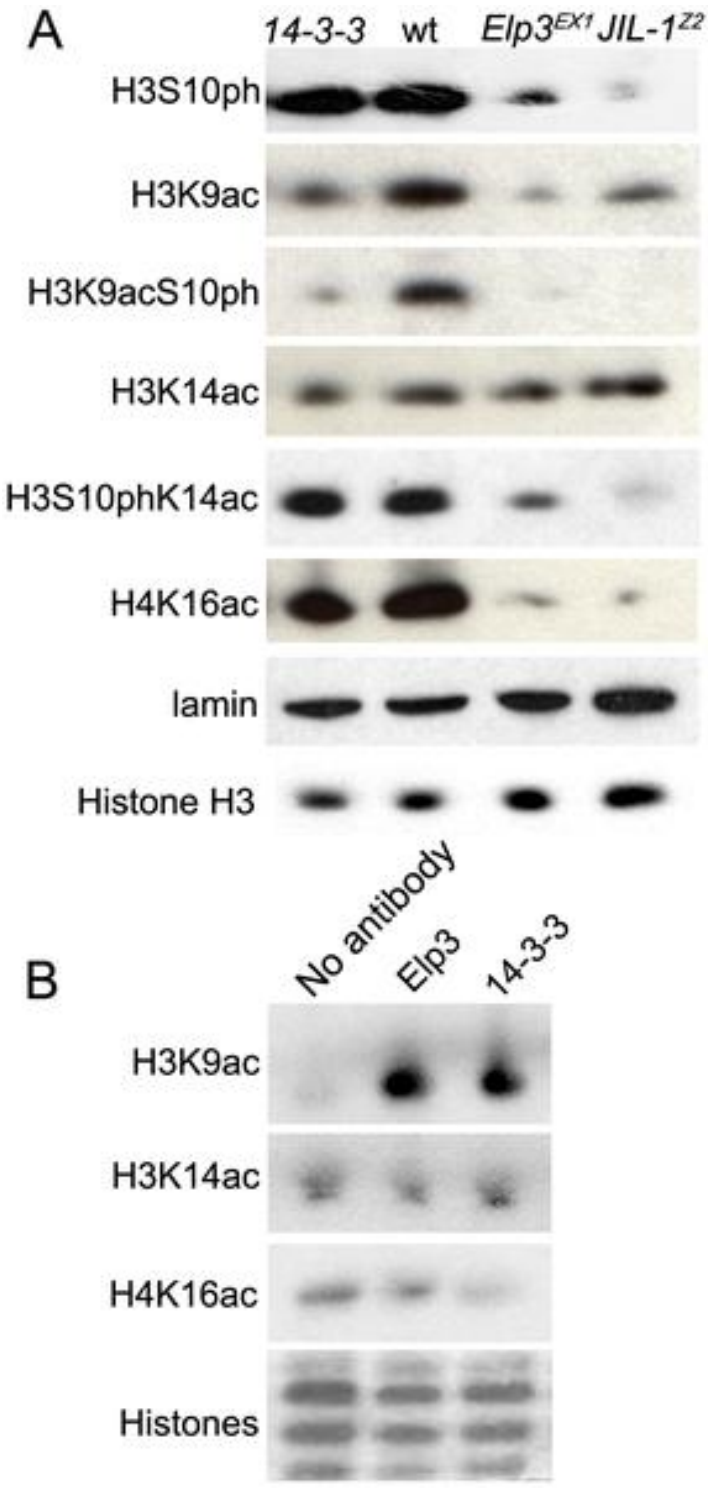

A
14-3-3
wt
Elp3EX1 JIL-1Z2
H3S10ph
H3K9ac
H3K9acS10ph
H3K14ac
H3S10phK14ac
H4K16ac
lamin
Histone H3
B
No antibody
Elp3
14-3-3
H3K9ac
H3K14ac
H4K16ac
Histones

Figure 2-9. Histone H3K9ac, a modification of active transcription carried out by Elp3, is dependent on JIL-1, 14-3-3, and Elp3.

Immunolocalization of H3K9ac (green) in wild type larvae, *JIL-1*^{Z2} or *Elp3*^{EX1}mutant larvae, or larvae expressing 14-3-3 RNAi. RNAP IIo^{ser5} (red) was used as an internal control and DAPI stains DNA (blue).

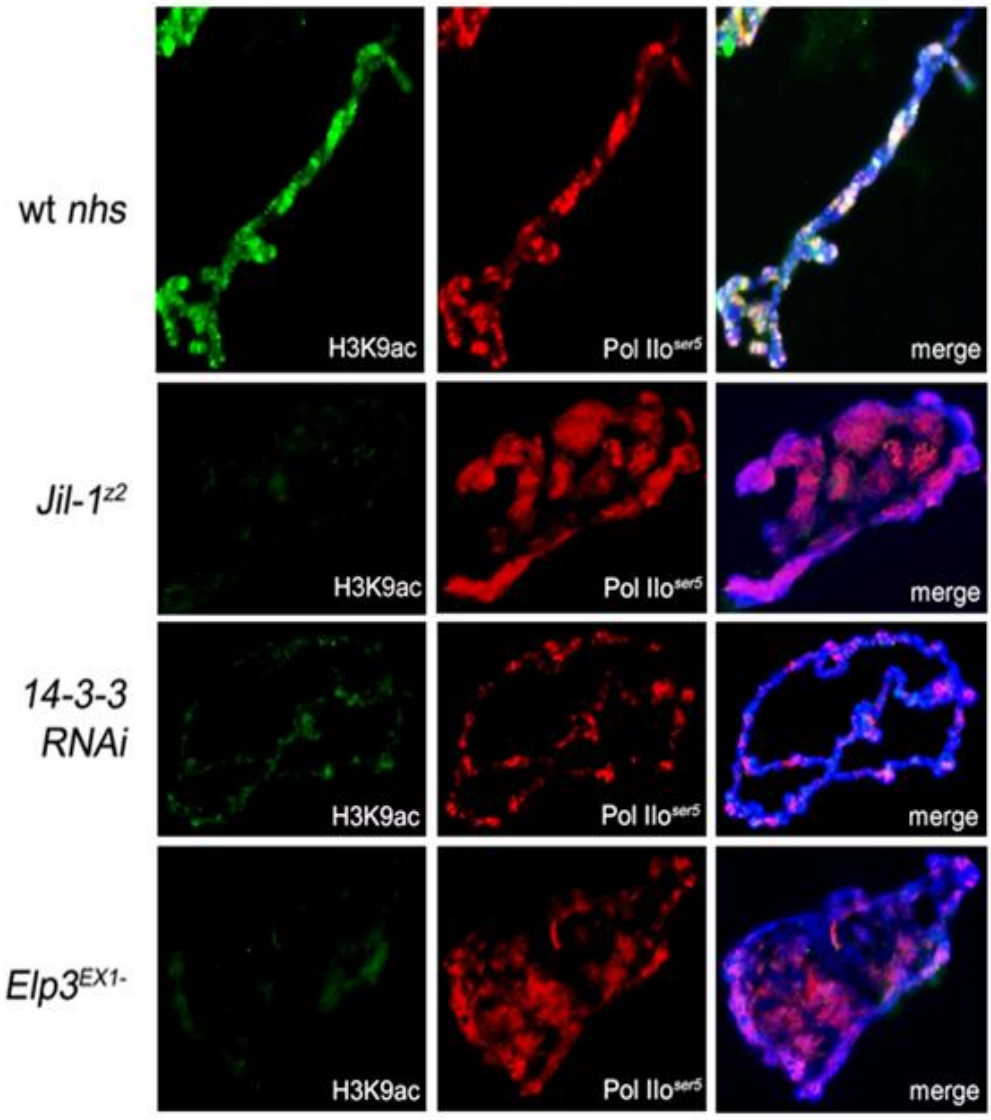

wt nhs
H3K9ac
Pol IIo ser5
merge
Jil-1 z2
H3K9ac
Pol IIo ser5
merge
14-3-3 RNAi
H3K9ac
Pol IIo ser5
merge
Elp3 EX1-
H3K9ac
Pol IIo ser5
merge

Figure 2-10. H4K16 acetylation is dependent on JIL-1 but not 14-3-3 in females.

Immunolocalization of H4K16ac (green) in female wild type larvae, *JIL-1*^{Z2} mutant larvae, larvae expressing 14-3-3 RNAi, or *Elp3*^{EX1} mutant larvae. Co-staining with RNAP IIo^{ser5} (red) used as an internal control and DAPI stains the DNA (blue).

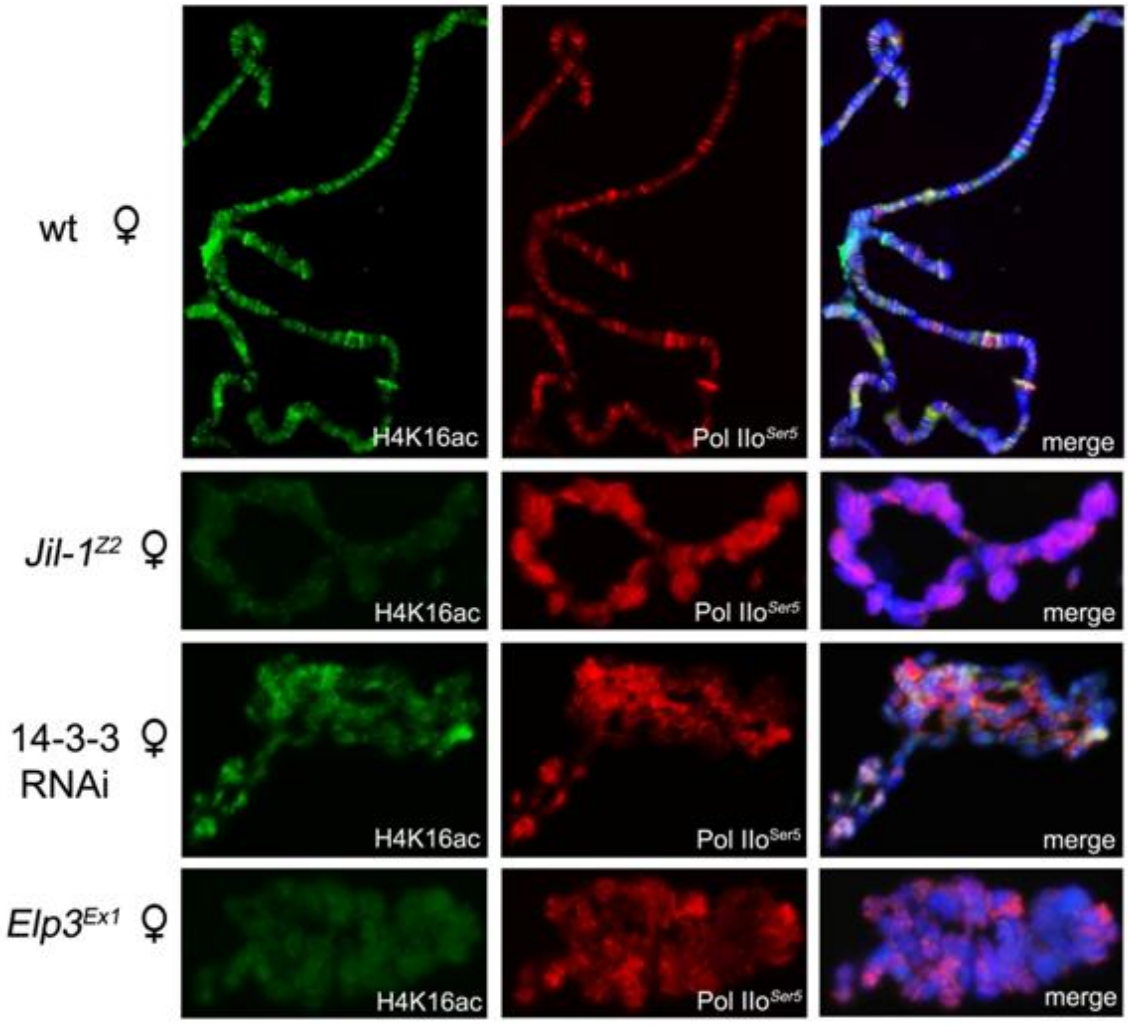
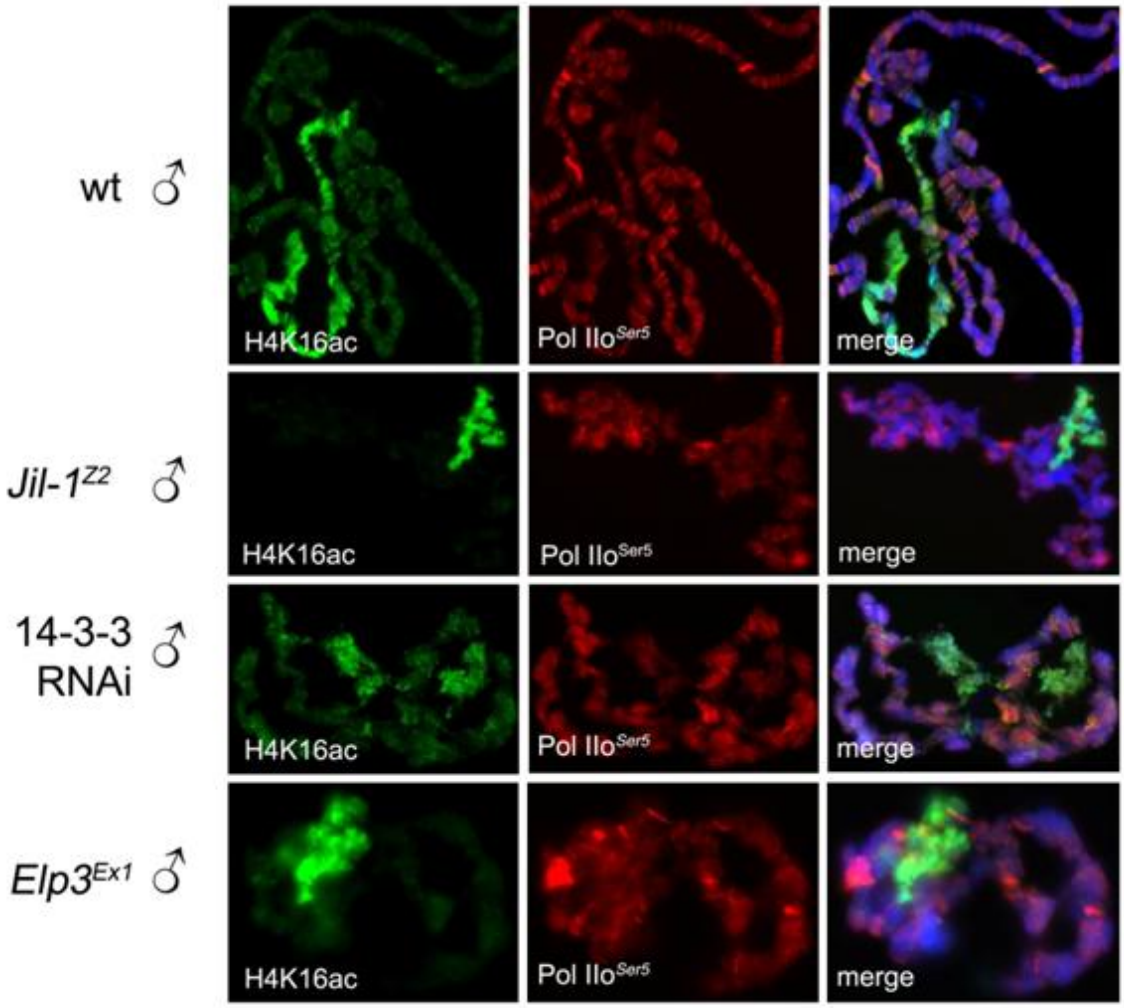

wt ♀
H4K16ac
Pol IIo Ser5
merge
Jil-1 Z2 ♀
H4K16ac
Pol IIo Ser5
merge
14-3-3 ♀
RNAi
H4K16ac
Pol IIo Ser5
merge
Elp3 Ex1 ♀
H4K16ac
Pol IIo Ser5
merge

Figure 2-11. H4K16 acetylation is dependent on JIL-1 but not 14-3-3 in males.

