The role of Spt6 in variant histone H3.3 deposition during transcription

Mikage Nakajima

Doctor of Philosophy

Department of Genetics
School of Life Science
The Graduate University for Advanced Studies

2008

Contents

Abstract---------- 2

Introduction---------- 7

Material & Methods---------- 11

Results---------- 18

Discussion---------- 26

Figures---------- 32

Acknowledgements---------- 48

References---------- 49

Abstract

Nucleosome is a fundamental unit of chromatin, consisting of 146bp of DNA wrapped around an octomer of four kinds of histones, H2A, H2B, H3 and H4. Because nucleosomes can be significant obstacles to transcription that are mediated by RNA polymerase II (Pol II), it is needed to destabilize nucleosomal structure by displacement of histones during Pol II passage. The destabilization of nucleosomes during transcription results in histone loss over the regions of heavily transcribed genes (Lee, C.K. et al. 2004). Histone loss sometimes causes aberrant transcription initiation from cryptic sites within coding regions (Kaplan, C.D. et al. 2003). To maintain the fidelity of transcription initiation, histone deposition behind Pol II passage is necessary.

Recent studies have revealed an interesting phenomenon regarding histone deposition during transcription in eukaryotes other than yeast. The phenomenon is that histone H3 variant, H3.3 is selectively incorporated into nucleosomes during transcription (Ahmad, K. and Henikoff, S. 2002a). In contrast to canonical histone H3, H3.3 is synthesized throughout the cell cycle and deposited onto DNA not only during the S phase but also during other phases (Ahmad, K. and Henikoff, S. 2002b, Tagami, H. et al. 2004). The selective deposition of H3.3 is a very interesting phenomenon, however, the underlying mechanisms as well as in vivo biological meanings have been elusive.

To elucidate the mechanisms I tried to identify a factor that mediates the deposition of H3.3. In this study I focused on a transcription elongation factor Spt6 as a candidate. Studies of yeast Spt6 have revealed that Spt6 plays a critical role in

maintaining normal chromatin structure during transcription elongation interacting with histone H3 (Bortvin, A. and Winstone, F. 1996, Kaplan, C.D. 2003). Although yeast has no transcription variant of histone H3, these findings in yeast studies led me to an idea that Spt6 might be involved in the variant histone H3.3 deposition during transcription in higher eukaryotes.

In order to understand the Spt6 function in higher eukaryotes, I investigated the in vivo role of Spt6 using *D. melanogaster* as a model organism. First, to investigate the localization of Spt6 on chromatin, I carried out ChIP-on-chip microarray analyses. By the analyses of ChIP-on-chip microarray, strong signals of Spt6 (probe set p-value < 0.001) were detected on about 30 genes within the half of the entire *Drosophila* genome. As these 30 genes are various in their functions, expression patterns and genome structures, any common features were not found among them. In most of the 30 genes, Spt6 was distributed throughout the gene body, especially in exons. I selected several genes such as *heat shock protein 83* (*hsp83*), *La related protein* (*larp*) and *thread* from these Spt6-localized genes for further analyses.

To investigate the function of Spt6 in vivo I generated and characterized a *spt6* null mutant. By P-element excision, I generated *spt6W40*. The *spt6W40* allele has a small deletion in the second exon of the *spt6* gene that causes a stop of the protein synthesis, hence is functionally null. Homozygotes of *spt6W40* show an embryonic lethal phenotype.

To examine the effects of the *spt6* mutation on transcription, I performed RT-PCR analyses using RNA from embryos to measure the amounts of mRNA. RT-PCR analyses with primers for 5' regions and 3' regions of *hsp83* and *larp* mRNA showed that the

levels of the mRNA were increased only at the 3' regions but not the 5' regions in the *spt6* mutant compared with the wild type. These results suggest that the transcription initiates from the middle of the coding region in the *spt6* mutant. To analyze the size of the transcripts from *hsp83*, I carried out a Northern blot analysis. The RNA probes synthesized over the coding sequence of *hsp83* detected not only expected 3 kbp transcripts but also shorter transcripts only in the *spt6* mutant. These shorter transcripts may be the products of aberrant transcription on *hsp83* gene in the *spt6* mutant. These results suggest that *spt6* mutation causes aberrant transcription initiation from cryptic sites within the coding region.

To examine the possibility that the aberrant transcription in the *spt6* mutant is due to changes in chromatin structure, I carried out a micrococcal nuclease (MNase) assay using *spt6W40* embryos. Although there was no difference in the patterns of bulk chromatin between the wild type and the *spt6* mutant, chromatin on *hsp83* exhibited an increased sensitivity to MNase in the *spt6* mutant compared with the wild type. This result indicates that the *spt6* mutation causes changes in the chromatin structure on the *hsp83* gene in vivo. Because the ChIP analyses with pan-H3 antibodies showed that the level of general H3 on *hsp83* was reduced in the *spt6* mutant, the changes in the chromatin structure on *hsp83* in the *spt6* mutant may be due to loss of histones.

Previously, examination of several *Drosophila* genes revealed that nucleosomes containing either canonical histone H3 or variant histone H3.3 were lost during transcription, and were selectively replaced with nucleosomes containing H3.3 (Wirbelauer, C. et al. 2005). Because the chromatin structure was changed by the loss of histones in the *spt6* mutant, I consider the possibility that Spt6 can help the

deposition of H3.3 during transcription. To observe the correlation of Spt6 with H3.3, I measured the levels of H3.3 on three Spt6-enriched genes (*hsp83*, *thread* and *larp*) and two non-enriched genes (*β-tubulin* and *cyp4d21*) by the ChIP analyses. The levels of H3.3 were higher on the Spt6-enriched genes rather than non-enriched genes. This result suggests that there is some correlation between Spt6 and H3.3 in their localization. For further examination of physical relationship between Spt6 and H3.3, I carried out a Re-ChIP assay to examine whether Spt6 localizes on nucleosomes containing H3.3 or H3. As the results, on *larp* and *hsp83*, Spt6 was efficiently immunoprecipitated with the nucleosomes containing H3.3 rather than H3. This indicates that Spt6 preferentially localizes on the nucleosomes containing H3.3 rather than H3.

In order to examine the functional relationship between Spt6 and H3.3, I used *spt6* RNAi lines obtained from NIG stock center. When *spt6* RNAi was ubiquitously induced by an *Ay-GAL4* driver, almost all the animals died before the pupal stage. If Spt6 is involved in the deposition of H3.3, a decrease in the efficiency of H3.3 deposition caused by *spt6* knockdown can be suppressed by over-expression of H3.3. To test this, H3.3 was over-expressed in the *spt6* RNAi line. As controls H3 or GFP was expressed instead of H3.3. The lethal phenotype of *spt6* knockdown was significantly suppressed only by the over-expression of H3.3. This result suggests that Spt6 is functionally related with H3.3 in vivo.

In summary I have found that Spt6 plays an important role in maintaining the chromatin structure during transcription elongation, thereby repressing production of aberrant transcripts in *D. melanogaster*. I also observed the correlation of Spt6 with

variant histone H3.3. This is the first report to suggest the relationship between Spt6 and H3.3. Further investigation will clarify the function of Spt6 in the H3.3 deposition, and it may provide new insights into chromatin regulations during transcription elongation.

Introduction

Nucleosome is a fundamental unit of chromatin, consisting of 146bp of DNA wrapped around an octomer of four kinds of histones, H2A, H2B, H3 and H4. There are 14 contact points between histones and DNA (Luger, K. et al. 1997). Because these multiple interactions make the nucleosome one of the most stable protein-DNA complexes under physiological condition, nucleosomes can be significant obstacles on all aspects of transcription that are mediated by RNA polymerase II (Pol II). In transcription experiments in vitro, elongation of the nascent RNA chains on nucleosomal templates was severely inhibited compared with elongation on naked DNA templates (Izban, M.G. and Luse, D.S. 1991, Chang, C.H. and Luse, D.H. 1997). Because it is difficult for RNA polymerase II to pass a nucleosome, it is needed to destabilize nucleosomal structure by displacement of histones during Pol II passage. The destabilization of nucleosomes during transcription results in histone loss over the regions of heavily transcribed genes (Lee, C.K. et al. 2004). Histone loss sometimes causes aberrant transcription initiation from cryptic sites within coding regions. This striking finding came from an observation of short transcripts at particular yeast genes in temperature-sensitive *spt6* mutant (Kaplan, C.D. et al. 2003). It turns out that nucleosome density is decreased in the ORFs of some highly transcribed genes in the absence of Spt6, and the transcription pre-initiation complexes was formed at cryptic TATA-like sequences in the ORFs. Aberrant transcripts then initiate inside the ORFs from these pre-initiation complexes. Their study revealed that nucleosome reassembly behind Pol II passage is critical to maintain the fidelity of transcription initiation.

Although the above studies have been carried out in yeast, recent studies have revealed an interesting phenomenon regarding histone deposition during transcription in eukaryotes other than yeast. The phenomenon observed recently is that histone H3 variant, H3.3 is selectively incorporated into nucleosomes during transcription (Ahmad, K. and Henikoff, S. 2002a). In contrast to canonical histone H3, H3.3 is synthesized throughout the cell cycle and deposited onto DNA both during and outside of S phase (Ahmad, K. and Henikoff, S. 2002b, Tagami, H. et al. 2004). These observations suggest that H3 containing nucleosomes deposited during S phase are lost during transcription and replaced with nucleosomes containing H3.3. Indeed, examinations of several *Drosophila* genes revealed that nucleosomes containing either H3 or H3.3 were lost during transcription, and were selectively replaced with nucleosomes containing H3.3 (Wirbelauer, C. et al. 2005). Because when an ectopic expression of H3 was induced after S phase H3 was not deposited onto DNA (Ahmad, K. and Henikoff, S. 2002a), H3 and H3.3 are distinguished in some way.

So far only one factor, HIRA is known as a histone chaperone to deposit histone H3.3. In vitro analysis revealed that HIRA specifically deposits H3.3-H4 dimers in a replication-independent pathway (Tagami, H. et al. 2004). However, recent study has reported that *Hira* mutant showed only the defect in assembly of paternal chromatin during male pronucleus formation (Bonnefoy, E. et al. 2007). In *Drosophila*, as in many animals, the condensed sperm chromatin contains protamines instead of histones. At fertilization, protamines of sperm chromatin were replaced with maternally provided histones. Previous studies showed that histone variant H3.3 is specifically incorporated into paternal chromatin at fertilization (Loppin, B. et al. 2005). Bonnefoy et al.

mentioned that the only crucial role of HIRA in *Drosophila* was to assemble nucleosomes containing H3.3 in the male pronucleus after the removal of protamines. They also suggested that HIRA-independent H3.3 deposition pathway might exist. Because there is no link between HIRA and transcription in higher eukaryotes in spite of the correlation between deposition of H3.3 and transcription, there may be another factor involved in the deposition of H3.3 during transcription. To identify the factor which deposits H3.3 is a very interesting and important theme in this field. It is necessary for understanding more detailed mechanisms of H3.3 deposition. Furthermore, analysis of the factor might help to understand the biological meanings of H3.3 deposition which is yet unknown.