Immunolocalization of H4K16ac in male wild type larvae, *JIL-1*^{Z2} or *Elp3*^{EX1} mutant larvae, or larvae expressing 14-3-3 RNAi. RNAP IIo^{ser5} was used as an internal control and DAPI stains the DNA (blue).


wt ♂
Jil-1Z2 ♂
14-3-3 RNAi ♂
Elp3Ex1 ♂
H4K16ac
Pol IIoSer5
merge

Chapter 3

Genome-wide Phosphoacetylation of Histone H3 at *Drosophil*a Enhancers and Promoters

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ABSTRACT

Transcription regulation is mediated by enhancers that bind sequence-specific transcription factors, which in turn interact with the promoters of the genes they control. Here I show that the JIL-1 kinase is present at both enhancers and promoters of ecdysone-induced *Drosophila* genes, where it phosphorylates the Ser10 and Ser28 residues of histone H3. JIL-1 is also required for CBP-mediated acetylation of Lys27, a well characterized mark of active enhancers. The presence of these proteins at enhancers and promoters of ecdysone-induced genes results in the establishment of the H3K9acS10ph and H3K27acS28ph marks at both regulatory sequences. These modifications are necessary for the recruitment of 14-3-3, a scaffolding protein capable of facilitating interactions between two simultaneously bound proteins. Chromatin conformation capture assays indicate that interaction between the enhancer and the promoter is dependent on the presence of JIL-1, 14-3-3 and CBP. Genome-wide analyses extend these conclusions to most *Drosophila* genes, showing that the presence of JIL1, H3K9acS10ph and H3K27acS28ph is a general feature of enhancers and promoters in this organism.

INTRODUCTION

Cell differentiation in multi-cellular organisms requires the creation of complex spatiotemporal patterns of gene expression during development. The establishment and maintenance of these transcription blueprints entails the precise orchestration of a complex set of interactions among regulatory sequences and their binding proteins. Enhancers are one of these regulatory sequences capable of binding combinations of transcription factors that then recruit chromatin remodeling enzymes and/or interact with components of the Mediator complex or TFIID to help recruit RNA polymerase II (RNAPII) (Ong and Corces, 2011). Transcription activation is accompanied by the establishment of a complex set of covalent histone modifications, such that the transcriptional status of a gene is determined by the presence of distinct modifications at enhancers and promoters. Well documented are the interconnected phosphorylation, acetylation, and methylation of histones at enhancers and promoters that are necessary for proper transcription of genes (Berger 2007; Guenther et al., 2007; Heintzman et al., 2009).

Genome-wide studies have identified histone modificaitons unique to enhancers, suggesting that the function of these regulatory elements may depend on combinations of specific covalent histone modifications. Enhancer regions are marked prior to activation by monomethylated H3K4 (H3K4me1) and acquire histone H3 acetylated on lysine 27 (H3K27ac) carried out by either CBP (CREB Binding Protein) or p300 (Heintzman et al., 2009). Studies of the FosL1 gene have shown that phosphorylation of Ser10 of histone H3 pre-acetylated in Lys9 (H3K9acS10ph) by the PIM1 kinase at enhancer sequences promotes binding of 14-3-3 and facilitates acetylation of histone H4 on Lys16 (H4K16ac) by the histone acetyltransferase MOF (Zippo et al., 2009). Similarly, the MSK1/2 kinases phosphorylate histone H3S10 at the c-Jun and c-Fos genes in response to serum stimulation (Soloaga et al., 2003; Vincent et al., 2005).

Studies in *Drosophila* indicate that the JIL-1 kinase, which is the homologue of the mammalian MSK1/2 kinases, is also responsible for phosphorylation of H3S10 (Wang et al., 2001), but its precise role in transcription remains controversial (Cai et al., 2008). I have proposed a direct role for JIL-1 in transcription and shown that JIL-1 is recruited to the promoter of the *hsp70* gene using standard ChIP assays. Previous studies show H3S10ph is associated with other actively transcribed genes and is required for the release of RNAPII from promoter-proximal pausing (Nowak et al., 2003; Ivaldi et al., 2007). H3S10ph then recruits 14-3-3 and the Elp3 histone acetyltransferase, which acetylates H3K9 and is necessary for the release of the paused polymerase (Karam et al., 2010).

In this study I analyze the role of JIL-1 in some of the events that take place before and after initiation of transcription and are necessary to proceed to productive elongation upon gene activation. To overcome concerns affecting the use of immunofluorescence techniques using acid-fixed polytene chromosomes (Cai et al., 2008), standard Chip and ChIP-seq assays using antibodies previously validated by ENCODE and modENCODE are used instead. I find that enhancers and promoters of ecdysone-induced genes undergo phosphorylation in the Ser10 and Ser28 residues of histone H3 by the JIL-1 kinase. The same histone tails are also acetylated at Lys9 and Lys27. These modifications are able to recruit 14-3-3, which I demonstrate is necessary to achieve a stable interaction between enhancer and promoter. I then extend these findings by examining the localization of these proteins genome-wide, and conclude that all or most *Drosophila* enhancers and promoters contain JIL-1, H3K9acS10ph and H3K27acS28ph. These results may offer important insights into the mechanisms used by cells to establish and/or maintain interactions between distant regulatory sequences in the genome.

RESULTS

JIL-1 is Responsible for H3S28 Phosphorylation During Transcription in *D. melanogaster*

To investigate whether H3S28 phosphorylation is involved in transcription in *Drosophila* cells and JIL-1 might be the kinase responsible for carrying out this modification, I examined polytene chromosomes from third instar larvae by immunofluorescence microscopy using antibodies specific to H3S28ph. The results of these experiments show that H3S28ph is present in interbands of polytene chromosomes, which contain actively transcribed genes, and appears at the heat shock puffs upon temperature elevation (Figure 3-1). Polytene chromosomes from *JIL-1* mutant larvae lack H3S28ph at the heat shock genes after exposure to elevated temperature. Lysates from salivary glands of *JIL-1* mutants also show a dramatic loss of H3S28ph (Figure 3-1C). Furthermore, *in vitro* kinase assays using JIL-1 immunoprecipitated from cell extracts show abundant phosphorylation of H3S28 (Figure 3-1D). These data suggest that JIL-1 is the kinase responsible for H3S28 phosphorylation upon transcriptional activation in *Drosophila*.

JIL-1 phosphorylation of H3S10 and H3S28 at promoters and enhancers is necessary for transcriptional activation of ecdysone responsive genes

To better understand the role of JIL-1 in transcription activation, I examined the presence of this protein at genes induced by the steroid hormone 20-hydroxyecdysone (20-HE) using ChIP experiments in Kc167 cells treated with this hormone. I analyzed three different ecdysone-induced genes, *Eip75B, Hsp27*, and *Eip71CD*, whose structure and organization is shown in Figure 3-2A (Riddihough and Pelham, 1987; Cherbas et al., 1991; Bernardo et al., 2009). Interestingly, all three genes contain a significant amount

of polymerase at the 5' end of the gene despite relatively low levels inside the gene suggesting these genes have a paused polymerase (Figure 3-2A). Chromatin immunoprecipitation (ChIP) assays performed on both untreated cells and cells treated with 20-HE for 3 hr demonstrate that the predicted enhancers for all three genes contain high levels of H3K4me1, a characteristic mark of enhancers, which increases upon activation. There is a small amount of initiated polymerase (RNAPII S5ph) at all three genes, in agreement with the fact that they are transcribed at low levels in untreated Kc cells (Gauhar et al., 2009; Wood et al., 2011). After induction, H3K4me3 increases at the promoter, while an increase of elongating RNA polymerase (RNAPII S2ph) is detected at promoters and coding regions, but not at enhancers (Figure 3-2B). These results verify that these promoter/enhancer pairs have the expected histone modifications after transcription activation.

I then examined whether JIL-1 is recruited to enhancers, promoters, and coding regions upon gene activation after 20-HE treatment. I also analyzed the presence of H3S10ph and H3S28ph, the two modifications carried out by JIL-1, at these regions when transcription is turned on. ChIP experiments using antibodies to JIL-1, H3S10ph and H3S28ph, are performed with and without 20-HE treatment of Kc167 cells. Results indicate that JIL-1 is recruited to both enhancers and promoters after 20-HE treatment and that H3S10ph and H3S28ph increase at these two sequences concomitant with the presence of JIL-1 (Figure 3-3A). In order to confirm that JIL-1 is responsible for the phosphorylation of H3 detected at the enhancers and promoters of the ecdysone responsive genes, I knocked down this enzyme in Kc167 cells using dsRNA and performed the same ChIP experiments after treatment with 20HE for 3 hr (Figure 3-3B). The JIL-1 knockdown cells show a severe growth delay and, at higher levels of knockdown, a lethal phenotype. The ChIP data demonstrate that loss of JIL-1 results in

loss of H3S10ph and H3S28ph from both the promoter and enhancer upon induction when compared to the β-gal knockdown used as a control. Additionally, the levels of initiated and elongating polymerase at the promoter and coding regions suggest that JIL-1 knockdown interferes with transcription elongation regardless of normal initiation of polymerase at the promoter (Figure 3-3B). A complete knockdown of JIL-1, manifested by cell growth defects, is necessary to observe these effects on transcription elongation. These data confirm earlier results suggesting that JIL-1 functions after transcription initiation but before elongation (Ivaldi et al., 2007). To confirm the transcription defects caused by lack of JIL-1, I isolated mRNA followed by reverse transcription to measure transcript levels of the *Eip75B* gene in JIL-1 knockdown versus β-gal knockdown cells. *Eip75B* encodes four transcripts named Eip75B–RA, -RB, -RC and -RD (Figure 3-2A); the first three transcripts undergo a large fold increase in expression upon hormone treatment whereas the Eip75B–RD transcript shows little or no increase. In cells lacking JIL-1, all three inducible transcripts show significantly loIr expression levels (Figure 3-3C). Therefore, JIL-1 is responsible for the H3S10ph and H3S28ph modifications observed at both the enhancer and promoter and lack of these modifications in JIL-1 knockdown cells correlates with failure to activate transcription.

CBP acetylation at promoters and enhancers is necessary for transcription activation of ecdysone-responsive genes

H3K27ac is a characteristic mark of active enhancers and is deposited by either CBP or p300 in mammals (Visel et al., 2009). In *Drosophila* there is no orthologue of p300, and CBP is the only reported acetyltransferase responsible for catalyzing this acetylation event (Tie et al., 2009). H3S28 phosphorylation facilitates loss of the

repressive methylation mark H3K27me3 in the neighboring residue and subsequent acetylation of the same lysine (Tie, Banerjee et al. 2009; Lau and Cheung 2011). Acetylation of H3K9 is enhanced by 14-3-3, a phospho-serine binding protein, via recruitment of the Elp3 acetyltransferase (Karam et al., 2010). *D. melanogaster* has two isoforms of 14-3-3, 14-3-3ζ and 14-3-3ε, both of which have been shown to bind H3S10ph and H3S28ph (Macdonald et al., 2005). Coimmunoprecipitation experiments with either H3S10ph or H3S28ph antisera demonstrate Jil-1 associates equally with H3S10ph and H3S28ph as expected since it is the enzyme responsible for carrying out both modifications . Western blots performed on the pulldown against the acetylated histones H3K9ac and H3K27ac demonstrate H3S10ph and H3S28ph modified histones associate with H3K9ac and H3K27ac at equal amounts. 14-3-3 associates more strongly with H3S28ph than H3S10ph, consistent with a higher predicted amino acid binding concensus for 14-3-3 (Figure 3-4).. I hypothesize that a similar process may take place at enhancers, with 14-3-3 binding to H3S28ph to recruit CBP and acetylate H3K27.

To confirm binding of CBP and 14-3-3 at enhancers and promoters, and acetylation of H3K27 upon transcriptional activation, I performed ChIP assays at the three selected ecdysone responsive genes. The 14-3-3 antibody detects both isoforms of *Drosophila* 14-3-3. Results show that CBP and 14-3-3 are recruited to both enhancers and promoters after 20-HE treatment. In addition, H3K27ac undergoes a significant increase at these two regulatory sequences upon gene activation (Figure 3-5A). ChIP analyses using antibodies against the dual modification H3K27acS28ph suggest that acetylation and phosphorylation occur on the same histone tail. These results indicate that most of the increase in H3K27ac detected upon transcription activation takes place in the tail of H3 carrying the H3S28ph mark at these enhancers

and promoters.

To confirm that CBP is the acetyltransferase responsible for acetylation of H3K27 during the ecdysone response, ChIP was performed in CBP knockdown cells (Figure 3-5C) at the *Eip75B* gene. Downregulation of CBP results in a dramatic decrease of H3K27ac and H3K27acS28ph at both promoter and enhancer (Figure 3-5C). Lack of CBP had no effect on the amount of initiating RNAPII but caused a reduction in the levels of elongating polymerase. The same experiments were then performed using knockdown cells for both isoforms of 14-3-3 (Figure 3-5D). Lack of 14-3-3 results in a reduction of H3K27ac upon hormone induction. However, there is not complete loss of H3K27ac, suggesting that the role of 14-3-3 in CBP activity may be partially redundant (Figure 3-5D). Normal levels of initiated polymerase are present at the promoter of *Eip75B* in both Kc cells treated with CBP or 14-3-3 dsRNAs, but loss of the elongating polymerase suggests that these proteins may function at the promoter-proximal pause step. The loss of elongating polymerase is confirmed by the significant decrease of all three 20-HE induced *Eip75B* transcripts in cells lacking CBP or 14-3-3 (Figure 3-5E). Neither mutation of CBP or 14-3-3 have an effect on the levels of H3S28ph (Figure 3-6). From these data I can conclude that CBP activity, enhanced by 14-3-3, can be detected at both enhancers and promoters and is necessary for transcription elongation.

14-3-3 recruitment and H3K27 acetylation are dependent on JIL-1 kinase at enhancers and promoters before release of polymerase from promoter-proximal pause.

If the hypothesis that H3K27 acetylation is facilitated by H3S28 phosphorylation and subsequent 14-3-3 recruitment is correct, then loss of H3S28ph should result in a

decrease of H3K27ac. To test this, JIL-1 is knocked down with dsRNAs and examined for the presence of H3K27ac and 14-3-3 using ChIP. It can be concluded from the dramatic decrease of both 14-3-3 and H3K27ac that the activity of CBP and 14-3-3 recruitment is dependent on JIL-1 at enhancers of ecdysone responsive genes (Figure 3-7A). Although there is also a significant decrease in H3K27acS28ph and 14-3-3 in promoter regions, the difference in H3K27ac before and after ecdysone induction is not statistically significant. Coimmunoprecipitation experiments demonstrate that CBP and JIL-1 physically interact and the interaction is facilitated by phosphorylation (Figure 3-7B). JIl-1 interacts with 14-3-3ε (top band); this is probably not the same 14-3-3 molecule associated with histones, since histones interact with both the epsilon and zeta isoforms equally (Figure 3-4).

Western blots were performed on lysates of all the knockdown cells to ensure that there is not a loss of any of the other proteins as a secondary effect of inhibition of transcription in these cells. Results show that levels of JIL-1 are normal in the 14-3-3 knockdown (Figure 3-7E).

To further test a possible effect of inhibition of transcription on protein levels, cells were treated with DRB, an inhibitor of the positive transcription elongation factor b (P-TEFb). DRB-treated Kc167 cells show normal levels of H3K27acS28ph as measured by western blots (Figure 3-7E) or ChIP analysis of the *Eip75B* gene upon 20-HE induction (Figure 3-7C), but fail to activate transcription of any of the *Eip75B* gene isoforms when measured by quantitative PCR (Figure 3-7D). These results demonstrate that H3S28 phosphorylation and H3K27 acetylation take place before the release of the polymerase, the step inhibited by DRB treatment.

JIL-1, 14-3-3, and CBP are required for enhancer-promoter interactions

I then used 3C (Dekker et al.,2002) to test the possibility that physical contacts between an enhancer and promoter of the *Eip75B* gene depend on the presence of the JIL-1, CBP or 14-3-3 proteins. The specific enhancer is located 37.5 kb downstream of the *Eip75B-RB* promoter and has been previously shown to be responsible for activating expression of this gene (Bernardo et al., 2009). The results show no interaction between the enhancer and the *Eip75B-RB* promoter in Kc167 cells (Figure 3-8). After a 3 hr ecdysone treatment, when expression of the *Eip75B-RB* gene is induced, I observe a dramatic increase in the interaction between the enhancer and the promoter of the gene (Figure 3-8). To determine the role of H3K27 acetylation and H3S10 and H3S28 phosphorylation in the establishment of these interactions, I next performed 3C on CBP or JIL-1 knockdown cells. In both cases, lack of the CBP or JIL-1 proteins results in a decrease in the strength or frequency of the interactions between the enhancer and the promoter, suggesting that the presence of the proteins themselves or the histone modifications they perform correlate with the establishment and/or maintenance of contacts between these two sequences. Since the role of H3S10ph and H3S28ph is to recruit 14-3-3 and this protein normally plays a role as a scaffold of large protein complexes, I examined whether enhancer-promoter interactions are affected in cells lacking both isoforms of 14-3-3. The results show a dramatic decrease in enhancer-promoter contacts detected by 3C in cells treated with ecdysone and lacking 14-3-3, suggesting an essential role for this protein in bringing together these two sequences in order to activate transcription (Figure 3-8).

Since knockdown of JIL-1, CBP or 14-3-3 inhibits transcription, it is possible that the observed effects on enhancer-promoter interactions are indirect and caused by lack

of expression of other factors important for transcription. To discard this possibility I examined the effect of DRB on promoter-enhancer interactions at the *Eip75B-RB* gene using 3C. DRB-treated cells, however, are able to achieve contacts between the promoter and the enhancer that are similar in frequency to those in untreated cells, even though transcriptional elongation of the gene is lost (Figure 3-8). These data suggest that promoter-enhancer contacts in the *Eip75B* gene depend on histone modifying enzymes that recruit 14-3-3, which in turn may serve as a physical bridge that stabilizes these interactions.

JIL-1 and phosphoacetylated H3 are present at promoters genome-wide and their quantity correlates with transcription levels

In order to investigate whether the presence of JIL-1, H3K9acS10ph and H3K27acS28ph at promoters is specific for ecdysone-inducible genes or is a general phenomenon, I carried out ChIP-seq experiments. Genome-wide datasets were generated in Kc cells not treated with ecdysone for H3K4me3, H3K4me1, H3K27ac, JIL-1, H3K9acS10ph, and H3K27acS28ph. Heatmaps of each modification around the TSS sorted by transcript levels show a correlation between the presence and amounts of JIL-1, H3K9acS10ph and H3K27acS28ph at gene promoters and levels of transcription (Figure 3-9A). A peak of JIL-1 is directly present over the TSS and drops within the first 200 bp inside the gene (Figure 3-9B). This distribution is consistent with the proposed association of JIL-1 with paused RNAPII. Examples of the distribution of JIL-1 with respect to genes based on these ChIP-seq data and in comparison with ChIP-chip datasets obtained by modENCODE are shown in Figure 3-10. Both H3K9acS10ph and H3K27acS28ph have very similar profiles as H3K4me3 suggesting these histone

modifications occur on the same nucleosome, the peak of which is located at the first nucleosome downstream from the TSS (Figure 3-9A). These results suggest that JIL-1 is present at the promoter region of actively transcribed genes and correlates with transcript levels, supporting the idea that JIL-1 plays a direct role in the transcription process.

JIL-1 and phosphoacetylated H3 are found at enhancers genome-wide prior to activation of transcription

To investigate a possible role for JIL-1 at enhancers genome-wide, I first identified regions with significant enrichment for H3K4me1 (15,369) and then subtracted any that were within 1kb of any TSS (10,655). Heatmaps generated for all the datasets were clustered to identify patterns between all the sets. Clustering appeared to be driven by the presence or absence of H3K27ac (Figure 3-11A). H3K27ac has been identified as a histone modification associated with active enhancers and it is the best characterized measure of enhancer activity (Heintzman et al., 2009; Creyghton et al., 2010; May et al., 2012). JIL-1, H3K9acS10ph and H3K27acS28ph appear to be present at gene enhancers irrespective of the levels of H3K27ac. Nevertheless, the cluster of genes containing high levels of H3K27ac appears to also have higher levels of JIL-1 and both phosphoacetylated forms of H3 (Figure 3-11B). Overall, the results suggest that JIL-1 and phosphoacetylated histone H3 are present at enhancers prior to activation and that their levels increase when enhancers are active.

DISCUSSION

Activation of transcription in higher eukaryotes requires the interaction between transcription factors bound to distal enhancers and proteins present at the promoter. Recent findings indicate that enhancers contain a variety of histone modifications that change during the establishment of specific cell lineages suggesting that these sequences may play a more complex role in transcription than previously thought (Ong and Corces, 2011). Given the presence of common as well as specific histone marks at enhancers and promoters, it is tempting to speculate that epigenetic modifications at these sequences serve to integrate various cellular signals required to converge in order to activate gene expression. Results described here support this hypothesis demonstrating the proteins that carry out these histone modifications are necessary to establish enhancer-promoter contacts and activate transcription of ecdysone-inducible genes.

The execution of this process in *Drosophila* requires the recruitment of JIL-1 by mechanisms that are not well understood. Although the direct involvement of JIL-1 in the transcription process has been brought into question due to the failure to observe recruitment of JIL-1 to heat shock genes in polytene chromosomes (Cai et al., 2008), results presented here clearly indicate that JIL-1 affects transcription at different steps in the transcription cycle. At the promoter region, phosphorylation of H3S10 by JIL-1 results in the recruitment of 14-3-3 and, subsequently, histone acetyltransferases Elp3 and MOF (Karam et al., 2010). Here I find that JIL-1 is also able to phosphorylate H3S28 at both promoters the enhancers. The establishment of the H3K9acS10ph and H3K27acS28ph modifications correlates with the recruitment of 14-3-3 to enhancers and promoters of ecdysone-induced genes. 14-3-3 has been implicated in numerous cellular processes, where it functions as a scaffold protein (Morrison 2009). 14-3-3 is found as dimers and multimers; each monomer is capable of binding two targets and can mediate

and stabilize interactions between two phosphoproteins. Additionally, acetylation facilitates the dimerization of 14-3-3 molecules and their ability to bind certain substrates (Tzivion et al., 1998; Choudhary et al., 2009). Binding assays have demonstrated that 14-3-3 interacts weakly with H3 tail peptides phosphorylated at S10 and S28 but strong binding is detected if the peptide is both phosphorylated and acetylated on the neighboring lysine residues (Walter et al., 2008). Given the ability of 14-3-3 to serve as a scaffold for large protein complexes, its demonstrated interactions with H3K9acS10ph and H3K27acS28ph and the presence of these two modifications at enhancers and promoters, it is possible that contacts between these two sequences are stabilized by 14-3-3. This hypothesis is supported by 3C experiments indicating that induction of transcription of the *Eip75B* gene is accompanied by strong enhancer-promoter interactions. These interactions are lost in JIL-1, CBP and 14-3-3 knockdown cells. Since these proteins act several steps downstream from transcription factor binding in the pathway leading to enhancer-promoter contacts, and loss of these proteins results in the abolishment of these contacts, it appears that these proteins, rather than specific transcription factors, may be responsible for enhancer promoter interactions at the ecdysone-inducible genes.

Genome-wide studies using ChIP-seq clearly show the presence of JIL-1, H3K9acS10ph and H3K27acS28ph at enhancers and promoters of most *Drosophila* genes. There is a clear correlation between the amount of JIL-1, H3K9acS10ph and H3K27acS28ph at promoters and the level of transcripts associated with the gene. These three marks are also present at enhancers defined by the occurrence of H3K4me1 and H3K27ac, suggesting that the JIL-1 kinase is a regulator of histone dynamics at enhancers and promoters genome-wide. JIL-1, H3K9acS10ph and H3K27acS28ph are found at low levels at enhancers before activation, which then

increase in intensity at the enhancer center and decrease in surrounding regions. These conclusions are different from those previously published examining the role of JIL-1 in transcription and dosage compensation (Regnard et al., 2011). The authors from this study conclude that JIL-1 binds active genes along their entire length and that the levels of JIL-1 are not associated with levels of transcription. The differences in the conclusions may be due to the different cell lines used, male S2 cells versus female Kc cells, and the emphasis of the analysis by Regnard et al on the expression of dosage-compensated genes in the male X-chromosome, which may contain JIL-1 throughout the genes as a consequence of their regulation at the elongation step. In addition, the study by Regnard et al. used ChIP-chip on custom tiling arrays of the X-chromosome plus cDNA arrays containing the whole genome. This strategy may bias the conclusions and suggest the presence of JIL-1 in the coding region of genes rather that at enhancers and promoters.

Results presented here extend the previous list of histone modifications characteristic of active enhancers to include H3K9acS10ph and H3K27acS28ph. Enhancers tend to be cell type specific and are determined during differentiation with the characteristic H3K4me1 modification (Visel et al., 2009; Taberlay et al., 2011). It is unclear how these regions are designated before activation and what keeps them in a poised state ready for activation upon receiving the proper signal from the cell. It is tempting to speculate that the presence of JIL-1 at enhancers prior to activation might play a role in maintaining the enhancer in this poised state. An important question for future studies is the mechanistic significance of the looping between enhancers and promoters in order to achieve transcription activation. One interesting possibility is that various signaling pathways in the cell contribute to building epigenetic signatures at enhancers and promoters in the form of histone acetylation and/or phosphorylation of various Lys/Ser/Thr residues. Acetylation marks at enhancers and promoters may then

cooperate to recruit Brd4 [Fs(1)h in *Drosophila*], which contains two bromodomains each able to recognize two different acetylated Lys residues (Vollmuth et al., 2009). The requirement for acetylation of histones at enhancers and promoters in order to recruit Brd4 would ensure that several different signaling events have taken place before recruitment of PTEF-b by Brd4 can release RNAPII into productive elongation.

MATERIALS AND METHODS

Treatment of cells with 20-HE, dsRNA, and DRB

Kc167 cells were grown in CCM3 serum-free insect media (HyClone SH30065.01) at 25ºC. For RNAi treatment, cells were plated at 0.5 x 10^6 cells/mL and incubated with dsRNA targeting either the *JIL-1, CBP, or 14-3-3ζ and 14-3-3ε* genes (Table 3-1) using Cellfectin II reagent (Invitrogen 10362-100). After addition of dsRNA, cells were grown for 4 days, re-plated at 2 x 10^6 cells/mL, re-treated with dsRNA as before and grown for 4 additional days. For control RNAi treatments, the same procedure was performed using dsRNA targeting the β-*gal* gene, but incubations were 2 days after each treatment instead of 4 days to collect a similar number of cells. All experiments labeled "Non" in the figures were treated with ethanol (the solvent used to prepare 20-HE solutions) for 3 hr and all experiments labeled 20-HE, JIL-1 KD, CBP KD, and 14-3-3 KD were treated with 20-HE (Sigma H5142) at a final concentration of 5 x 10^{-7} M for 3 hr. For DRB (5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside; Sigma D1916) treatment, cells grown to 2 X 10^6 cells/ml were pretreated for 5 min at a concentration of 100 µM; 20-HE was then added to the media for an additional 3 h incubation as described above.

Antibodies and Co-IP experiments

All antibodies to histone modifications used in the studies described here have been tested by modENCODE and shown to specifically recognize appropriate epitopes (http://compbio.med.harvard.edu/antibodies/). The following antibodies were used in this study. Histone H3 (Abcam ab1791), H3K4me3 (Abcam ab8580), H3K4me1 (Abcam ab8895), RNAPII S5ph (Covance MMS-134R), RNAPII S2ph (Covance MMS-129R), H3S28ph (Mwellipore 07-145), H3K27acS28ph (Mwellipore 05-896), H3K27ac (Abcam ab4729), JIL-1 (Karam, Kellner et al. 2010), H3S10ph (Mwellipore 05-806), 14-3-3 (Santa Cruz Biotechnology sc-629), CBP (M. Mannervik, Stockholm University). Specificity of the antibodies against H3K27ac and H3K27acS28 was examined using dot blots of the appropriate peptides and it is shown in Figure S5. For immunoprecipitation experiments, 2 X 10^6 Kc167 cells were lysed in cell lysis buffer (5 mM PIPES (pH8.0), 85 mM KCl, 0.5% TX-100), nuclei were spun down for lysis in 1 ml RIPA buffer containing protease inhibitor complex (Roche 04 693 159 001) and briefly sonicated to ensure lysis. Nuclear lysates are treated with 5 µl DNase I (Sigma D5319) plus 6 mM $MgCl_2$ at 25ºC for 20 min to obtain single nucleosomes. Antibodies are incubated in lysate at 4ºC for 6 hr and precipitated with 50/50 mixture of Protein A and Protein G beads. After 4 washes in RIPA buffer, bound proteins are eluted in 1x Laemmli buffer and analyzed by western blot.