I took notice of Spt6 protein as a candidate because Spt6 is clearly involved in transcription elongation interacting with histone proteins. *spt6* was originally identified as one of Suppressor of Ty (*SPT*) genes through a genetic screen for mutations in *Saccharomyces cerevisiae* that restore gene expression disrupted by the insertion of the transposon Ty (Winston, F. et al. 1984). Studies in *S. cerevisiae* and *D. melanogaster* have shown that Spt6 localizes with elongating RNA polymerase II (Pol II) (Andrulis, E.D. et al. 2000, Kaplan, C.D. et al. 2000). Spt6 facilitated transcription elongation on naked DNA templates in vitro (Endoh, M et al. 2004). Studies of yeast Spt6 have revealed that Spt6 plays a critical role in maintaining normal chromatin structure during transcription elongation interacting with histone H3 (Bortvin, A. and Winstone, F. 1996, Kaplan, C.D. 2003). Although yeast has no transcription variant of histone H3, these findings in yeast studies led me an idea that Spt6 might be involved in the variant histone H3.3 deposition during transcription in higher eukaryotes.

In order to understand the Spt6 function in higher eukaryotes, I investigated the in

vivo function of Spt6 using *D. melanogaster* as a model organism. First, I analyzed the mutant effects of *spt6* on chromatin structure and transcription. The analyses of the *spt6* mutant revealed that *Drosophila* Spt6 is necessary to keep the fidelity of transcription through maintaining chromatin structure. Second, I examined the relationships between Spt6 and H3.3. The results of my study suggested that Spt6 is related with H3.3 in vivo. Based on these findings, I propose a possible model that Spt6 plays an important role in maintaining chromatin structure during transcription through the deposition of H3.3.

Material & Methods

Fly strains

Flies were raised on a standard agar/ cornmeal/yeast medium at 25℃. The *spt6* RNAi line, 12225R and *yw ; UAS-GFPN* were obtained from NIG stock center. The GAL4 driver lines, *Ay-GAL4/TM6B* was obtained from the Bloomington Stock Center. The transgenic flies expressing FLAG-H3 and FLAG-H3.3 were gifted by T. Nakayama (Nakayama, T. et al. 2007) and were used for the re-ChIP assay. The *spt6* mutant line, *PL83* was gifted by A. Vincent (Bourbon, H.M. et al. 2002). Balancer line, *N1/FM7c, GAL4-twi.,UAS-2xEGFP* were obtained from the Bloomington Stock Center. The *spt6* null mutant, *spt6W40* was obtained by excision of a P-element from *PL83* in the presence of the P transposase Δ 2-3.

Generation of transgenic flies

To do a rescue experiment of the *spt6* mutant, I generated some *spt6* transgenic flies. A fragment containing the entire *spt6* coding sequence was cloned and placed downstream of the *hsp83* promoter in a pCaSpeR transformation vector (Pirrotta, V. 1988). *hsp83-spt6* transgenic flies were produced by P-element mediated transformation using *yw* strain as a host, and several independent lines were obtained. For the obtained *hsp83-spt6* transgenic lines, expression levels of Spt6 were examined by Western blot analyses. A line expressing Spt6 in an appropriate level was selected for the mutant rescue experiment.

To carry out in vivo functional experiments using *spt6* RNAi, I generated some

transgenic flies expressing FLAG-H3 or FLAG-H3.3. A fragment containing FLAG-tagged H3 coding sequence or FLAG-tagged H3.3 coding sequence was cloned and placed downstream of the UAS-*hsp70* promoter in a pUAST transformation vector (Brand, A.H. and Perimon, N. 1993). Several independent transgenic lines were obtained by P-element mediated transformation. After the expression check by Western blot, two lines were selected respectively for the functional experiments.

Antibody generation

The DNA fragment encoding amino acid residues 1349-1831of *Drosophila* Spt6 polypeptide was cloned into a pQE82L (QIAGEN). The protein fragments expressed in *E. coli* BL21(DE3)CodonPlus-RIL (Stratagene) at 37 ℃ were purified by an affinity chromatography on the Ni-NTA resin (Sigma). The purified protein fragments were immunized a rabbit to generate polyclonal antibodies. Crude antiserum was purified by an affinity chromatography using the antigen polypeptides coupled to CNBr-activated Sepharose 4B (Amersham Biosciences). All immunological experiments were performed with the affinity purified anti-Spt6 antibodies.

Western blotting

Western blot analyses were performed by a standard protocol. Proteins from homogenized *Drosophila* embryos (collected 10-16 hours after egg laying) were separated by SDS-PAGE, transferred to a PVDF membrane (Roche), probed with the rabbit antibodies against Spt6, followed by horseradish peroxidase-linked anti-rabbit IgG and detected using SuperSignal (Pierce).

Immunostainig of polytene chromosomes

Salivary gland polytene chromosomes from third instar larvae were fixed for 90 seconds in 45% acetic acid, 1.85% formaldehyde and stained as described previously (Lori A. P. and Wassarman D. A. 2002). The anti-Spt6 antibodies were used at 1:200 dilutions. As a secondary antibody Alexa fluor™488 anti-rabbit IgG (Molecular Probes) was used. Finally, the stained chromosomes were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories).

Chromatin immunoprecipitation (ChIP)

The nuclei were isolated from embryos (collected 2-22 hours after egg laying) in nuclear isolation buffer (0.3M sucrose, 15mM pH7.4 Tris-HCl, 60m MKCl, 15mM NaCl, 5mM $MgCl_2$, 0.1mM EGTA, 0.5mM DTT, 0.3% NP-40) containing a protease inhibitor cocktail (Sigma). Chromatin of the isolated nuclei was cross-linked with Fix buffer (1% Formaldehyde, 50mM HEPES, 100mM NaCl, 0.1mM EDTA, 0.5mM EGTA) at RT for 10 min. Stop solution (150mM Glycine, PBS) was added to stop the cross-linking. DNA of the cross-linked chromatin was sheared by a BRANSON model 250 sonicator. The sheared chromatin was incubated with antibody-conjugated Protein-A Agarose beads (Upstate) in RIPA buffer (10mM pH8.0 Tris-HCl, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 0.1% SDS, 0.1% Sodium Deoxycholate, 1% Triton X-100) over night at 4℃. A portion of chromatin containing 75 μg DNA was used per IP. Subsequently, the beads were washed five times with RIPA buffer, once with RIPA High Salt buffer (0.5M NaCl in RIPA buffer), LiCl buffer (10mM pH8.0 Tris-HCl, 250mM LiCl, 1mM EDTA, 0.5% Sodium Deoxycholate,

0.5% NP-40) and TE. After RNase A treatment, bound DNA fragments were eluted by reverse cross-link treatment in Reverse Cross-link buffer (50mM pH8.0 Tris-HCl, 0.3M NaCl, 1mM EDTA, 0.5% SDS) containing Protenase K at 65℃ over six hours. The precipitated DNA fragments were amplified by PCR with Blend Taq (TOYOBO). The primer sequences for PCR are listed in the primers list. The PCR products were resolved by gel electrophoresis, stained by SYBR green (Molecular Probes) and analyzed by a LAS-4000 luminescent image analyzer (FUJIFILM). The enrichment ratio was gained by calculating the ratio of the signal intensity of immunoprecipitated product with specific antibodies to that with control IgG (Mock).

ChIP-on-chip microarray analysis

The procedure of ChIP-on-chip analysis was based on Agilent Mammalian ChIP-on-chip protocol version 9.1 (Agilent Technology) with minor modifications. Immunoprecipitated DNA fragments and control input DNA were amplified by Ligation-Mediated PCR. The ends of DNA were blunted by T4 DNA polymerase (TaKaRa), the blunted DNA ends were ligated with linker oligo nucleotides using T4 DNA ligase (TaKaRa) and the ligated DNA was amplified by PCR with Taq DNA polymerase (TaKaRa). The amplified DNA was fluorescent labeled by cyanine3-dUTP for input DNA and cyanine5-dUTP for immunoprecipitated DNA using a BioPrime Array CGH Genomic Labeling kit (Invitrogen). The labeled DNA (0.5 μg) was hybridized to a microarray. The microarray used here was Drosophila Whole Genome ChIP-on-chip Microarray slide2 (slide ID 14817) covering a half of the entire *Drosophila* genome at a density of about 1 oligo per 233bp (Agilent Technology). The microarray signals scanned by an Agilent

Scanner as TIFF images were extracted by the Feature Extraction Software as a Text file. Then the data were analyzed by the ChIP Analytics Software and Excel. The enrichment of the protein was expressed as a log ratio of the intensity in the immunoprecipitated DNA channel to that in the input DNA channel. A whole chip error model (Hughes, T.R. et al. 2000) was used to calculate the confidence values for each spot. To automatically determine the bound regions in the datasets, an algorithm to incorporate information from neighboring probes (Boyer, L.A. et al. 2005) was used. In the algorithm the average p-values of three adjoined probes (probe set p-values) were calculated. If the probe set p-value was less than 0.001, the three probes were marked as potentially bound. The location of all bound regions were compared to the genome database of Fly Base (http;//flybase. Bio.Indiana.edu/).

RT-PCR

Total RNA was extracted using Sepazol RNA1 (Nakarai) from 100 embryos (collected 10-16 hours after egg laying). The RNA was treated with DNase I (TaKaRa). cDNA was synthesized using a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche) with random primers. The cDNA was amplified by PCR. The primer sequences for PCR are listed in primers list. The PCR products were resolved by gel electrophoresis, stained by SYBR green (Molecular Probes) and analyzed by a LAS-4000 luminescent image analyzer (FUJIFILM).

Northern blot analysis

Total RNA was extracted using Sepazol RNA1 (Nakarai) from 200 embryos (collected

10-16 hours after egg laying). The RNA was treated with DNase I (TaKaRa). The total RNA was purified and resolved by 1.2% agarose gel electrophoresis. Northern Blot analysis was performed according to the manufacture's protocol, Techniques for Hybridization of DIG-labeled Probes to a Blot (Roche). The labeled RNA probes were generated on *hsp83* and *β-tubulin* by in vitro transcription from DNA templates using a DIG Norhtern Starter Kit (Roche).

MNase assay and Southern blot analysis

The isolated nuclei from embryos (collected 10-16 hours after egg laying) were digested with MNase at 37℃ for 5 min. After RNase A (Fermentas) and Proteinase K (Roche) treatments, the DNA was purified and resolved by 2% agarose gel electrophoresis. Southern blot analysis was performed according to the manufacture's protocol, Techniques for Hybridization of DIG-labeled Probes to a Blot (Roche). DIG-labeled probes were produced on *hsp83* gene by PCR labeling using a PCR DIG Probe Synthesis Kit (Roche).

Re-ChIP assay

In this assay, the ChIP method was the same as the normal ChIP assay described above. The chromatin samples were prepared from embryos of transgenic fly expressing FLAG tagged H3 or FLAG tagged H3.3. The chromatin samples were immunoprecipitated with anti-FLAG antibodies (Sigma, F3165) in the first step. The bound chromatin was eluted by FLAG peptide (Sigma). After the DNA concentration of each eluted chromatin was adjusted, the chromatin samples were re-immunoprecipitated with anti-Spt6 antibodies.

The following processes were the same as the normal ChIP assay.

spt6 RNAi and histone over-expression

spt6 RNAi line, 12225R (*UAS-IRspt6*) was crossed with *UAS-H3-FLAG, UAS-H3.3-FLAG* or *UAS-GFPN* to generate *UAS-IRspt6;UAS-H3-FLAG, UAS-IRspt6;UAS-H3.3-FLAG* or *UAS-IRspt6;UAS-GFPN*. These *spt6* RNAi lines were crossed to *Ay-GAL4/TM6B* to observe phenotypes of the progenies. The phenotypes of the progenies were evaluated by counting the number of pupae and calculating the ratio of RNAi induced flies (*Tb+*) to no-induced flies (*Tb-*).

Primers list

ChIP, RT-PCR

larp	5'-GCAGCATAGAAGCGGGCACG-3'	5'-CACCACCGCCACCATTTGAG-3'
hsp83	5'-GTTCGAGAGCCTGTGCAAGC-3'	5'-CCGAACTGCGAAGTGACAATG-3'
β-tubulin	5'-CATGTTGCTCTCGGCCTCGG-3'	5'-GTGCGATCGGGACCTTTCGG-3'
cyp4d21	5'-CGATATCCAGACATCCCAAGG-3'	5'-CATCGTCTCCTTGATCACGC-3'
thread	5'-CGCCGTAGCAGATCTTGCAC-3'	5'-CAGCCACACGCATCTTCAAC-3'
hsp83-5'	5'-GAGATTTTCCTGCGCGAGTTG-3'	5'-CTTGATGTACAGCTCCTTGCCAG-3'

Results

Generation and analysis of anti-Spt6 antibodies

Spt6 protein contains an acidic region at the N terminus, and also contains a Tex homology domain which is homologous to the shared domain of a family of bacterial proteins implicated in transcription elongation (Fuchs, M.E. et al. 1996), a DNA binding helix-hairpin-helix (HhH) domain and an RNA binding S1 domain. At the C terminus there is a specific amino acid-rich region, SPTG rich domain. The function of SPTG rich domain is unknown. All the domains are evolutionarily conserved from yeast to human, except that yeast Spt6 lacks the C terminal SPTG domain (Figure 1A, Kaplan, C.D. et al. 2000).

To investigate in vivo function of Spt6, first, Spt6 antibodies were raised in a rabbit. I generated Spt6 polyclonal antibodies against the C terminal 483 amino acid fragment of *Drosophila* Spt6 (Figure 1A). Western blotting of *WT* whole embryos showed that these Spt6 antibodies specifically recognized a protein band of about 210 kD (Figure 1B). This size is in agreement with the predicted molecular mass of Spt6, which is 209 kD. To verify that these Spt6 antibodies recognize Spt6 protein on chromatin, I carried out immunofluorescence staining of polytene chromosomes. As previously reported (Andrulis, E.D. et al. 2000; Kaplan, C.D. et al. 2000), Spt6 signals were detected in diffuse uncoiled regions of polytene chromosomes called puffs, which are known to be sites of active transcription (Figure 2A-C). After 10 min heat shock, Spt6 was accumulated on heat shock puffs, especially on the *hsp70* loci (Figure 2D-E). These results were consistent with the results previously reported (Andrulis, E.D. et al. 2000; Kaplan, C.D. et al. 2000), therefore I concluded that these antibodies can specifically

recognize Spt6 protein.

Spt6 is enriched on a subset of genes

The Immunostainig of polytene chromosomes with the Spt6 antibodies showed that Spt6 localizes on transcriptionaly active chromatin (Figure 2), however detailed localization of Spt6 on the *Drosophila* genome was unknown. To investigate the localization of Spt6 on chromatin, I carried out ChIP-on-chip microarray analyses. Chromatin from *WT* embryos was immunoprecipitated with the anti-Spt6 antibodies. The immunoprecipitated products were fluorescent labeled and hybridized to *Drosophila* Whole Genome ChIP-on-chip Microarray slides covering half of the entire *Drosophila* genome, which contains chromosome X and III. By the analysis of ChIP-on-chip microarray, strong signals of Spt6 (probe set p-value < 0.001) were detected on about 30 genes (Table 1). As these 30 genes are various in their functions, expression patterns and genome structures, any common features were not found among them. Among these genes, I chose *La related protein* (*larp*) and *heat shock protein 83* (*hsp83*) for further analyses because they are ubiquitously expressed. We usually use whole embryos as a material for chromatin analyses such as ChIP, therefore ubiquitously expressed genes are convenient. Hsp83 is a protein folding factor which is not only responsive to heat shock but also expressed ubiquitously (FlyBase, http;//flybase. Bio.Indiana.edu/). Larp is involved in the biological processes such as centrosome separation and mitotic chromosome condensation, but its molecular function is unknown (FlyBase, http;//flybase. Bio.Indiana.edu/). Regarding detailed distribution of Spt6 on the *larp* and *hsp83* genes, significant signals of Spt6 were distributed throughout each

gene, especially in exons (Figure 3). In most of the 30 genes, Spt6 was enriched in exons but not in introns. The significant signals of Spt6 were not detected on the *β-tubulin* gene, therefore *β-tubulin* was used as a negative control for further experiments.

In order to confirm the results of the ChIP-on-chip experiments, I performed conventional ChIP analysis using PCR primers generated at the positions shown by red bars (Figure 3). Chromatin from *WT* embryos was immunoprecipitated with the antibodies against Spt6, RNA polymerase II or control IgG. The DNA purified from the chromatin immunoprecipitated products were amplified by PCR. As expected, signals of RNA polymerase II were detected on all three genes, *larp*, *hsp83* and *β-tubulin* because they are transcribed (Figure 4). Although all three genes are transcribed, the signals of Spt6 were detected by PCR only on *larp* and *hsp83*, but not on *β-tubulin* (Figure 4). These results are consistent with the results of ChIP-on-chip analyses. All of these experiments revealed that Spt6 localizes on the chromatin of a subset of genes.

Generation and characterization of *spt6* mutant

To investigate the function of Spt6 in vivo I generated and characterized the *spt6* null mutant. The *Drosophila* genome contains a single *spt6* gene CG12225. The *spt6* mutant, *PL83* gifted by A. Vincent (Bourbon, H.M. et al. 2002) is a P-element inserted allele in the second exon of the *spt6* gene (Figure 5-A). *PL83* homozygotes show an embryonic lethal phenotype. In order to gain *spt6* deletion mutants, I carried out P-element excision from *PL83*. When the P-element was precisely excised from *PL83*, the lethality of *PL83* was rescued. This means that the lethality of *PL83* was caused by the insertion of P-element in the *spt6* gene. By imprecise excision, *spt6W40* was generated.

spt6W40 allele has a small deletion in the second exon of the *spt6* gene that causes a stop of the protein synthesis, hence is functionally null (Figure 5-A). Homozygotes of *spt6W40* show an embryonic lethal phenotype (data not shown). The lethal phenotype of *spt6W40* was rescued by expression of Spt6 from a transgene. This indicates that the lethality of *spt6W40* was caused by the *spt6* mutation. To confirm that Spt6 was not expressed in *spt6W40*, western blot analysis of *spt6W40* homozygous embryos was performed with the Spt6 antibodies. The expression of Spt6 was not detected in *spt6W40* homozygotes (Figure 5-B). I decided to use *spt6W40* as an *spt6* null mutant for further analyses.

spt6 **mutant produces aberrant shorter transcripts**

Because previous studies have provided evidence that Spt6 is involved in transcription elongation, I examined the effects of the *spt6* mutation on transcription. First, I performed RT-PCR analyses using RNA from embryos to measure the amounts of mRNA. The cDNA synthesized with random primers was used as a template for PCR. The PCR was performed with the primers synthesized for the 3' regions of *larp* and *hsp83* mRNA (Figure 3-a). The RT-PCR experiments presented unexpected results that the levels of mRNA from *larp* and *hsp83* genes were not decreased but increased in the *spt6* mutant (Figure 6-A). Previous studies have shown that yeast *spt6* mutant permits aberrant transcription initiation from cryptic sites within the coding region of some genes (Kaplan, C.D. et al. 2003). To investigate whether the increase of mRNA in the *spt6* mutant was due to aberrant transcripts, I carried out RT-PCR analyses with primers for 5' region of each mRNA (Figure 3-a). In contrast to the results for the 3' region of

each mRNA, the levels of the 5' region were not increased in the *spt6* mutant compared with the wild type (Figure 6-B). These results suggest that the transcription initiates from the middle of the coding region in the *spt6* mutant. To analyze the size of the transcripts from *hsp83*, I carried out a Northern blot analysis of RNA from embryos. The RNA probes synthesized over the coding sequence of *hsp83* detected the expected 3kbp transcripts both in the wild type and the *spt6* mutant (Figure 7). The *hsp83* probes also detected other shorter transcripts only in the *spt6* mutant. These shorter transcripts may be the products of aberrant transcription on *hsp83* gene in the *spt6* mutant. However, any quantitative relations cannot be discussed in this experiment because the amounts of total RNA loaded on the gel were different between the wild type and the *spt6* mutant. In contrast, the control *β-tubulin* probes detected only one normal transcript (1.8 kbp) both in the wild type and the *spt6* mutant. These results suggest that *spt6* mutation causes aberrant transcription initiation from cryptic sites within the coding region.

***spt6* mutation causes changes in chromatin structure in vivo.**

To examine the possibility that the aberrant transcription in the *spt6* mutant is due to changes in chromatin structure, I carried out a micrococcal nuclease (MNase) assay using *spt6W40* embryos. After digestion with MNase, chromatin DNA was stained with ethidium bromide to observe the bulk chromatin structure. There was no difference in the patterns of bulk chromatin stained with ethidium bromide between the wild type and the *spt6* mutant (Figure 8-A). To observe the chromatin structure on the Spt6-enriched *hsp83* gene, DNA transferred on a membrane was probed with *hsp83*. The

result showed that the rate of generation of mono-nucleosomes was increased in the *spt6* mutant (Figure 8-B). This means that the chromatin in the *spt6* mutant exhibited an increased sensitivity to MNase. This result indicates that the *spt6* mutation causes changes in the chromatin structure on the *hsp83* gene in vivo.