ChIP and 3C Analysis

ChIP experiments are performed on 2X10^7 Kc167 cells as described previously (Wood et al., 2011). ChIPs are quantified as percent of input on a standard curve using SYBR real-time PCR. n= 5 biological replicates of the β-gal knockdown samples labeled Non and 20HE, n= 3 biological replicates for JIL-1 KO, CBP KO, 14-3-3 KO, and DRB samples. For ChIP-seq experiments, libraries are sequenced on an Illumina GAII or

HiSeq system and reads are aligned to the *Drosophila* dm3 genome using Bowtie v0.12.7. Peak files and wiggle files are generated using MACS v1.4.1. 3C-qPCR analysis was done as described (Wood, et al. 2011). Primers used for anchor and bait fragments can be found in Table 3-2. Error bars represent the standard deviation of the mean of 3 biological replicates.

Expression Analysis

Cells (1×10^6) are collected, RNA extraction was carried out using the RNeasy kit (Qiagen) and the RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Transcript specific primers spanning exon junctions for the *Eip75B* gene are as described (Wood, Van Bortle et al. 2011). All samples are normalized to mitochondrial gene product myt:Col and the Non sample set to 1 for comparison. Error bars represent the standard deviation of the mean of 3 biological replicates for the JIL-1, CBP and 14-3-3 knockdowns and 5 biological replicates of the β-gal knockdown.

Figure 3-1. JIL-1 kinase is necessary for H3S28 phosphorylation at actively transcribed regions of polytene chromosomes

(A) Immunolocalization of JIL-1 and H3S28ph on wild type polytene chromosomes before heat shock.

(B) Immunolocalization of JIL-1 and H3S28ph on polytene chromosomes before and after heat shock in wild type and *JIL-1* mutants (nhs, non heat-shocked; hs, after heat-shock).

(C) western blot of salivary gland protein extracts from wild type and *JIL-1* mutant larvae showing levels of H3S28ph.

(D) *In vitro* JIL-1 kinase assay of histones. Immunoprecipitates of either IgG or JIL-1 incubated with ATP and histones. The reaction is run on a gel for western blot analysis using antibodies against H2S28ph. A coommassie staining of the histone region is included to ensure equal histone concentrations in the assay. Lysate used for immunoprecipitates was included for reference of H3S28ph location. This demonstrates JIL-1 phosphorylates H3S28 *in vitro*.

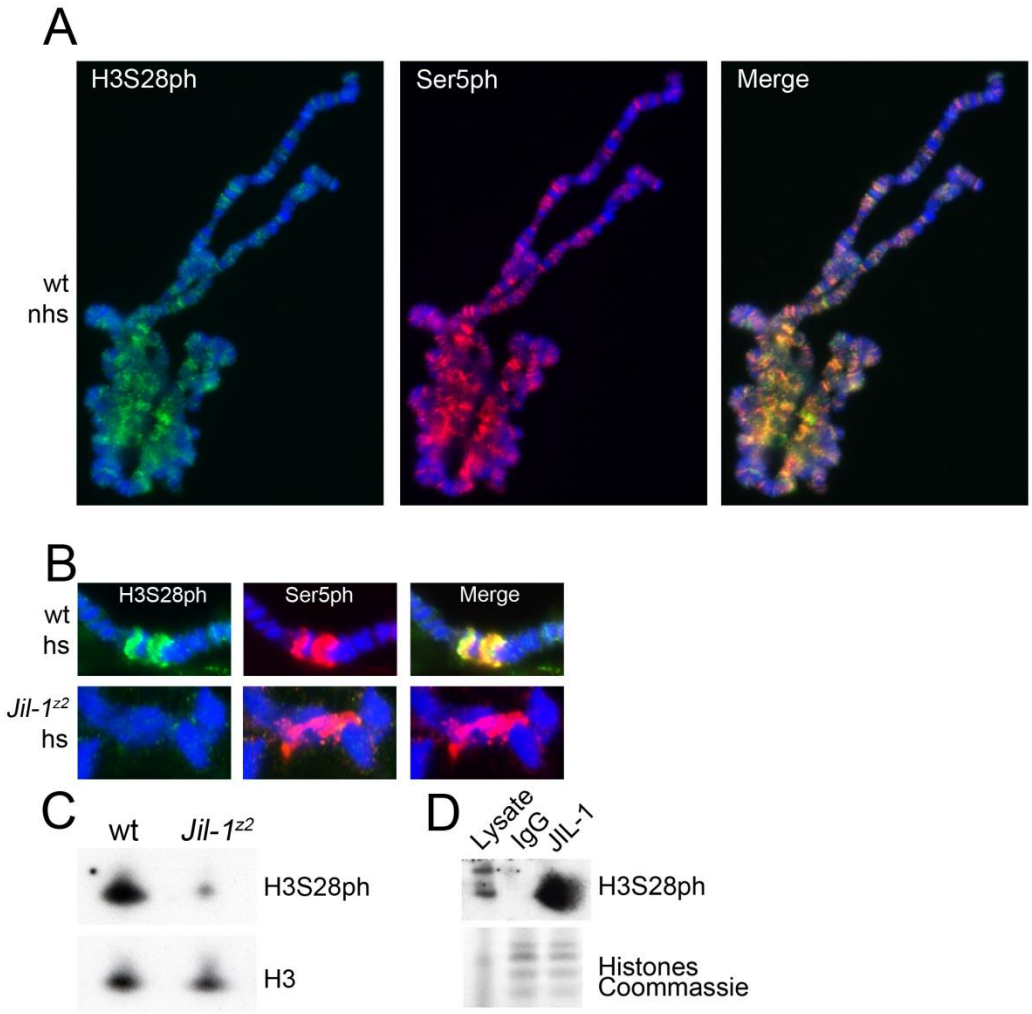

A
H3S28ph
Ser5ph
Merge
wt
nhs
B
H3S28ph
Ser5ph
Merge
wt
hs
Jil-1z2
hs
C
wt
Jil-1z2
H3S28ph
H3
D
Lysate
IgG
JIL-1
H3S28ph
Histones
Coommassie

Figure 3-2. JIL-1 phosphorylation of H3S10 and H3S28 at promoters and enhancers is necessary for transcriptional activation of ecdysone responsive genes

(A) Structure of three early 20-HE responsive genes with previously characterized enhancers showing the location of promoter, enhancer and coding sequences used in ChIP analyses.ChIP-chip (modENCODE) profile of RNAPII large subunit indicated in green above each gene.

(B) ChIP analysis of Kc167 cells treated for 3 hr with ethanol (Non) or 20-HE (20HE). Antibodies used for pull-down are indicated along the X-axis (RNAPII phosphorylated on serine 5 of CTD=Ser5ph, RNAPII phosphorylated on serine 2 of CTD= Ser2ph). DNA was quantified by real-time PCR using primers designed to amplify the promoter, enhancer, and coding regions of each of the 3 genes.

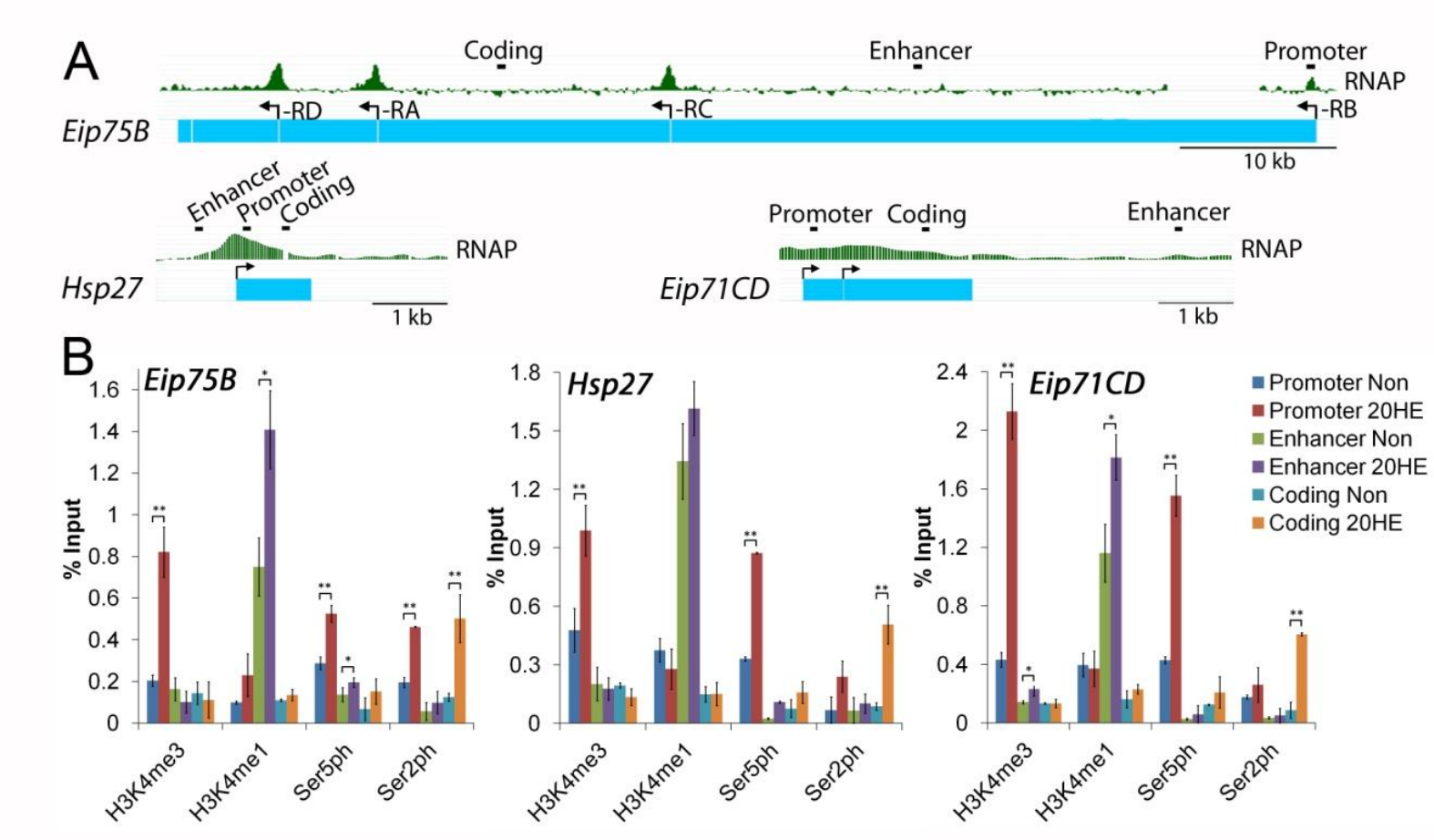

A
Coding
Enhancer
Promoter
RNAP
-RD
-RA
-RC
-RB
Eip75B
10 kb
Enhancer
Promoter
Coding
RNAP
Hsp27
1 kb
Promoter
Coding
Enhancer
RNAP
Eip71CD
1 kb
B
Eip75B
Hsp27
Eip71CD
% Input
Promoter Non
Promoter 20HE
Enhancer Non
Enhancer 20HE
Coding Non
Coding 20HE
H3K4me3
H3K4me1
Ser5ph
Ser2ph

Figure 3-3. JIL-1 is required for phosphorylation at enhancers and promoters upon ecdysone transcriptional activation

(A) ChIP analysis of Kc167 cells treated for 3 hrs with ethanol (Non) or 20-HE (20HE). Antibodies used for pull-down are indicated along the X-axis (Pol II phosphorylated on serine 5 of CTD= Ser5ph, Pol II phosphorylated on serine 2 of CTD= Ser2ph). DNA was quantified by real-time PCR using primers designed to amplify the promoter, enhancer, and coding regions of each of the 3 genes.

(B) ChIP analysis in Kc167 cells in which *JIL-1* or β-*gal* expression was inhibited by RNAi. Cells are treated with 20-HE for 3 hrs. Antibodies used are indicated along the X-axis (Pol II phosphorylated on serine 5 of CTD= Ser5ph, Pol II phosphorylated on serine 2 of CTD= Ser2ph). DNA was quantified by real-time PCR and results are reported as percentage of input.

(C) RNA expression analysis in cells treated with dsRNAs corresponding to the *JIL-1* or β-*gal* genes. Cells are treated with ethanol (Non) or 20-HE (20HE) for 3 hrs. RNA levels are determined by qPCR using primers specific to each of the four Eip75B transcripts. All samples are normalized to the mitochondrial gene myt:Col and the Non sample was set to 1 for comparison.

(D) western analysis of dsRNA-treated cells showing undetectable levels of JIL-1. Error bars represent the standard deviation of the mean of 3 biological replicates (***** $P < 0.05$, ****** $P < 0.01$).