The increase of MNase sensitivity in the *spt6* mutant could be caused by either loss of nucleosomes or altered nucleosome structure. To distinguish between these possibilities, I measured the levels of general histone H3 on *hsp83* and control gene *cyp4d21* by ChIP analysis in the wild type and the *spt6* mutant. *cyp4d21* is not transcribed during the embryonic stage. Spt6 is not enriched on *cyp4d21*. The results showed that the level of general H3 on *hsp83* immunoprecipitated with pan-H3 antibodies was reduced in the *spt6* mutant compared with the wild type (Figure 9). Such reduction of H3 was not detected on the control gene *cyp4d21*. These results demonstrate that the chromatin structure on *hsp83* was changed by the loss of histones in the *spt6* mutant.

Spt6 localizes on the chromatin containing H3.3

Previously, examination of several *Drosophila* genes revealed that nucleosomes containing either canonical histone H3 or variant histone H3.3 were lost during transcription, and were selectively replaced with nucleosomes containing H3.3 (Wirbelauer, C. et al. 2005). Because the chromatin structure was changed by the loss of nucleosomes in the *spt6* mutant, I consider the possibility that Spt6 can help the deposition of H3.3 during transcription. To observe the correlation of Spt6 with H3.3, I performed ChIP analysis of H3.3 using embryos expressing FLAG-tagged H3.3. Because

H3.3 differs at only five amino acid positions compared with canonical histone H3, it is difficult to generate specific antibodies that discriminate between them. From this reason, I used FLAG-tagged histone expressing lines. I measured the levels of H3.3 on three Spt6-enriched genes (*hsp83*, *thread* and *larp*) and two non-enriched genes (β-*tubulin* and *cyp4d21*). Thread is a ubiquitin-protein ligase involved in regulation of apoptosis. The levels of H3.3 were higher on the Spt6-enriched genes rather than non-enriched genes (Figure 10). This result suggests that there is some correlation between Spt6 and H3.3 in their localization.

For further examination of physical relationship between Spt6 and H3.3, I carried out a Re-ChIP assay using embryos expressing FLAG-tagged H3.3 or FLAG-tagged H3. In this assay, first, mono-nucleosomes produced by micrococcal nuclease (MNase) were immunoprecipitated with anti-FLAG antibody to collect nucleosomes containing H3.3 or H3 specifically. Then, immunoprecipitated nucleosomes were re-immunoprecipitated with anti-Spt6 antibody to examine whether Spt6 localizes on nucleosomes containing H3.3 or H3. On *larp* and *hsp83*, Spt6 was efficiently immunoprecipitated with the nucleosomes containing H3.3 rather than H3 (Figure 11A). As a control, the same experiment was carried out using antibodies against RNA polymerase II (Pol II) instead of Spt6. Pol II was immunoprecipitated with the nucleosomes containing H3.3 and H3 at the same extent (Figure 11B). These results indicate that Spt6 preferentially localizes on the nucleosomes containing H3.3 rather than H3.

Phenotype of *spt6* RNAi knockdown is suppressed by over-expression of H3.3

To further explore the in vivo function of Spt6, I observed the phenotypes of *spt6* RNAi. RNAi lines were gifted by R. Ueda. When *spt6* RNAi was ubiquitously induced by an *Ay-GAL4* driver, almost all the animals died before the pupal stage (Table 2-B). Western blot analysis showed that the level of Spt6 was reduced to 35 % of that in control upon RNAi knockdown of *spt6* (Figure 12A).

If Spt6 is involved in the deposition of H3.3, a decrease in the efficiency of H3.3 deposition caused by *spt6* knockdown can be suppressed by over-expression of H3.3. To test this, FLAG-tagged H3.3 was expressed in the *spt6* RNAi line. As controls FLAG-H3 or GFP was expressed instead of FLAG-H3.3. Two independent lines were tested for each expressed histone. I confirmed that the expression levels of exogenous histones were almost the same between FLAG-H3 lines and FLAG-H3.3 lines by Western blot analysis (Figure 12B). In this experiment, over-expression of histones in control did not affect the viability of flies (Table 2-A). In the *spt6* RNAi, most animals died before the pupal stage. When FLAG-H3.3 was over-expressed in the *spt6* RNAi, the viability of pupae was increased (Table2-B). The viability was not increased by the over-expression of GFP. While the viability was slightly increased by the over-expression of FLAG-H3, the increase in the viability was much smaller than that of FLAG-H3.3. Statistically, the level of increase in the viability by FLAG-H3.3 over-expression was significantly ($p < 0.001$) higher than that by H3. In brief, the lethal phenotype of *spt6* knockdown was significantly suppressed only by the over-expression of H3.3. This result supports the idea that Spt6 is involved in the deposition of histone H3.3 in vivo.

Discussion

Spt6 is involved in the maintenance of chromatin structure through interaction with H3.3

In this study I investigated the in vivo function of Spt6 related to chromatin structure in *D. melanogaster*. I analyzed the chromatin structure in the *spt6* null mutant. MNase assay and ChIP analysis of chromatin from mutant embryos revealed that the chromatin structure of Spt6-enriched gene was changed by the loss of histones. (Figures 8,9) Moreover, Northern analysis of RNA from mutant embryos showed that the aberrant transcripts were produced in the *spt6* mutant (Figure 7). This is the first demonstration of aberrant transcription initiation by a mutation of transcription elongation factor in eukaryotes except yeast. These results suggest that Spt6 is necessary to keep the fidelity of transcription through maintaining chromatin structure. To understand the change of chromatin structure in the *spt6* mutant and the molecular mechanisms of Spt6 function, I took notice of the phenomenon that variant histone H3.3 is specifically deposited on to the nucleosomes during transcription. I considered the possibility that Spt6 can help the deposition of H3.3 during transcription. The results of this study showed that Spt6 has some relationships with H3.3 physically and functionally. Spt6 was efficiently immunoprecipitated with the nucleosomes containing H3.3 rather than H3, suggesting that Spt6 physically interacts with the nucleosomes containing H3.3 (Figure 11). As I examined only by the re-ChIP assay, it is impossible to determine that the physical relationship between Spt6 and H3.3 is direct or not. To test the possibility, further experiments such as in vitro binding assay with purified Spt6 and

recombinant histones will be needed. In regard to functional analysis between Spt6 and H3.3, I referred to the yeast study by Bortvin, A. and Winston, F. In their study over-expression of H3 suppressed the lethality of the *spt6* ts mutant (Bortvin, A. and Winston, F. 1996). In my study the lethal phenotype caused by the *spt6* knockdown was suppressed by the over-expression of H3.3 but not H3 (Table 2). This result supports the idea that Spt6 is involved in the deposition of H3.3. To confirm the point, I need to examine whether the levels of H3.3 on chromatin are reduced in the *spt6* mutant. To test this I made the *spt6* mutant line expressing FLAG-H3.3. However, expression level of the FLAG-H3.3 transgene was not stable and increased in the *spt6* mutant but not in the wild type during propagation of the lines. Under these conditions, it is difficult to compare the precise levels of H3.3 on chromatin between the *spt6* mutant and the wild type. Because H3.3 is deposited on to nucleosomes both during transcription and replication, the basal levels of H3.3 on chromatin would be changed by an increase of H3.3 expression. I need to construct another transgene that is not affected by the *spt6* mutation.

In summary results of my study suggest that Spt6 plays an important role in maintaining the chromatin structure during transcription elongation, thereby repressing production of aberrant transcripts through interaction with histone variant H3.3 (Figure 13).

Spt6 is not a general transcription factor

To identify Spt6-dependent genes, I carried out the analyses of the ChIP-on-chip microarray. This is the first genome wide survey on the distribution of Spt6 in

eukaryotes. Strong signals of Spt6 were detected on only about 30 genes within the half of the entire *Drosophila* genome that contains 13600 genes (Table 1). This result is surprising because Spt6 has been thought to be a more general transcription elongation factor in *Drosophila* (Andrulis, E.D. 2000, Kaplan, C.D. 2000). One reason is because I used whole embryos collected 2-22 hours after egg laying as a material for ChIP, it is difficult to detect the cell type specific or temporary localization of Spt6 on chromatin. Another possible reason is that because I chose Spt6 signals using so stringent criteria (probe set p-value < 0.001) that I could not extract all the signals. However, even if I extracted signals using less stringent criteria Spt6 signals were still detected on a limited number of *Drosophila* genes. This result shows that Spt6 is not a general transcription factor, but has some target genes.

Another surprising result from the ChIP-on-chip microarray analyses is that most Spt6 signals were detected in exons but not in introns. This result may be related to the fact that Spt6 is involved in the mRNA processing. In eukaryote cells, transcription elongation is tightly coupled to mRNA processing (5'-end capping, splicing, cleavage and polyadenylation), surveillance and export (Bentley, D.L. 2005). *Drosophila* Spt6 was co-purified with the exosome, a complex of 3' to 5' exoribonuclease that is involved in the processing of structural RNA and in the degradation of improperly processed pre-mRNA (Andrulis, E.D. et al. 2002). A point mutation of murine Spt6 (R1358K) showed splicing defects (Yoh, S.M. et al. 2007). Taken together these findings suggest that Spt6 may be working on exons connecting transcription elongation with mRNA processing, surveillance and export.

Biological roles of histone H3.3 deposition

In this study I suggested that Spt6 is involved in the deposition of variant histone H3.3 during transcription. Regarding H3.3, many previous examinations revealed that H3.3 is specifically incorporated into nucleosomes during transcription. However, biological meanings of H3.3 deposition are still elusive. I will discuss the biological meanings of variant histone H3.3 deposition. First, I will consider the meanings of H3.3 from a transcriptional point of view. Do H3.3-containing nucleosomes possess unique properties that would affect transcription? Recent study has shown that H3.3-containing nucleosomes isolated from vertebrates are unusually sensitive to salt-dependent disruption. It means that H3.3-containing nucleosomes are intrinsically less stable than those containing H3, and that this may reduce the energy required to move or displace nucleosomes from promoters, enhancers, and gene-coding regions (Jin, C. and Felsenfeld, G. 2007). This result suggests that H3.3 plays an active role in maintaining accessible chromatin structures in transcribed regions. To make this speculation more certain, in vitro transcription experiments will be needed.

Second, I will consider the meanings of H3.3 from an epigenetical point of view. Histone proteins are well known to be modified by covalent modifications such as methylation, acetylation and phosphorylation. These modifications can affect the various stages of gene expression by altering structure of chromatin. According to the recent studies, variant histone H3.3 specifically contains marks associated with transcriptionally active chromatin such as K36,K79 methylation and K9,K27 acetylation, whereas canonical histone H3 contains silent modifications such as K9,K27 methylation (McKittrick,E. et al. 2004, Hake, S.B. et al. 2006). These findings lead us a suggestion

that exchange of histone variant during transcription can become a quick switch for histone modification to change the states of chromatin structure and gene expression. Furthermore, these histone modification marks can be inherited to daughter cells through DNA replication. Now, different models have been proposed to explain how epigenetic memory can be achieved. One is the conservative inheritance model, the other is the semi-conservative inheritance model. The conservative inheritance model proposes that H3-H4 tetramers are distributed on daughter strands in a random fashion during replication (Baxevanis,A.D. et al. 1991). Histone chaperons deposit new H3-H4 tetramers onto daughter strands to fill in the gaps. In this model the maintenance of epigenetic inheritance is difficult. In contrast, the semi-conservative inheritance model proposed by Tagami, H. et al. (Tagami, H. et al. 2004) is that nucleosomes are separated into two H3-H4 dimers and distributed equally onto daughter strands during replication. Histone chaperons deposit new H3-H4 dimers to inherited H3-H4 dimers on daughter strands forming histone octamers. If the semi-conservative model is true, distributed H3-H4 dimer and H3.3-H4 dimer can become epigenetic memory through replication.

The function of Spt6 in higher eukaryotes

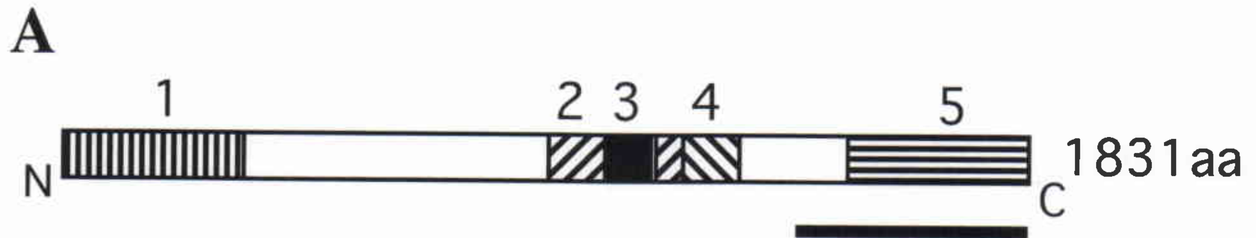
In this study I investigated the functions of Spt6 in *D. melanogaster*. However, most of the previous studies on Spt6 have been carried out in *S. cerevisiae.* Although, *S. cerevisiae* is a useful model animal to study transcription mechanisms, it is different from other eukaryote in some respects. One example is the variant histone H3.3. Higher eukaryotes such as *H. sapiens, D. melanogaster* and *A. thaliana* possess both canonical

histone H3 (H3.1 for *H. sapiens*) and variant histone H3.3. In contrast, *S. cerevisiae* possesses only one kind of histone H3 except the centromere-specific histone variant. H3 sequence of *S. cerevisiae* is similar to H3.3 sequence of other organisms rather than canonical histone H3. The fact that higher eukaryotes possess both silent chromatin-related H3 and active chromatin-related H3.3, whereas *S. cerevisiae* possesses only one H3 similar to H3.3 is consistent with that *S. cerevisiae* has the gene-rich genome and lacks the machinery for facultative heterochromatin formation such as H3K27 methylase and PcG. Moreover, because higher eukaryotes need the different principles to govern the proliferation and differentiation, it may be reasonable to use two different histones. In regard to the structure of Spt6 protein, *S. cerevisiae* Spt6 lacks the C-terminal domain conserved in other organisms (Figure 1A). Although the function of the C-terminal domain of Spt6 is unknown, this domain might provide Spt6 with a further function to distinguish H3.3 from H3 in higher eukaryotes.

Conclusion

In this study I have found that Spt6 plays an important role in maintaining the chromatin structure during transcription elongation, thereby repressing production of aberrant transcripts in *D. melanogaster*. I also observed the correlation of Spt6 with variant histone H3.3. This is the first report to suggest the relationship between Spt6 and H3.3. Further investigation will clarify the function of Spt6 in the H3.3 deposition, and it may provide new insights into chromatin regulations during transcription elongation.

A



B

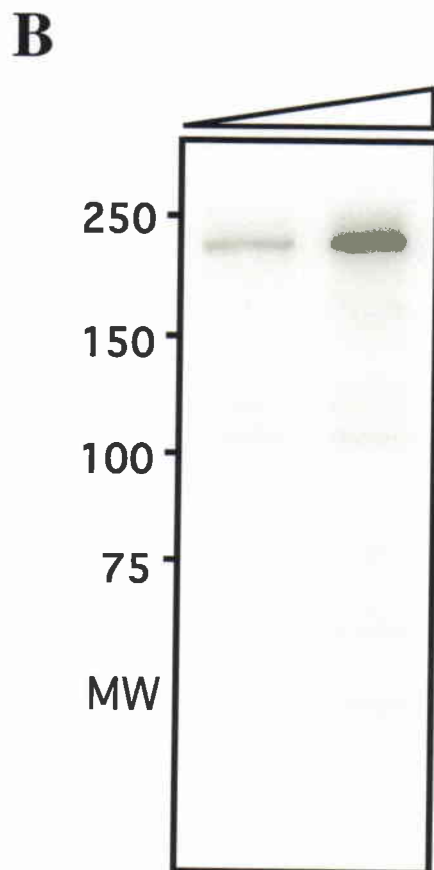


Figure 1 Generation and analysis of anti-Spt6 antibodies

A; Structure of Spt6 protein. Spt6 contains five conserved domains. 1;acidic region, 2;Tex homology, 3;HhH domain, 4;S1 domain, 5;STPG rich domain. The bar indicates the region against which antibodies were raised.

B; Western blot analysis of *WT* whole embryos with anti-Spt6 antibodies.

A band of about 210 kD was recognized by anti-Spt6 antibodies.

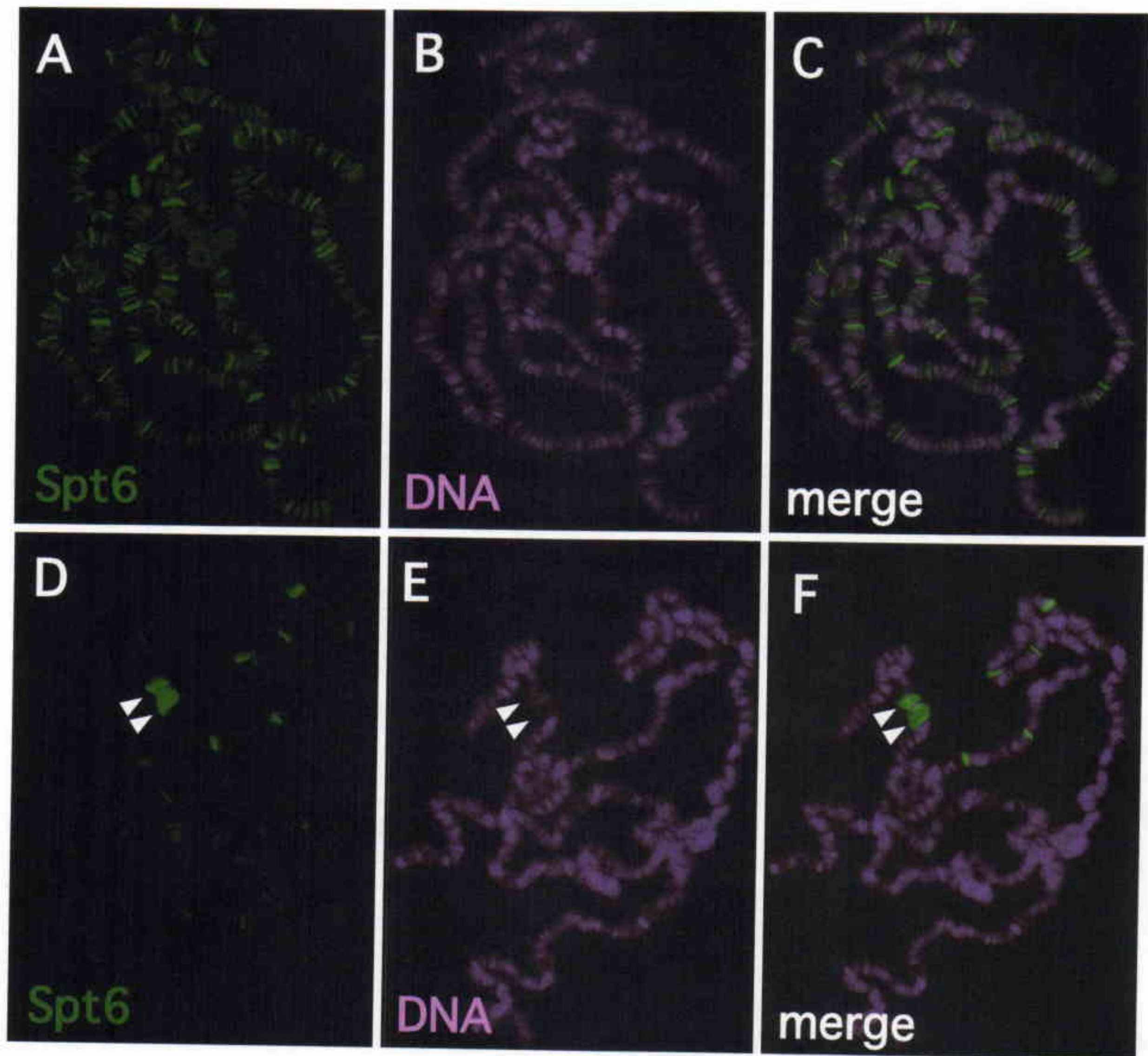


Figure 2 Polytene chromosome staining with anti-Spt6 antibodies

A-C; before heat shock, D-F; after 10 min heat shock. A and D; Spt6 (green), B and E; DNA (magenta), C; overlay of A and B, F; overlay of D and E. Arrow heads show the location of the *hsp70* loci. Before heat shock Spt6 signals were detected in chromosomal puffs. After heat shock Spt6 signals were accumulated on the heat shock gene loci.