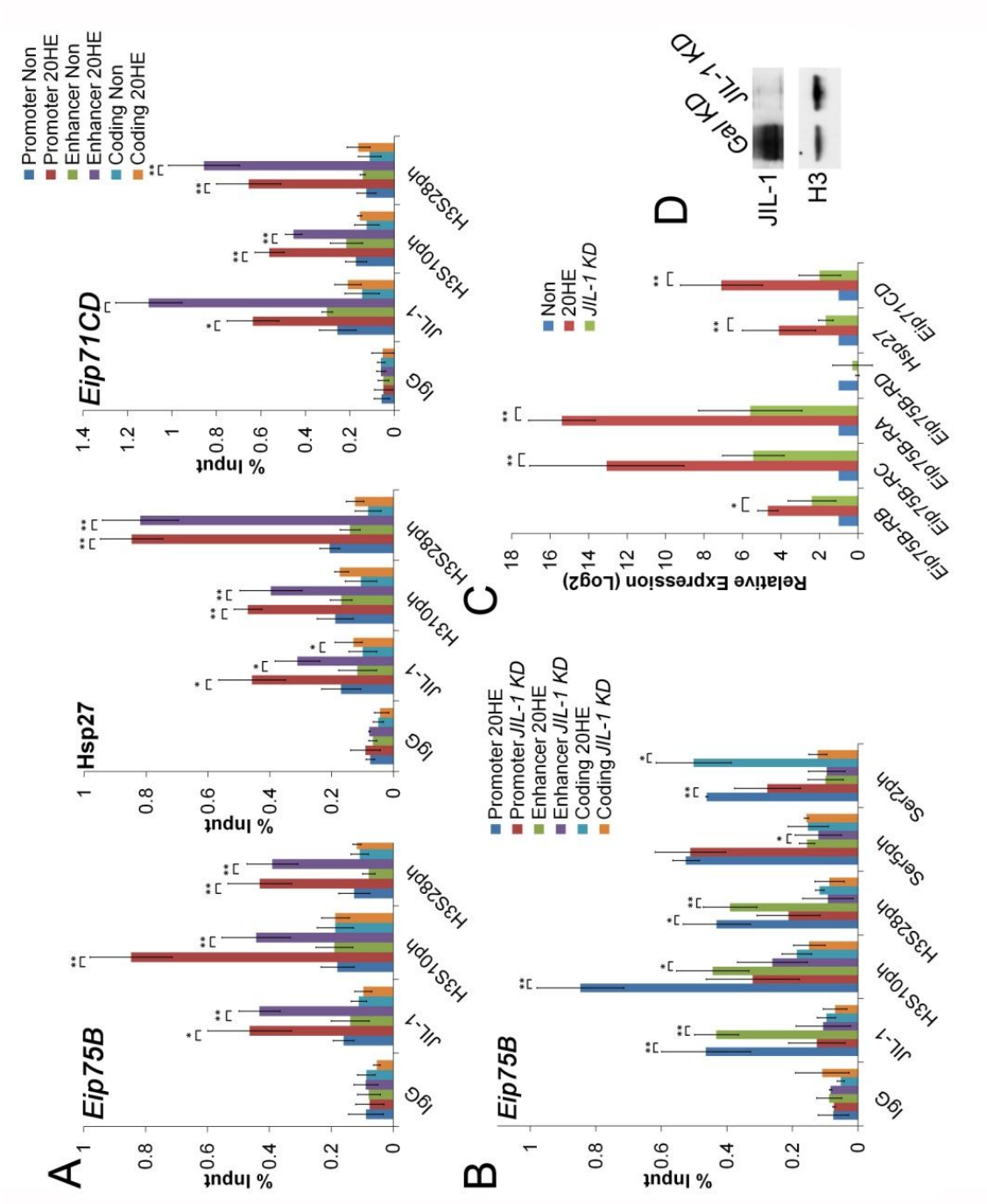

A
Eip75B
Hsp27
Eip71CD
% Input
IgG
JIL-1
H3S10ph
H3S28ph
Promoter Non
Promoter 20HE
Enhancer Non
Enhancer 20HE
Coding Non
Coding 20HE
B
Eip75B
Promoter 20HE
Promoter JIL-1 KD
Enhancer 20HE
Enhancer JIL-1 KD
Coding 20HE
Coding JIL-1 KD
Ser5ph
Ser2ph
C
Relative Expression (Log2)
Non
20HE
JIL-1 KD
Eip75B-RB
Eip75B-RC
Eip75B-RA
Eip75B-RD
Hsp27
Eip71CD
D
Gal KD
JIL-1 KD
JIL-1
H3

Figure 3-4. Either of the phosphorylation marks pulls down equal amounts of acetylated histone

Lysates of Kc167 cell lysates immunoprecipitated with antibodies against H3S10ph or H3S28ph loaded as a 1:10 ratio. Western blot analysis using antibodies against JIL-1, 14-3-3, H3K9ac or H3K27ac was performed on the immunoprecipites. Coommassie stain of the histones from the pull-down is included as a loading control. H3S10ph immunoprecipitation demonstrates equal amounts of H3K9ac and H3K27ac as the H3S28ph immunoprecipitation. 14-3-3 is more strongly associated with H3S28ph, reported to have a better consensus sequence for binding. JIL-1 is equally associated with H3S10ph and H3S28ph modified on the same nucleosomes as H3K9ac and H3K27ac.

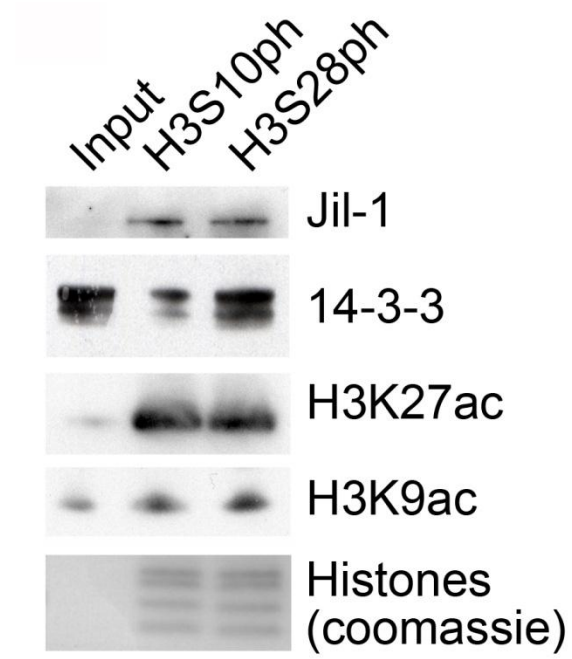

Input
H3S10ph
H3S28ph
Jil-1
14-3-3
H3K27ac
H3K9ac
Histones
(coomassie)

Figure 3-5. CBP acetylation and 14-3-3 recruitment at promoters and enhancers is necessary for transcription activation of ecdysone-responsive genes

(A) ChIP analysis of Kc167 cells treated for 3 hr with ethanol (Non) or 20-HE (20HE) was performed using the antibodies indicated along the X-axis (RNAPII phosphorylated on serine 5 of CTD= Ser5ph, RNAPII phosphorylated on serine 2 of CTD= Ser2ph). DNA was quantified by real-time PCR using primers designed to amplify the promoter, enhancer, and coding regions of each of the 3 ecdysone-induced genes.

(B) Western analysis of lysates from cells treated with CBP (top) or 14-3-3 (bottom) dsRNAs. Levels of either protein are undetectable. Error bars represent the standard deviation of the mean of 3 biological replicates (***** $P < 0.05$, ****** $P < 0.01$).

(C) ChIP analysis of Kc167 cells non-treated and treated with CBP dsRNA compared to β-gal dsRNA. The cells are also incubated with 20-HE for 3 hr and chromatin was immunoprecipitated using the antibodies indicated along the X-axis. DNA was quantified by real-time PCR and the result is reported as a percent of the input at the *Eip75B* gene.

(D) Similar as in panel B but cells are treated with dsRNA to 14-3-3.

(E) RNA expression analysis in cells treated with dsRNAs corresponding to the *CBP, 14-3-3* or β-*gal* genes. Cells are treated with ethanol (Non) or 20-HE (20HE) for 3 hr. RNA levels are determined by qPCR using primers specific to each of the four *Eip75B* transcripts. All samples are normalized to the mitochondrial gene myt:Col and the Non sample was set to 1 for comparison.

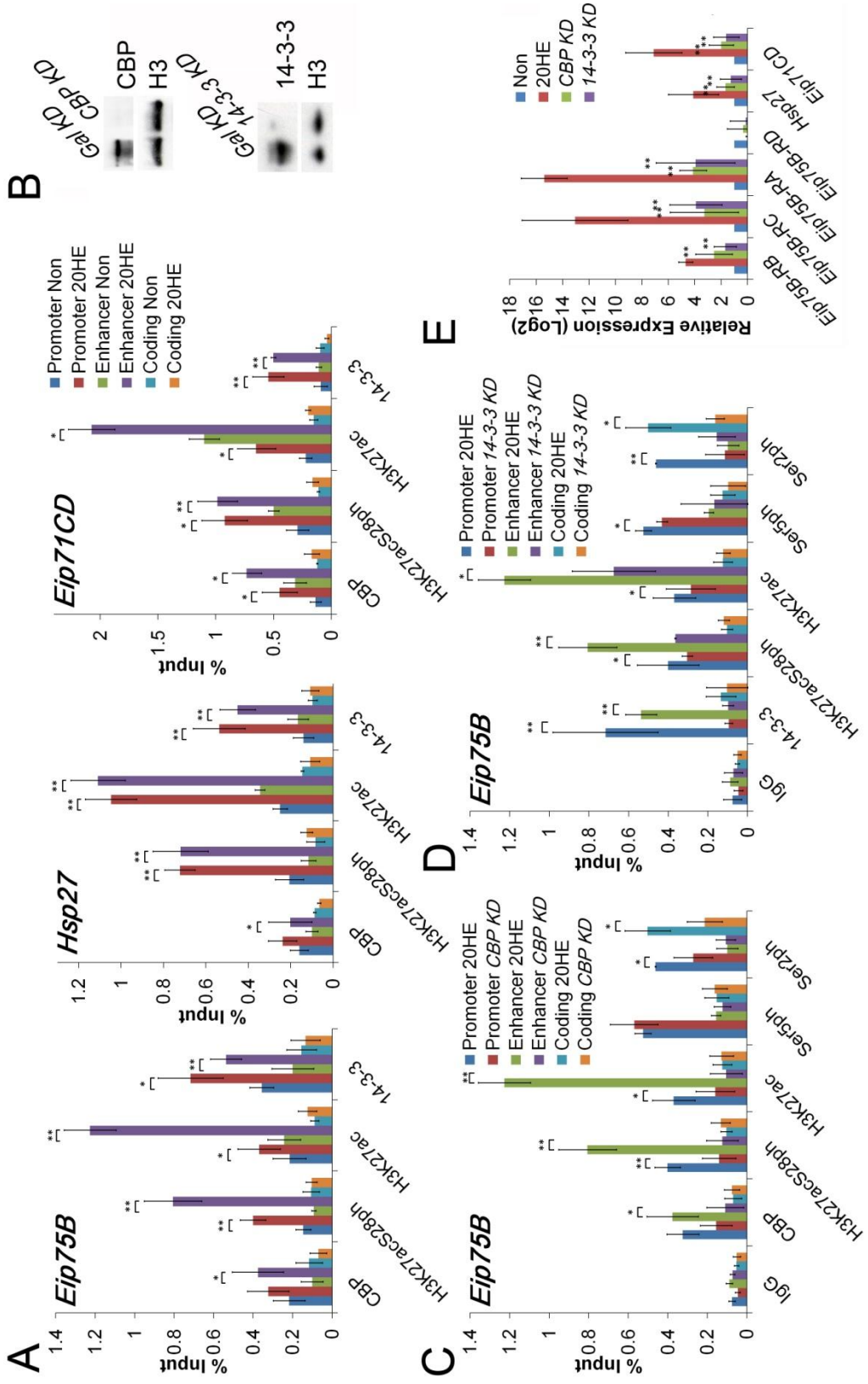

A
Eip75B
Hsp27
Eip71CD
% Input
Promoter Non
Promoter 20HE
Enhancer Non
Enhancer 20HE
Coding Non
Coding 20HE
CBP
H3K27acS28ph
H3K27ac
14-3-3
B
Gal KD CBP KD
CBP
H3
Gal KD 14-3-3 KD
14-3-3
H3
C
Eip75B
Promoter 20HE
Promoter CBP KD
Enhancer 20HE
Enhancer CBP KD
Coding 20HE
Coding CBP KD
IgG
CBP
H3K27acS28ph
H3K27ac
Ser5ph
Ser2ph
D
Eip75B
Promoter 20HE
Promoter 14-3-3 KD
Enhancer 20HE
Enhancer 14-3-3 KD
Coding 20HE
Coding 14-3-3 KD
IgG
14-3-3
H3K27acS28ph
H3K27ac
Ser5ph
Ser2ph
E
Relative Expression (Log2)
Non
20HE
CBP KD
14-3-3 KD
Eip75B-RB
Eip75B-RC
Eip75B-RA
Eip75B-RD
Hsp27
Eip71CD

Figure 3-6. Knockdown of CBP or 14-3-3 does not affect H3S28ph levels

ChIP analysis of Kc167 cells treated for 3 hr with ethanol (Non) or 20-HE (20HE) and treated with CBP or 14-3-3 dsRNAs under the same 20-HE conditions was performed using the antibodies against H3S28ph. DNA was quantified by real-time PCR using primers designed to amplify the promoter, enhancer, and coding regions of the *Eip75B* gene. Results are reported as a percent of the input at the *Eip75B* gene. Differences between samples from 20-HE-treated cells in wild type or CBP and 14-3-3 mutant backgrounds are not statistically significant.

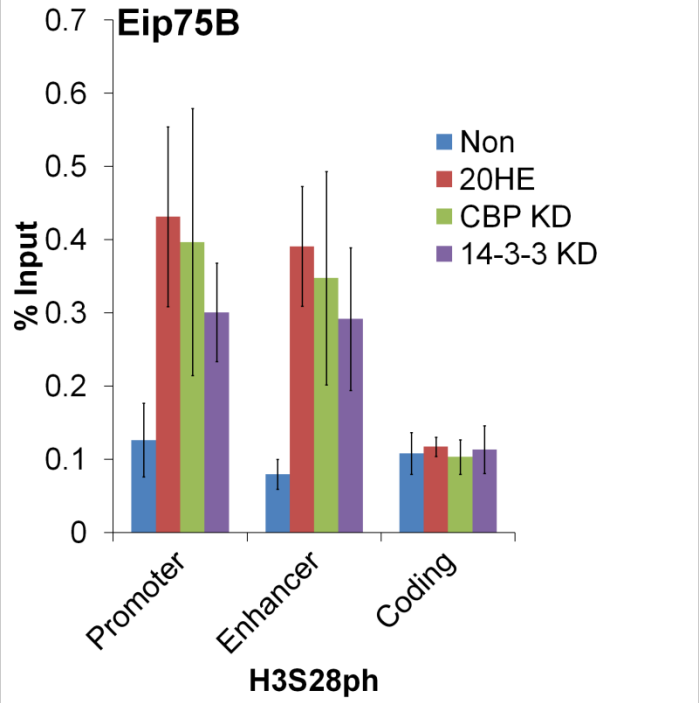

Eip75B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
% Input
Non
20HE
CBP KD
14-3-3 KD
Promoter
Enhancer
Coding
H3S28ph

Figure 3-7. 14-3-3 recruitment and H3K27 acetylation are dependent on JIL-1 kinase at enhancers and promoters

(A) Kc167 cells treated with dsRNAs to β-*gal* or *JIL-1* are incubated with 20-HE for 3 hr and subjected to ChIP analysis using the antibodies indicated along the X-axis. DNA was quantified by real-time PCR and reported as a percent of the input.

(B) Kc167 cell lysates with and without phosphatase inhibitors (PPI) are immunoprecipitated using antibodies against JIL-1, loaded 1:10 with respect to input, and subjected to Western analysis using antibodies against JIL-1, CBP, and 14-3-3.

(C) Using Kc167 cells pretreated for 5 min with 100 μM DRB followed by 3 hr treatment with 20-HE (20HE), ChIP was performed using the antibodies indicated along the X axis and quantitated by realtime PCR using primers designed to amplify the promoter, enhancer, and coding regions of the *Eip75B* gene.

(D) Relative expression analysis of the DRB treated cells compared with normal 20-HE induction using primers specific to each of the 4 transcripts of *Eip75B*. All samples are normalized to mitochondrial gene product myt:Col and the Non sample set to 1 for comparison. Error bars represent the standard deviation of the mean of 3 biological replicates.