Location	Gene name	Function
5C7	*Actin 5C (Act5C)*	structural constituent of cytoskeleton
68C10	*charybde (chrb)*	unknown, negative regulation of growth
100C7	*Cyclin G (CycG)*	cyclin-dependent protein kinase regulator
64E5	*DnaJ-like-1 (DnaJ-1)*	heat shock protein binding
61C9	*extra macrochaetae (emc)*	transcription corepressor activity
66D10	*hairy (h)*	transcriptional repressor activity
10E3-10E4	*Heat shock protein cognate 3*	ATPase activity response to heat
67B2	*Heat shock protein 26 (Hsp26)*	unknown, response to heat
63B11	*Heat shock protein 83 (Hsp83)*	ATPase activity, response to heat
93D4	*Heat shock RNA* ω *(Hsr*ω*)*	unknown, response to heat
99B10-99C1	*kayak (kay)*	RNA polymerase II transcription factor activity
98C3-98C4	*la related protein (larp)*	unknown, centrosome separation
4F3	*rugose (rg)*	protein kinase A binding
66D6-66D7	*rhea*	actin binding
68B4-68C1	*scylla (scyl)*	unknown, negative regulation of growth
3A8-3B1	*shaggy (sgg)*	protein serine/threonine kinase activity
70B2	*stv*	protein binding, apoptosis
72D1	*thread (th)*	ubiquitin-protein ligase activity
67F1	*tonalli (tna)*	zinc ion binding, maintenance of transcription
77C1	*tribbles (trbl)*	protein serine/threonine kinase activity
100D1	*tramtrack (ttk)*	RNA polymerase II transcription factor activity
62F4	CG2083	unknown
67C4-67C5	CG6767	ribose phosphate diphosphokinase activity
64B4-64B5	CG11347	unknown
4F10	CG15784	unknown
61B2	CG16971	unknown
75F2	CG18135	unknown
61B2	CR32477	unknown

Table 1 Spt6 enriched genes

Table of genes on which Spt6 signals (probe set p-value < 0.001) were detected by ChIP-on-chip analyses. Location, gene name and function were based on the information from FlyBase (http://flybase.bio.indiana.edu/).

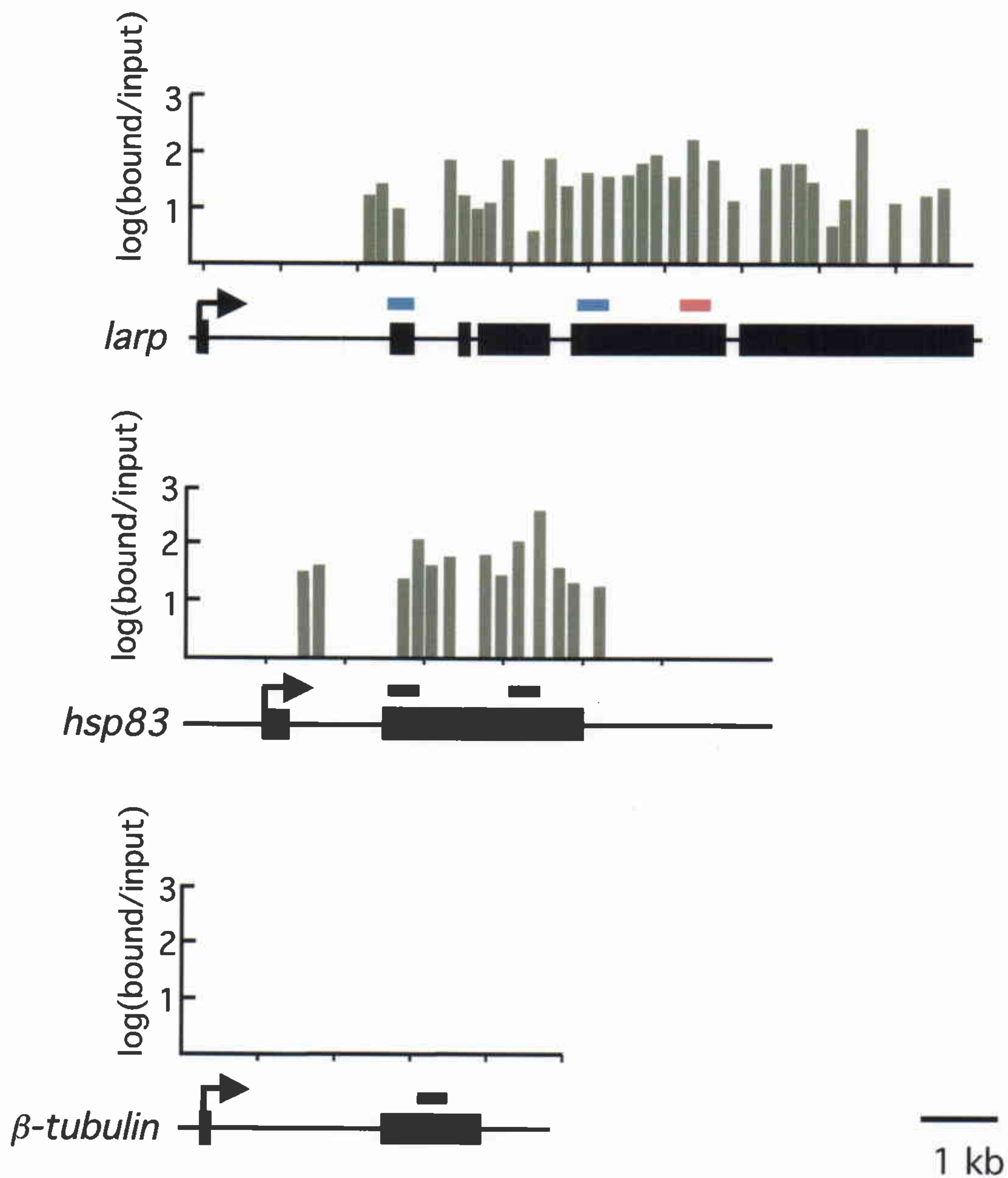


Figure 3-a Signals of Spt6 were distributed in the entire gene, especially exons
Enrichment of Spt6 across *larp*, *hsp83* and *β-tubulin*. Enrichment was calculated as the log ratio of immunoprecipitated DNA (bound) to input DNA (input). Red bars show the positions of PCR primers for conventional ChIP analyses (Figure 4,9 and 10) and RT-PCR (Figure 6). Blue bars show the positions of PCR primers for RT-PCR (Figure 6).

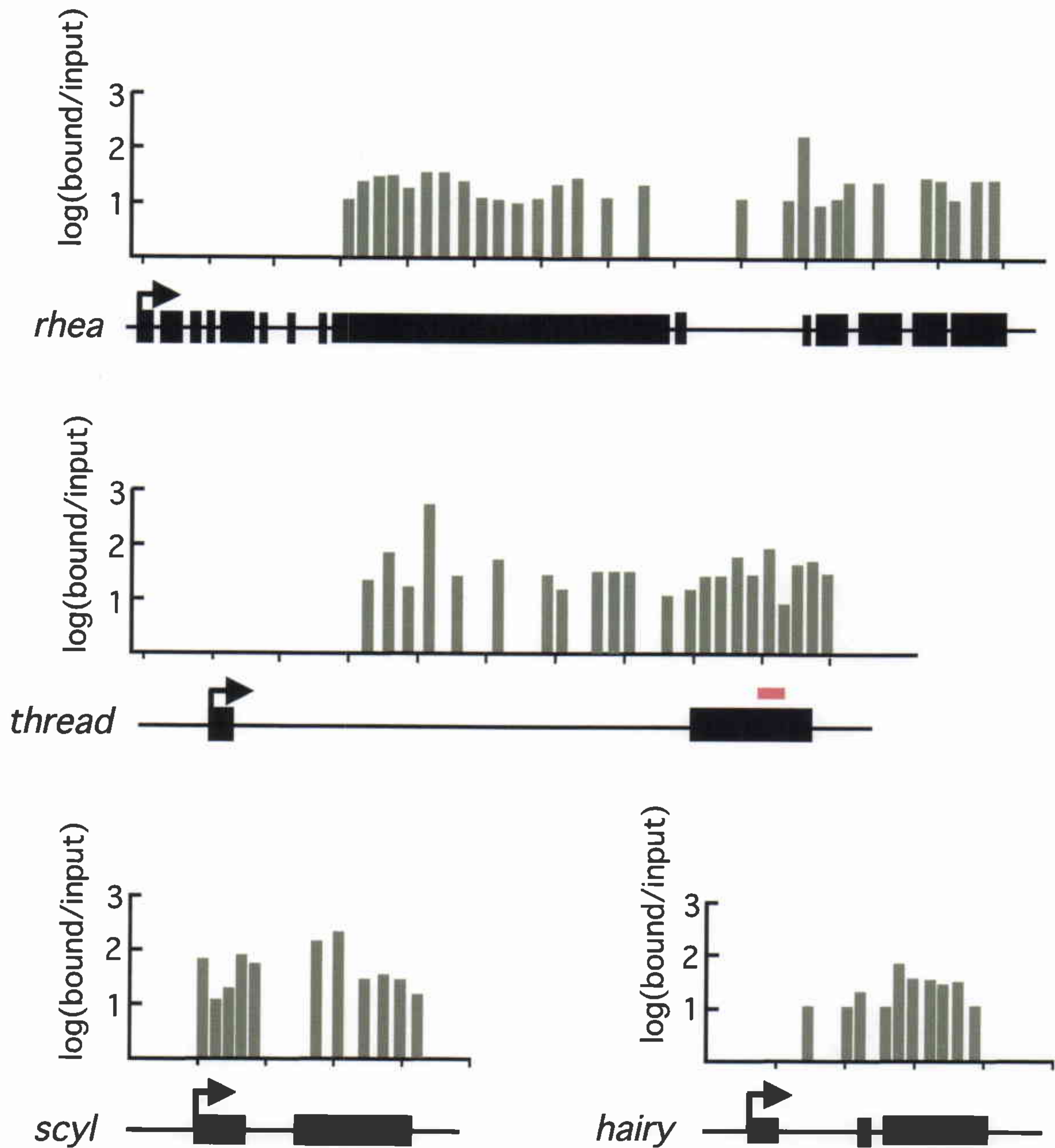


Figure 3-b Signals of Spt6 were distributed in the entire gene, especially exons
Enrichment of Spt6 across *rhea, thread, scyl* and *hairy*. Enrichment was calculated as the log ratio of immunoprecipitated DNA (bound) to input DNA (input). Red bar shows the position of PCR primers for the ChIP analysis (Figure 10).