(E) Western analysis of lysates from Kc167 cells treated with dsRNAs corresponding to the *JIL-1*, *CBP, 14-3-3* or β-*gal* genes; cells are pre-treated with DRB for 5 min and with 20-HE for 3 hr. Error bars represent the standard deviation of the mean of 3 biological replicates (***** $P < 0.05$, ****** $P < 0.01$).

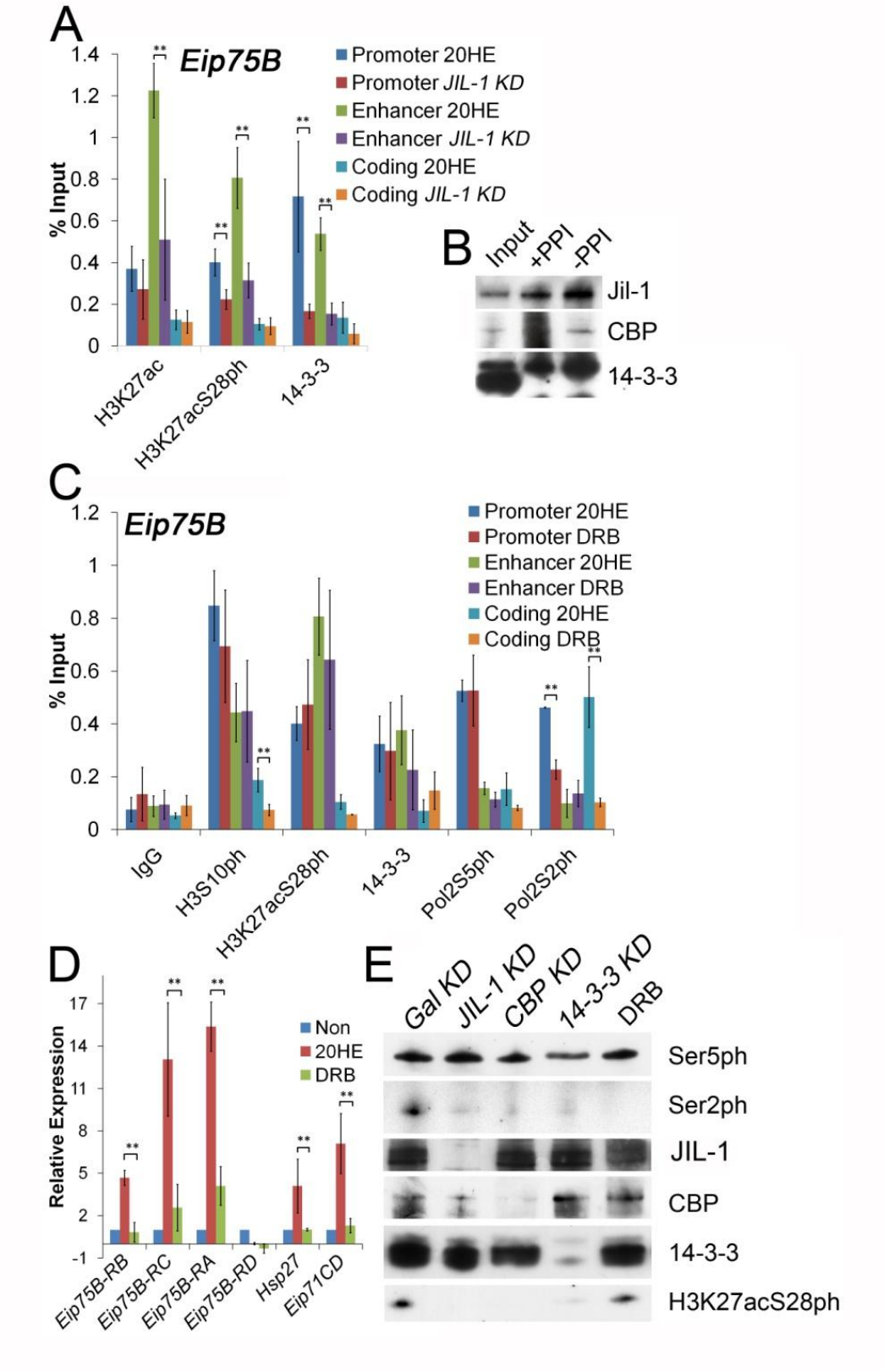

A
Eip75B
Promoter 20HE
Promoter JIL-1 KD
Enhancer 20HE
Enhancer JIL-1 KD
Coding 20HE
Coding JIL-1 KD
% Input
H3K27ac
H3K27acS28ph
14-3-3
B
Input
+PPI
-PPI
Jil-1
CBP
14-3-3
C
Eip75B
Promoter 20HE
Promoter DRB
Enhancer 20HE
Enhancer DRB
Coding 20HE
Coding DRB
% Input
IgG
H3S10ph
H3K27acS28ph
14-3-3
Pol2S5ph
Pol2S2ph
D
Non
20HE
DRB
Relative Expression
Eip75B-RB
Eip75B-RC
Eip75B-RA
Eip75B-RD
Hsp27
Eip71CD
E
Gal KD
JIL-1 KD
CBP KD
14-3-3 KD
DRB
Ser5ph
Ser2ph
JIL-1
CBP
14-3-3
H3K27acS28ph

Figure 3-8. JIL-1, 14-3-3, and CBP are required for enhancer-promoter interactions

3C analysis of Kc167 cells after β-gal knockdown and treatment with ethanol (Non) or 20-HE (20HE) for 3hr was performed in normal cells as Well as cells in which the expression of the *JIL- 1*, *CBP, 14-3-3* or β-*gal* genes was inhibited by treatment with the corresponding dsRNAs. 3C analysis was also done on cells treated with DRB for 5 min. Crosslinking efficiencies are 27 reported after normalizing to a BAC clone with an insert of 170 KB containing the entire *Eip75B* locus. Restriction digest of this DNA gives 112 fragments numbered 1 to 112, beginning with the first fragment of the BAC. Error bars represent the standard deviation of the mean of 3 biological replicates.

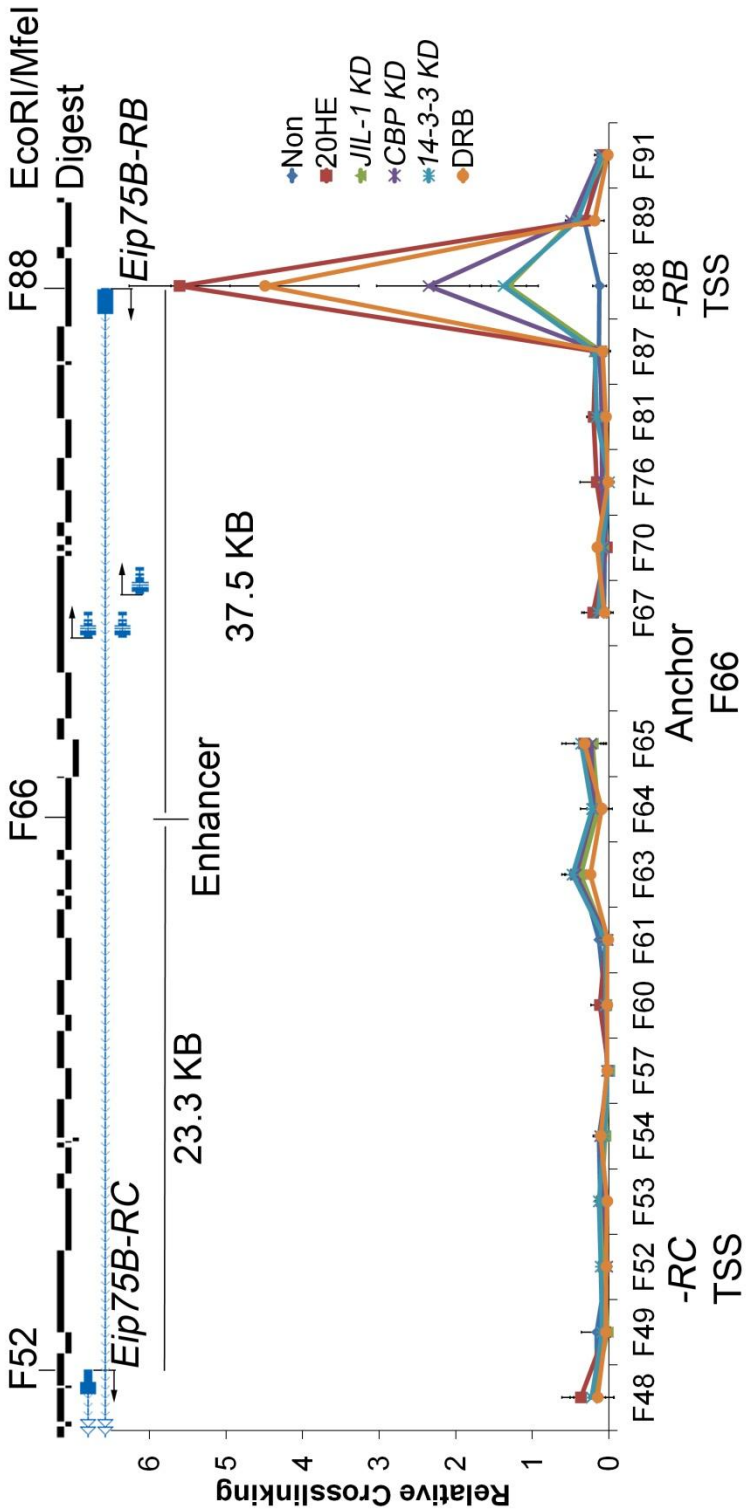

F52
F66
F88
EcoRI/MfeI Digest
Eip75B-RB
Eip75B-RC
23.3 KB
Enhancer
37.5 KB
Non
20HE
JIL-1 KD
CBP KD
14-3-3 KD
DRB
Relative Crosslinking
0
1
2
3
4
5
6
F48 F49 F52 F53 F54 F57 F60 F61 F63 F64 F65 F67 F70 F76 F81 F87 F88 F89 F91
-RC TSS
Anchor F66
-RB TSS

Figure 3-9. JIL-1 and histone phosphoacetylation at promoters correlate with transcript levels genome-wide

(A) RNA expression levels for Kc167 cells are obtained from modENCODE and the TSS was sorted by expression level from highest to loIst and given up/downstream orientation according to strand. The total number of reads is plotted for each ChIP-seq dataset in 20 bp bins covering 1 kb upstream and downstream from each TSS and vieId in Java Treeview.

(B) All TSSs are broken down into 5 equal bins according to expression levels and the average number of reads for each 20 bp region for all the TSS in that bin is plotted 1 kb upstream and downstream for each bin.

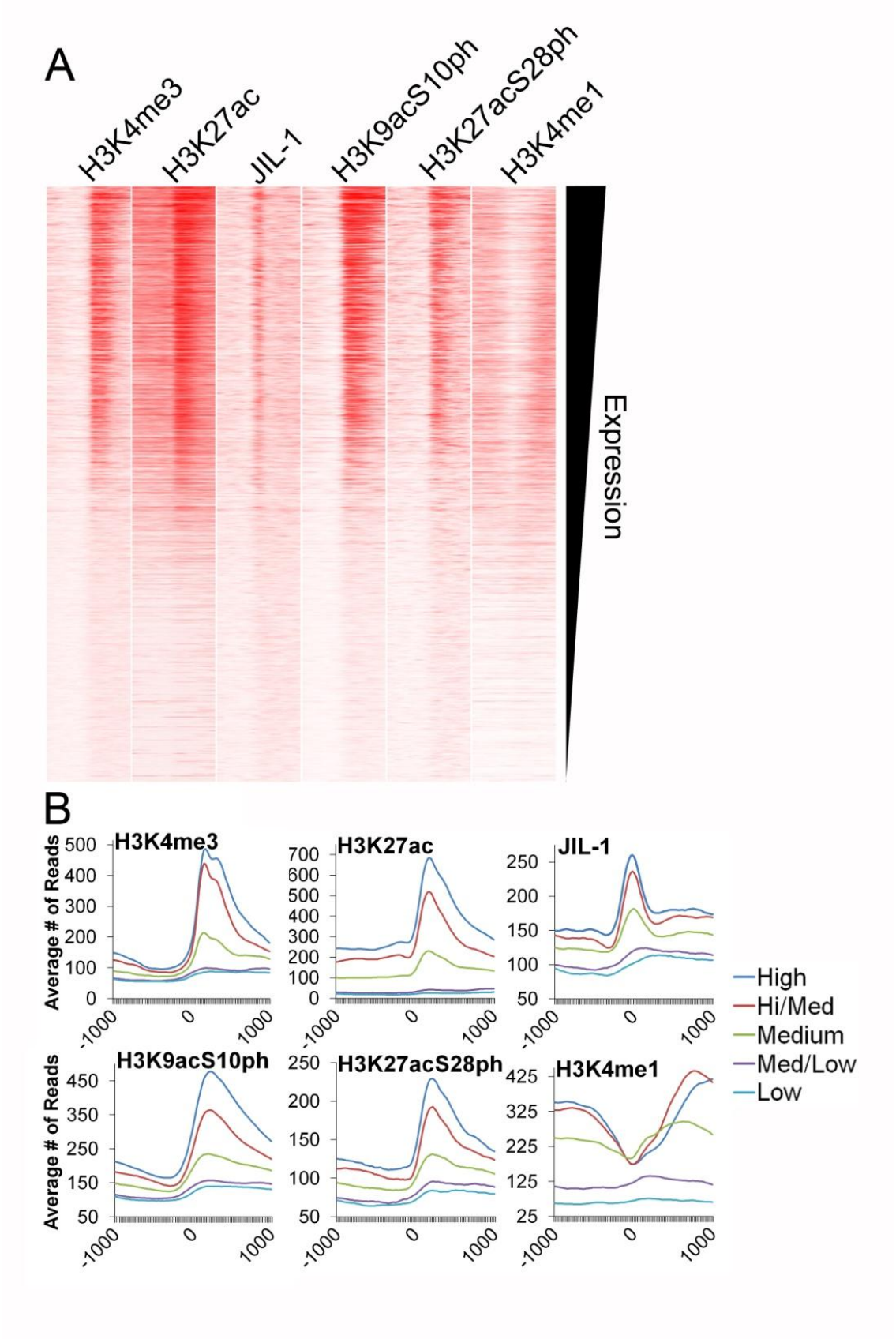

A
H3K4me3
H3K27ac
JIL-1
H3K9acS10ph
H3K27acS28ph
H3K4me1
Expression
B
H3K4me3
H3K27ac
JIL-1
H3K9acS10ph
H3K27acS28ph
H3K4me1
Average # of Reads
-1000
0
1000
High
Hi/Med
Medium
Med/Low
Low

Figure 3-10. Distribution of JIL-1 with respect to genes

Panels A and B show the distribution of JIL-1 obtained by ChIP-seq (red) comapared to results obtained by ChIP-chip (green) in two regions of chromosome 3L. The ChIP-seq data suggest higher levels of JIL-1 at promoters with respect to the coding region of genes.