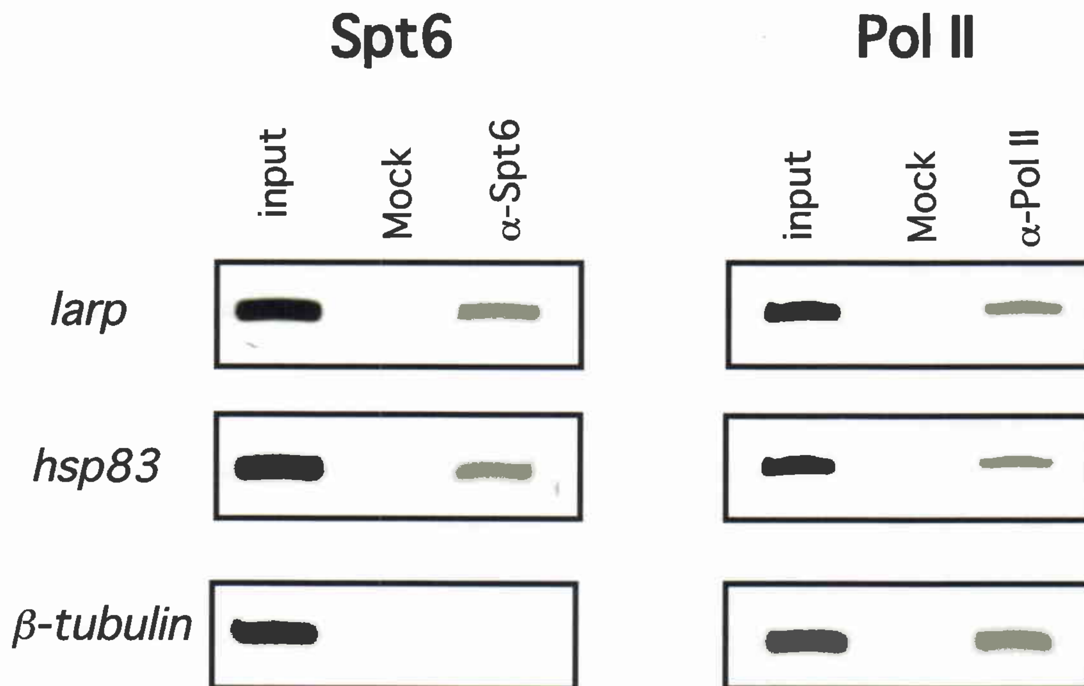
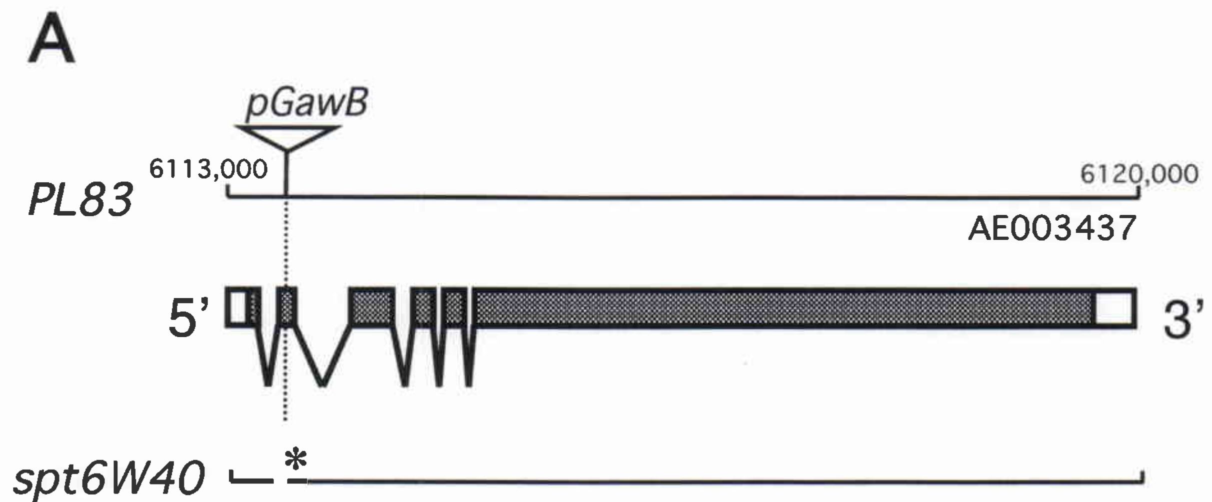


Figure 4 Conventional ChIP analyses of Spt6 (left panel) and RNA polymerase II (right panel)

Input; 0.5% of input DNA, Mock; IP with control IgG. Poll II was detected by PCR on *larp*, *hsp83* and *β-tubulin*. Spt6 was detected on *larp* and *hsp83*, but not on *β-tubulin*.



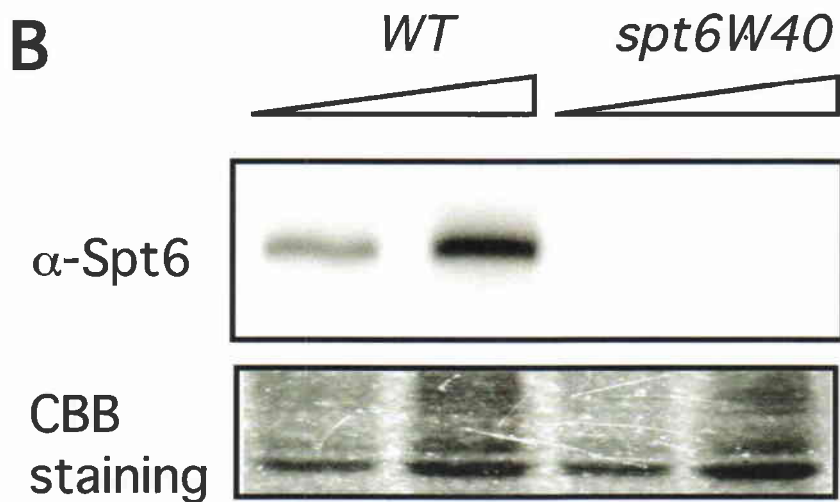


Figure 5 Characterization of *spt6* mutant

A; *PL83* is a P-element inserted allele in the second exon of the *spt6* gene. *spt6W40* is a P-element imprecisely excised allele from *PL83*. *spt6W40* has a small deletion in the second exon of the *spt6* gene caused a stop of the protein synthesis(*).

B; Western blot analysis of embryos with Spt6 antibodies. The expression of Spt6 protein was not detected in the *spt6W40* homozygotes.

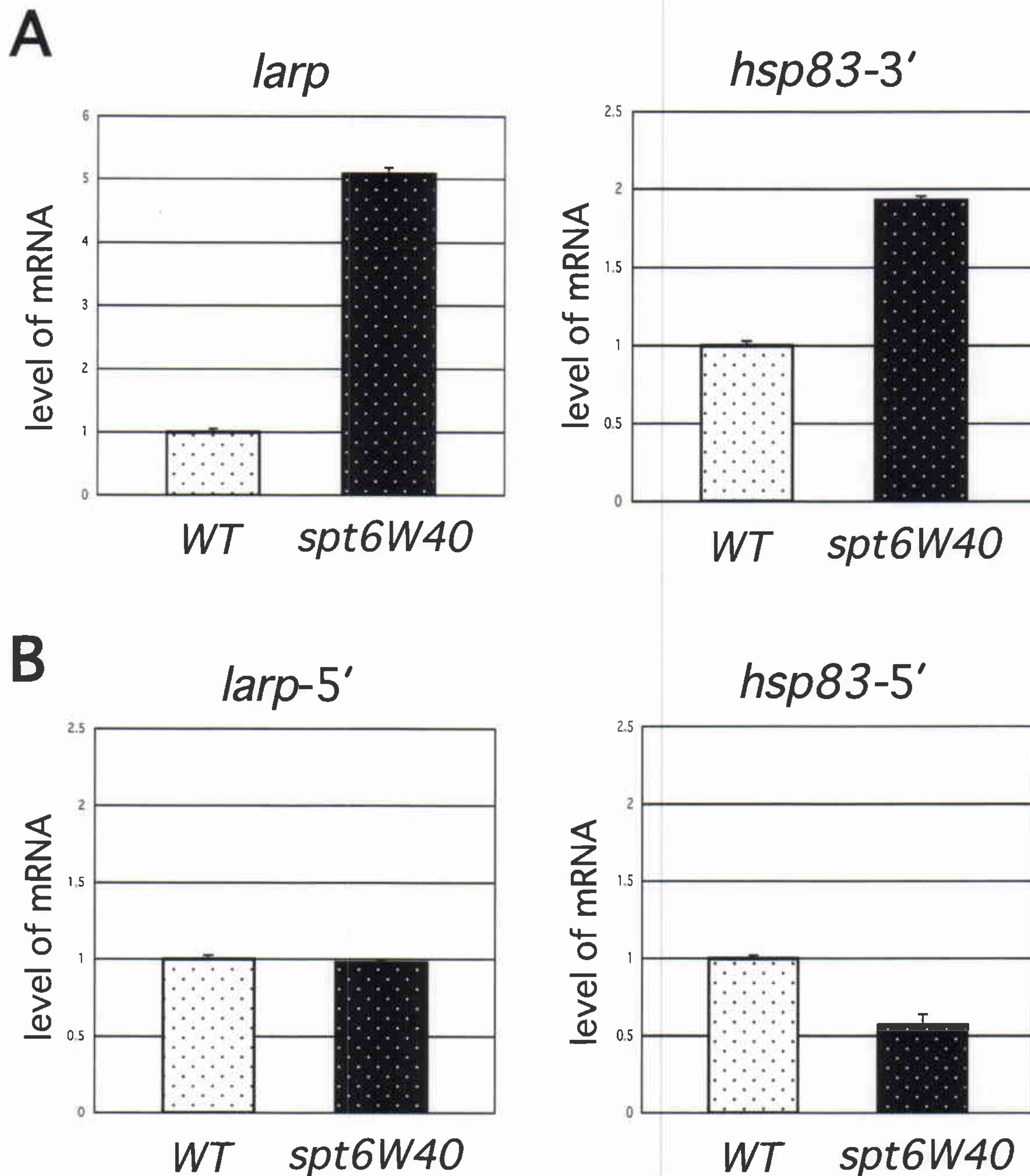


Figure 6 Transcription may initiate from the middle of the coding region in the *spt6* mutant

A; RT-PCR analyses with RNA from embryos were carried out on *larp* and *hsp83*. PCR was performed with the primers synthesized for 3' regions of *larp* and *hsp83* mRNA (Figure 3-a). Level of mRNA indicates the ratio of the amount of target mRNA to that of control *β-tubulin* mRNA. The level of *WT mRNA* was set to 1. The levels of *larp* and *hsp83* mRNA were increased in the *spt6* mutant.
B; RT-PCR analyses with the primers synthesized for 5' regions of *larp* and *hsp83* mRNA (Figure 3-a). The levels of the 5' region of each mRNA were not increased in the *spt6* mutant.