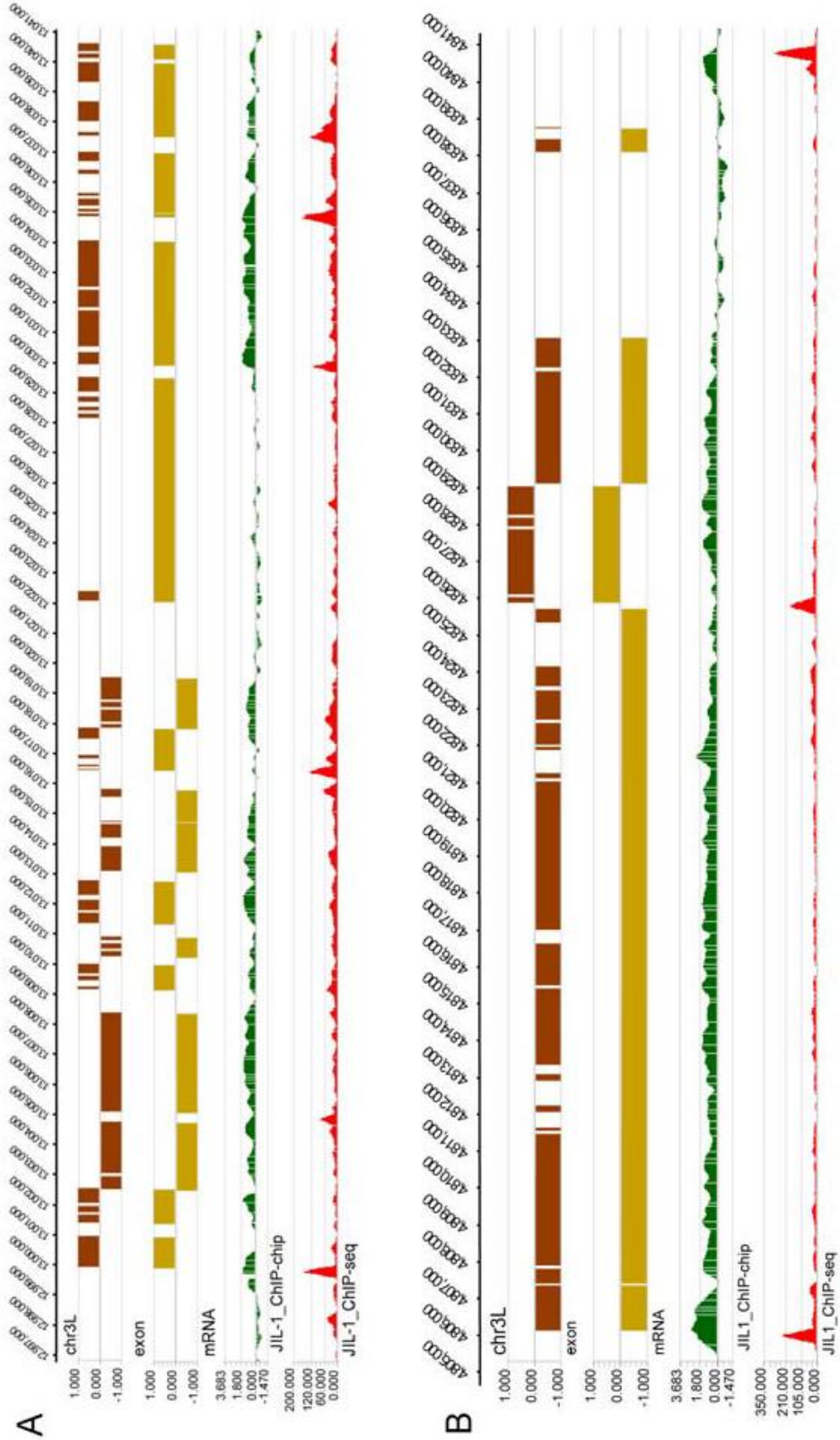

A
chr3L
1.000
0.000
-1.000
exon
1.000
0.000
-1.000
mRNA
3.683
1.800
0.000
-1.470
JIL-1_ChIP-chip
200.000
120.000
60.000
0.000
JIL-1_ChIP-seq
B
chr3L
1.000
0.000
-1.000
exon
1.000
0.000
-1.000
mRNA
3.683
1.800
0.000
-1.470
JIL1_ChIP-chip
350.000
210.000
105.000
0.000
JIL1_ChIP-seq

Figure 3-11. JIL-1, H3K9acS10ph and H3K27acS28ph are present at enhancers

Enhancers are selected by the presence of H3K4m1 and clustered into 2 clusters using Cluster 3.0. (A) The total number of reads is plotted for each ChIP-seq dataset in 20 bp bins covering 1 kb upstream and downstream from the H3K4me1 peak summit and vieId in Java Treeview. (B) The average number of reads plotted for every H3K4me1 peak in the inactive or active cluster in 20 bp bins covering 1 kb upstream and downstream from the summit of H3K4me1.

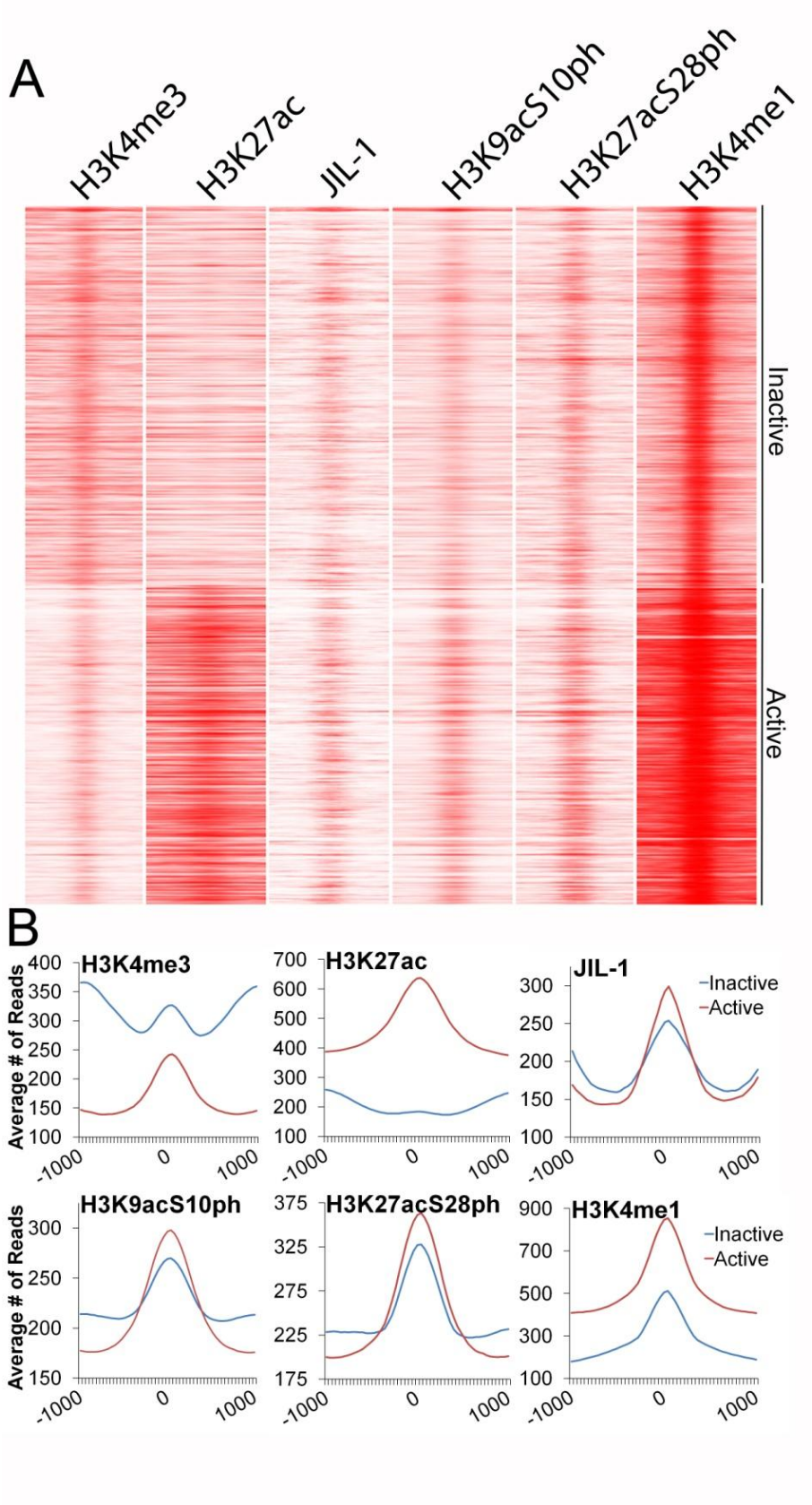

A
H3K4me3
H3K27ac
JIL-1
H3K9acS10ph
H3K27acS28ph
H3K4me1
Inactive
Active
B
H3K4me3
H3K27ac
JIL-1
Inactive
Active
H3K9acS10ph
H3K27acS28ph
H3K4me1
Average # of Reads

Table 3-1. Double stranded RNA Primers for gene knock down analysis

Double stranded RNA was produced using MEGAscript (Ambion AM1334) according to the manufacturer's protocol using the primers listed above. dsRNAs for each target are mixed before transfection with cell culture media to a concentration of 4 µg/mL and pre-incubated 30 min with 200 µL media and 8 µL/ml Cellfectin (Invitrogen 10362-100).

3C: fragment 48	GCGAGCAAAAGTTCAAGAGCCGC	Forward
3C: fragment 49	GCTCAGTCGCCTCGGTGGTG	Forward
3C: fragment 52	TTTGTGGGAATGTGGGAGCTGTTT	Forward
3C: fragment 53	TGAGGCCTGGCTGGCTGTTACT	Forward
3C: fragment 54	GGATCTCTCGACTACCGTGCTTACA	Forward
3C: fragment 57	TTTCGCGTTGCACTCCGCATTTG	Forward
3C:fragment 60	ACTGAGTCACAATGGGAAGTCAGGT	Forward
3C: fragment 61	AGTCTCTTTCGGCCCCTTTTCTTTC	Forward
3C: fragment 63	AAAAGCGGCCCCACACCCCT	Forward
3C: fragment 64	GCGCATAAAGTTCCCAGCCAACC	Forward
3C: fragment 65	TTGCATAGAGCGTAGCGCTGGT	Forward
3C: fragment 66	GCCATTCACTTGCACCAGTTAG	Forward
3C: fragment 67	GGGAAATTCGTTGCCGCTTGTGG	Forward
3C: fragment 69	ACCTATGTGCATTGTAAGCGAAATTCGT	Forward
3C: fragment 70	CTCAAGCTGGCATAAACTCTGCGCT	Forward
3C: fragment 76	TCTGGCTCAGTTCTTGTTCTCGAGA	Forward
3C: fragment 81	CCCCCACTCTCCTCTCTTAGAGTTTCG	Forward
3C: fragment 87	GCAGCACTGGTTTGGATTGGATTCG	Forward
3C: fragment 88	TCGTATGGCCAACAGCTGAGGGT	Forward
3C: fragment 89	GCGAGTCGGTTCTCGCGCAA	Forward
3C: fragment 91	ACCACTTATTCGGACGCATCACGC	Forward

Table 3-2: 3C Primers

Quantitative PCR reactions are performed using TaqMan Universal PCR Master Mix (Applied Biosystems 4324018) using an anchor primer (F66) in combination with a primer from the fragment of interest. All primers are designed to be complementary to the same strand in the same orientation to ensure the product is made only when the DNA is digested and ligated.

siJil-1	5’	TAATACGACTCACTATAGGGAGACCGACATTTGGTTAGCTGGT
siJil-2	3’	TAATACGACTCACTATAGGGAGATCGTGCACTATCTCGTCGTC
siJil-3	5’	TAATACGACTCACTATAGGGAGAAACCACCAAGCGAGAAGAGA
siJil-4	3’	TAATACGACTCACTATAGGGAGAATAAAGTTGACGCATTGCC
siCBP	5’	TAATACGACTCACTATAGGGAGATCAGCATTCTGGCGCCGCAA
siCBP	3’	TAATACGACTCACTATAGGGAGAGCGACAATTGGCCATCGCCG
siCBP	5’	TAATACGACTCACTATAGGGAGATCGTTGCCAACTCGCGTGCA
siCBP	3’	TAATACGACTCACTATAGGGAGACGGCGGCCGATTGACGACTG
si14-3-3zeta	5’	TAATACGACTCACTATAGGGAGAGTCCGACACCCAAGGCGACG
si14-3-3zeta	3’	TAATACGACTCACTATAGGGAGAGGCCGGCATCTGTCCAAGCA
si14-3-3zeta	5’	TAATACGACTCACTATAGGGAGAGATAATCCAGGCGAGAGCAG
si14-3-3zeta	3’	TAATACGACTCACTATAGGGAGACGGAGCATCTAGAGTTTGGC
si14-3-3epsilon	5’	TAATACGACTCACTATAGGGAGAAGCTGACCGTCGAGGAGCGA
si14-3-3epsilon	3’	TAATACGACTCACTATAGGGAGATGCCAAGCCCAAACGGATGGG
si14-3-3epsilon	5’	TAATACGACTCACTATAGGGAGAGAGGAGCGAAATCTGCTGTC
si14-3-3epsilon	3’	TAATACGACTCACTATAGGGAGAACACCGAGAAGTTCAATGCC

Chapter 4

Discussion

Regulation of promoter-proximal pausing

Promoter-proximal pausing appears to be a widely used mechanism of transcription regulation in eukaryotic cells. The steps necessary for release of RNAP II into transcriptional elongation are not well understood. The presence of RNAP II initiated at the majority of genes whether they are transcribed or not, suggests that the rate limiting step is release of the polymerase into elongation (Adelman et al., 2007; Nechaev et al., 2010). Evolving technologies offer more sensitive approaches to detect RNAP II initiation and transcription of the first 20-50 base pairs of the gene with techniques like RNA-seq and Global Run-on sequencing (GRO-seq) (Core et al., 2008; Nechaev et al., 2010). Recent studies demonstrate that the first 20-50 bp of a gene are transcribed several hundred times more that sequences further downstream in paused genes. Even more interesting, is the existence of high levels of transcription that take place on the antisense strand of the promoter (Core et al., 2008) as well as bidirectional transcription at enhancers (Kim et al., 2010), none of which result in productive elongation. This suggests that the mechanism of release from the promoter proximal pause provides strand orientation and processivity that is absent in the first 50 bp of a gene and at enhancers. Processivity and strand orientation can be partially explained by DSIF, which plays a role in negatively regulating RNAP II when associated with NELF. When not associated with NELF, DSIF forms a clamp around the DNA/RNA duplex stabilizing the interaction of DNA with RNAP II and facilitating long transcripts that are not capable without DSIF (Missra and Gilmour, 2010). Additionally, RNAP II pausing is associated with loading of elongation factors such as the RNA capping complex and the splicing complex necessary for producing a stable RNA molecule that can be transported outside of the nucleus for translation (Kim et al., 2004; Lenasi et al., 2011). The fact that promoter-proximal pausing is a regulated process necessary to load the proper machinery for producing mRNA, might explain the relatively small transcripts produced

from the sense strand (~25-50 bp) compared to the other transcripts from the antisense strand and enhancer sequences (~300-400 bp). RNAP II on the sense strand of promoters has a mechanism to inhibit progression to make longer transcripts that is lacking in either antisense or bidirectional RNAP II. However, recent studies demonstrate that coding exons or alternate promoters from one cell type can function as enhancers in a different cell type (Birnbaum et al., 2012; Kowalczyk et al., 2012). These studies blur the lines in the definition of enhancers or promoters, which ultimately depends on the utilization of the sequence in a given context. With this in mind, it is not surprising that I find similar mechanisms of regulation occurring at enhancers and promoters. The mechanisms that determine which specific sequences well function as an enhancer and which genes will have RNAP II initiated and poised for transcription in a given cell type are currently unknown.

Evidence provided here demonstrates that mutations in some of the proteins necessary for release of the paused polymerase result in genome-wide depletion of elongation complexes. Therefore, even at genes where there is no evidence of promoter-proximal pausing, these steps might still be necessary to achieve elongation. The presence of significantly higher levels of non productive transcripts produced from the first 20-50 base pairs than full-length transcripts from genes classified as not paused lends support that the RNAPII is regulated at the same step even these genes, but not as the rate limiting step (Nachaev et al., 2010). It is likely that the events described here represent a more general mechanism used by all RNAP II transcription and it is the kinetics of these events that determine if the genes are paused or not. This is supported by the fact that transcription for virtually all mRNA would require factors demonstrated to be loaded at the pausing step, such as the capping and splicing complexes (Glover-Cutter et al., 2008; Anderson and Jensen, 2010).

Enhancer regulated transcription

A large body of evidence suggests that enhancers regulate transcription levels, ultimately determining cell type and response to stimuli. Given the fact that RNAP II is initiated at significant levels at the majority of genes, and the data I present here demonstrate that many of the proteins necessary for release of RNAP from the promoter-proximal pause also are bound at enhancers, suggest that enhancers function to regulate transcription by facilitating steps necessary for release from the promoter-proximal pause. Therefore, it is not surprising that phosphorylation and acetylation of histones for Brd4 and P-TEFb recruitment take place at the enhancer upon activation. One explanation is that the proteins needed for release from the promoter proximal pause might first be recruited to the enhancer and then become available at the promoter upon contact with the enhancer by looping. The fact that enhancer to promoter contacts tend to have a higher frequency at genes that have high levels of transcription than ones that have lower levels, supports this explainantion. In addition, without the phosphorylation and acetylation of histones shown to be necessary for release of RNAP II from the promoter-proximal pause, the enhancer to promoter contact is not made and transcriptional activation is not achieved. However, this does not rule out the possibility that the enhancer provides a second source of RNAP II components, so that more is available upon enhancer contact with the promoter once the paused RNAP II moves into elongation. Future studies will be needed to better understand the function of RNAP II at enhancers and what an enhancer provides to promoters to promote elongation.

In addition, evidence presented here show some of the same events regulating transcription at the promoter-proximal pause, such as histone phosphorylation and subsequent histone acetylation, also take place at enhancers. It is enticing to speculate a common role for these same events at both locations. One possibility is the proteins

necessary for release of the polymerase are bound at the enhancer and, upon contact of the enhancer with the promoter, become available to the paused polymerase at the promoter. For example, experiements recruiting P-TEFb to an enhancer results in activating transcription at the promoter of the gene it regulates (Taube et al., 2002; Giacinti et al., 2007). If P-TEFb is recruited to enhancers by Brd4/Fs(1)h proteins as reported, this would explain the necessity for histone hyperacetylation at enhancers (Zippo et al., 2009). An exhaustive review of the literature reveals no evidence of binding assays of Brd4 or P-TEFb with an H3K27 acetylated peptide. H3K27ac is a modification better correlated with enhancer activity compared with H3K9/K14 acetylation or H4K5/8/12/16 acetylation (Wang et al., 2008). Additionally, another lysine residue, H3K23, is four residues from H3K27, a very similar distance between acetylated residues reported to bind Brd4/Fs(1)h (Morinière et al., 2009). It remains to be tested whether Brd4/Fs(1)h can bind H3K23/K27 when acetylated as another possible combination of residues like K5/8/12/16 of histone H4 that have been reported as binding combinations for Brd4/Fs(1)h. It is not clear whether Brd4/Fs(1)h recruitment of P-TEFb is the limiting factor provided by the enhancer upon contact with the promoter. Additional experiments are required to determine the nature of the signal for transcription and any factors needed at the promoter that are provided by the enhancer.

Enhancer-promoter contacts are required for activation

The signal that is provided from distal enhancers to the promoter in order to achieve transcription elongation is dependent on enhancer to promoter contact. Evidence presented in this study demonstrates that histone phosphorylation of both the enhancer and promoter followed by 14-3-3 recruitment is necessary to facilitate this contact. In addition, acetylation, in particular H3K27ac performed by CBP, is also necessary to form a contact of the enhancer with the promoter. While this acetylation

modification has not been investigated for Brd4/Fs(1)h binding, I show that it is still necessary for promoter to enhancer contact. Investigations of histone acetylation have demonstrated that if lysine residues H3K9/K14 neighboring phosphorylated H3S10 are acetylated, there is more recruitment of 14-3-3 than if there is only phosphorylation of H3S10 (Macdonald et al., 2005; Winter et al., 2008; Walter et al., 2008). 14-3-3 is well characterized as binding phosphoylated residues on proteins to facilitate interactions between the proteins to which it binds. Interactions between 14-3-3 molecules are dependent on acetylation of lysine residues on 14-3-3 itself in order to form functional dimmers and multimers. If the acetyltransferases are also capable of acetylating the lysine residues of 14-3-3 necessary for forming multimers, it would explain why higher levels of 14-3-3 are recruited when H3K9/K14 are acetylated. This phenomenium is likely a result of forming larger interacting complexes between 14-3-3 binding proteins such as histone acetyltransferases and histones. Additionally, since this takes place at both the enhancer and promoter, and I have demonstrated that histone phosphorylation, acetylation and 14-3-3 are necessary for interaction between the enhancer and promoter, 14-3-3 might play a role in facilitating the interaction.

JIL-1 is present at enhancers prior to activation

Most surprising from this study was the finding that JIL-1 and the histone phosphorylation it performs are present at enhancers prior to activation. A previous study reported that H3S28 phosphorylation leads to a destabilized nucleosome and replacement with histone variant 3.3 (Sun et al., 2007). Enhancers have a well characterized histone-free region that expands upon activation. It is possible that JIL-1 plays a role in maintaining the histone free region at enhancers to make the DNA accessible for transcription factor binding upon activation. It would be logical to investigate a possible role for JIL-1 in maintaining the histone free region at enhancers

and whether this protein is necessary for the maintenance of enhancers prior to activation. In addition, there must be some way to recruit JIL-1 to enhancers that has not yet been elucidated . Defining the mechanisms that determine a set of enhancers for a given cell will likely give insight into cell differentiation since enhancers play a role in specifying cell type. Future experiments could be designed using the JIL-1 knockdown cells characterized here or the JIL-1 mutant flies to look for abnormalities in tissue differentiation and signaling responses between cells. Ideally these investigations would lead to pathways and proteins involved in determining a given set of enhancers for a cell type and the biological implications when any of these proteins are perturbed.

Fs(1)h characterization in *Drosophila*

Fs(1)h, the *Drosophila* Brd4 orthologue, is not well characterized and no studies have been performed investigating its role in P-TEFb recruitment. A single study looking at Fs(1)h regulation of the *Ultrabiothorax* gene demonstrates that it is necessary for proper transcriptional regulation at the promoter region (Chang et al., 2007). Other than this study, a transcriptional role of Fs(1)h in *Drosophila* has not been reported. If Fs(1)h performs the same functions as its homologue Brd4 in mammalian cells, then it is expected that ChIP experiments would show recruitment upon transcriptional activation at promoters and enhancers. I have already performed this experiment using the ecdysone response in Kc cells and results show a recruitment of Fs(1)h to ecdysone responsive enhancers and promoters. Additionally, if Fs(1)h is required for recruitment of P-TEFb to enhancers, it would be expected that P-TEFb fails to be recruited in order to facilitate release of RNAP II into elongation in *Fs(1)h* mutants or RNAi knockdown cells. On the same note, knockdown of different histone acetyltransferases reported to acetylate lysine residues important for Brd4/Fs(1)h binding can be performed to determine if all the acetylated lysines are important or if there are redundancies.

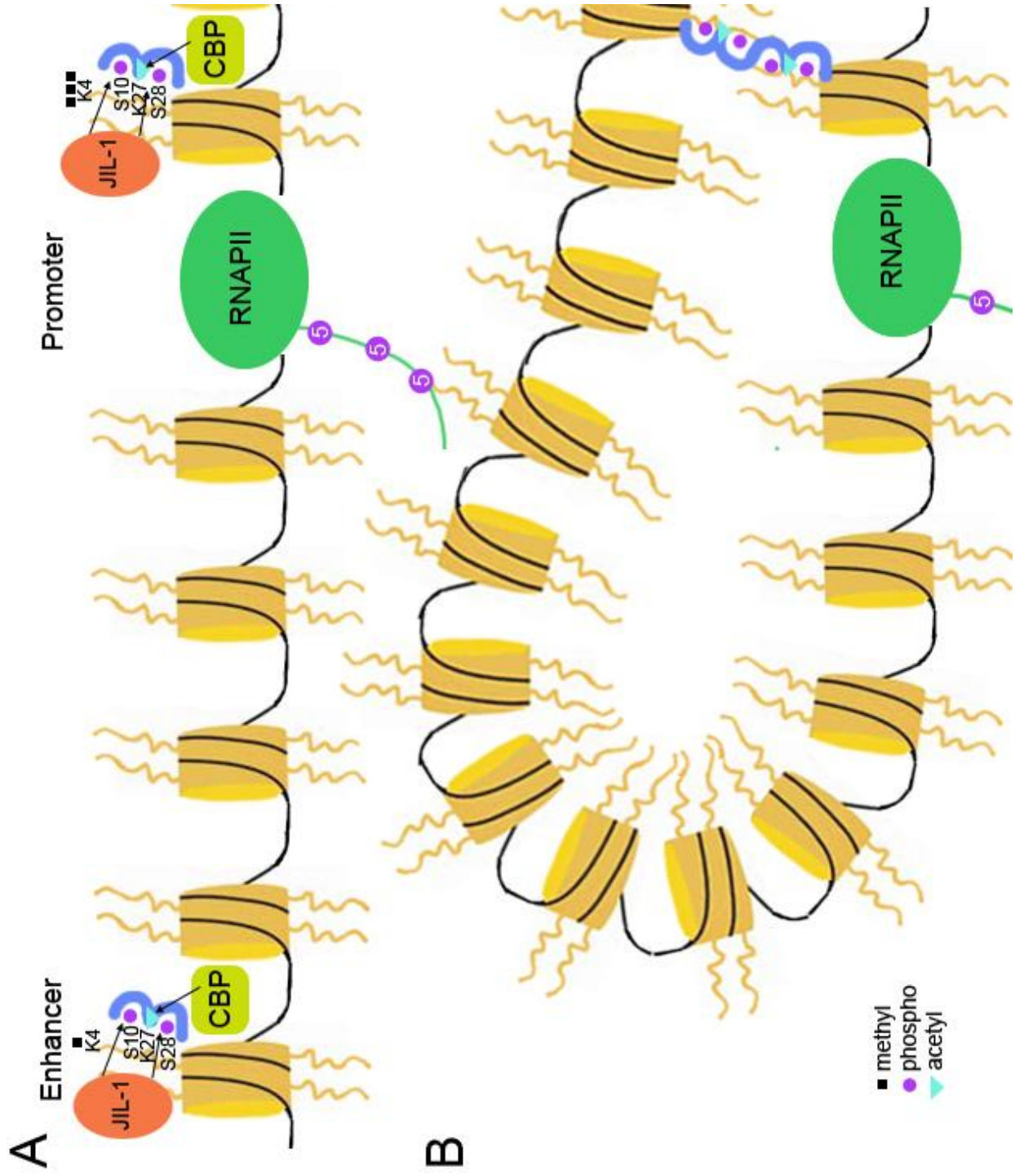
Because Fs(1)h is the only BET family member in *Drosophila,* analysis of this protein in this organism makes genome-wide ChIP analysis simple and comprehensive. The Fs(1)h gene encodes two isoforms that arise by alternative splicing, long [Fs(1)h L] and short [Fs(1)h S], both of which contain the tandem double bromodomains and the ET domain. The short isoform is identical to the long, but the long isoform contains an additional carboxy-terminal motif (CTM) that is essential for normal development of *Drosophila* (Digan et al., 1986). It has not been determined if the two have overlapping or separate functions, but an Fs(1)h L isoform specific mutant does not have homeotic transformations. This phenotype characterized by the majority of other mutants that effect both the long and short isoforms, suggests distinct roles between the two isoforms. ChIP-seq experiments with antibodies recognizing only Fs(1)h L or both isoforms have already been performed and are currently being analyzed for differences in binding patterns between the two isoforms and the genes they regulate. Hopefully, these datasets will help illuminate possible differences in function by the location in the genome to generate hypotheses about functional differences that can be tested by other experiments.

If Fs(1)h binding at enhancers and promoters is demonstrated to be a genome-wide phenomenon, it would explain the need for genome-wide phosphorylation and 14-3-3 recruitment needed for histone acetyltransferase activity to achieve hyperacetylation at enhancers and promoters upon activation. Data presented in this study demonstrate that these steps are necessary at ecdysone responsive genes to achieve release of RNAP II from the promoter-proximal pause. This is a novel finding of events at the enhancer that are necessary for release of the RNAP II from the promoter-proximal pause, lending support for a role for enhancers in the release of the polymerase into elongation.

Figure 4-1. Phosphoacetylation at enhancers and promoters is necessary for contact.

(A) At genes where there is a paused polymerase, upon activating signals JIL-1 phosphorylates H3S10 and H3S28 at the promoter and enhancer. 14-3-3 binds these phosphorylation modifications and mediates CBP acetylation of H3K27 at both enhancer and promoter.

(B) Contact made between the enhancer and the promoter and is dependent on JIL-1 phosphorylation, 14-3-3 binding, and CBP acetylation.


A
Enhancer
Promoter
JIL-1
K4
S10
K27
S28
CBP
RNAPII
5
B
methyl
phospho
acetyl

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