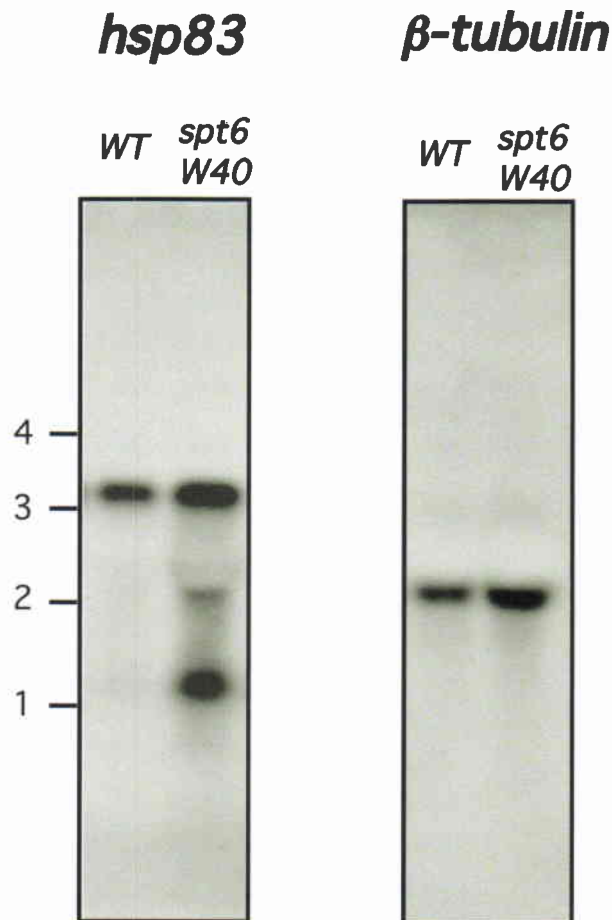


Figure 7 The shorter transcripts were detected in the *spt6* mutant

The results of the Northern blot analysis of RNA from embryos. The left panel; RNA probed *with hsp83*.The right panel; RNA probed with control *β-tubulin*. *hsp83* probes detected expected 3 kbp transcripts both in *WT* and the *spt6* mutant. The *hsp83* probes also detected shorter transcripts in the *spt6* mutant. The control *β-tubulin* probes detected expected 1.8 kbp transcripts both in *WT* and the *spt6* mutant. Any quantitative relations cannot be discussed in this experiment.

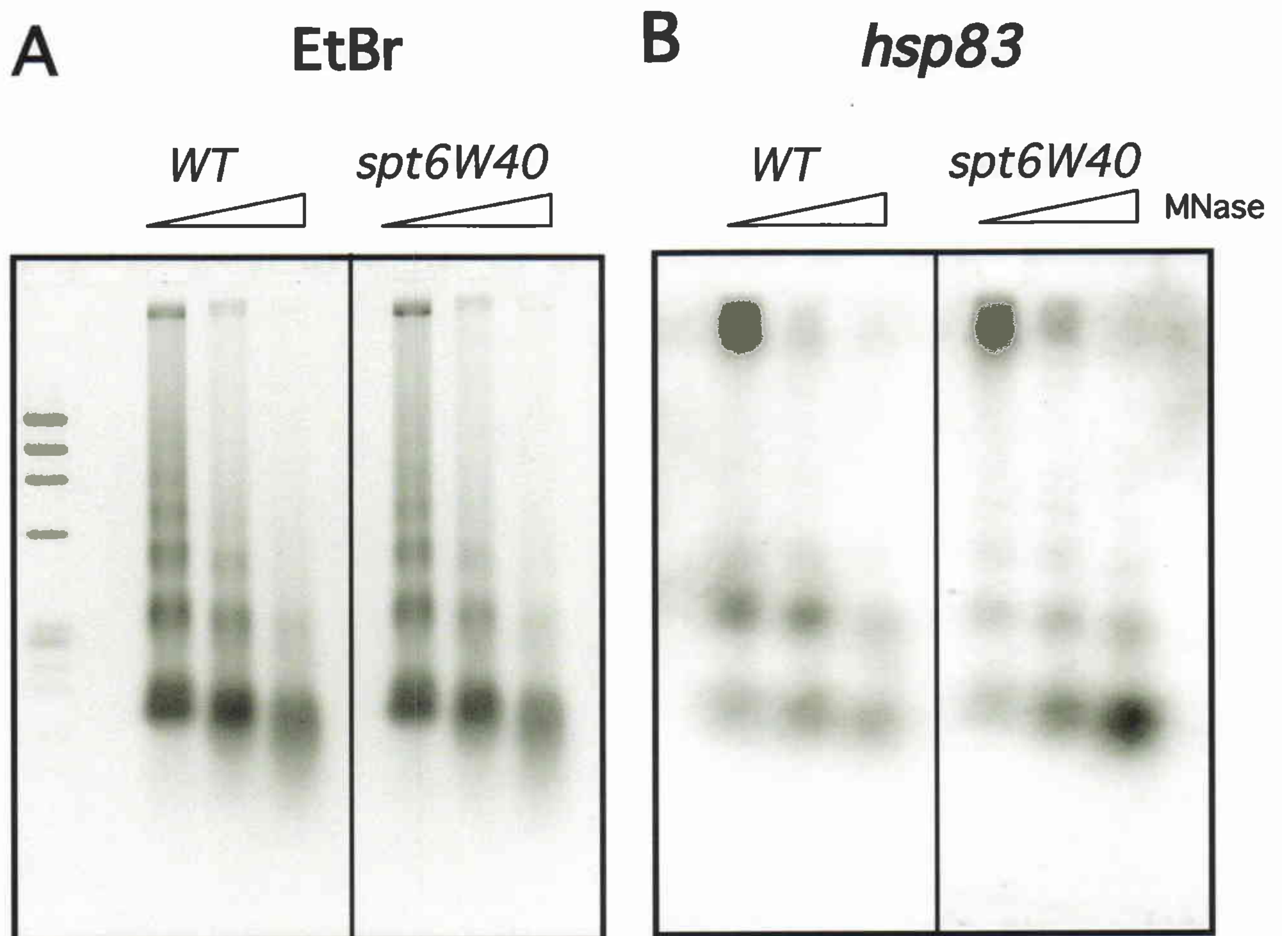


Figure 8 Chromatin structure was changed in the *spt6* mutant

The chromatin DNA from *WT* and *spt6W40* embryos was digested with increasing concentrations of MNase.

A; DNA stained with ethidium bromide.

B; DNA probed with *hsp83.*

The chromatin on *hsp83* exhibited an increased sensitivity to MNase in the *spt6* mutant .

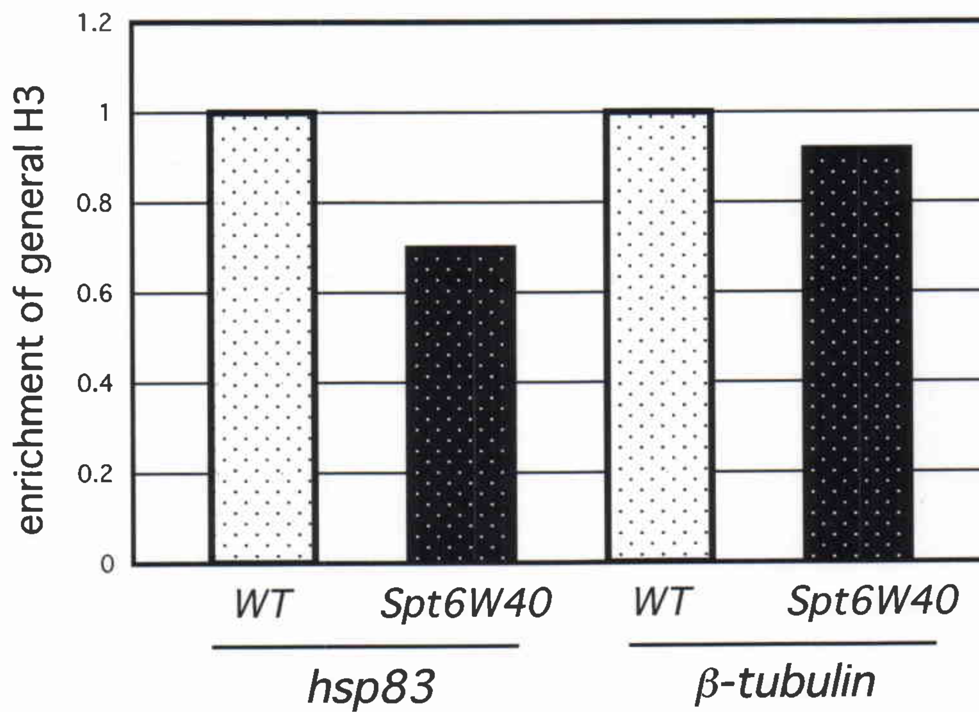


Figure 9 The level of general H3 on *hsp83* was reduced in the *spt6* mutant
The results of the ChIP analysis with pan-H3 antibodies on *hsp83* and control *β-tubulin*. The bars show the average enrichment of two independent experiments. The level of H3 on *hsp83* was reduced in the *spt6* mutant.

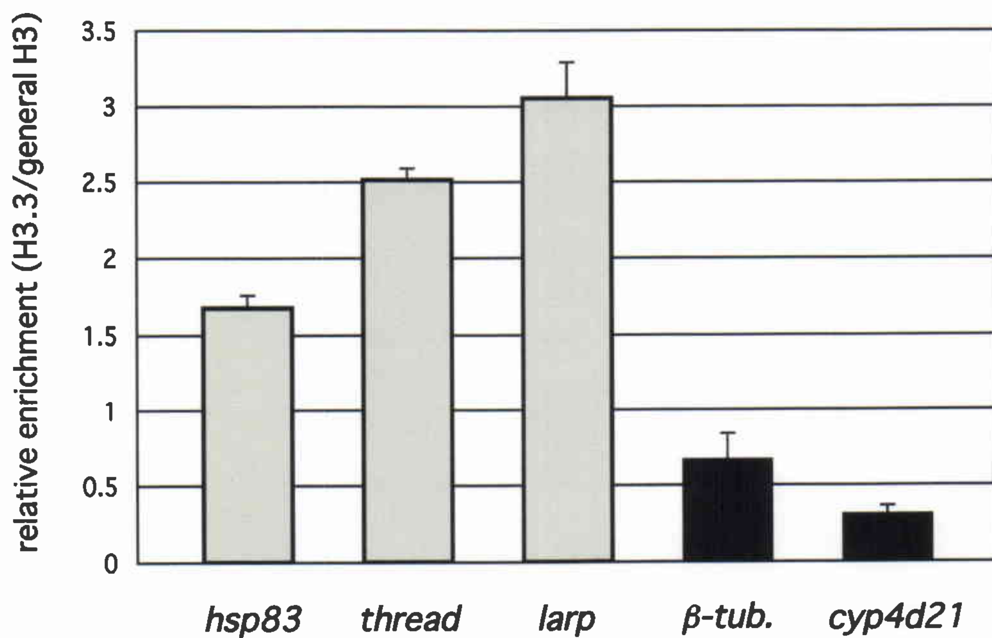


Figure 10 The levels of H3.3 were higher on Spt6-localized genes
Results of the ChIP analyses of FLAG-H3.3 line. Relative enrichment shows the ratio of H3.3 enrichment to general H3 enrichment. Grey bars show the results on Spt6-enriched genes. Black bars show the results on control genes. Error bars indicate standard deviations from three independent experiments. The levels of H3.3 were higher on Spt6-enriched genes.

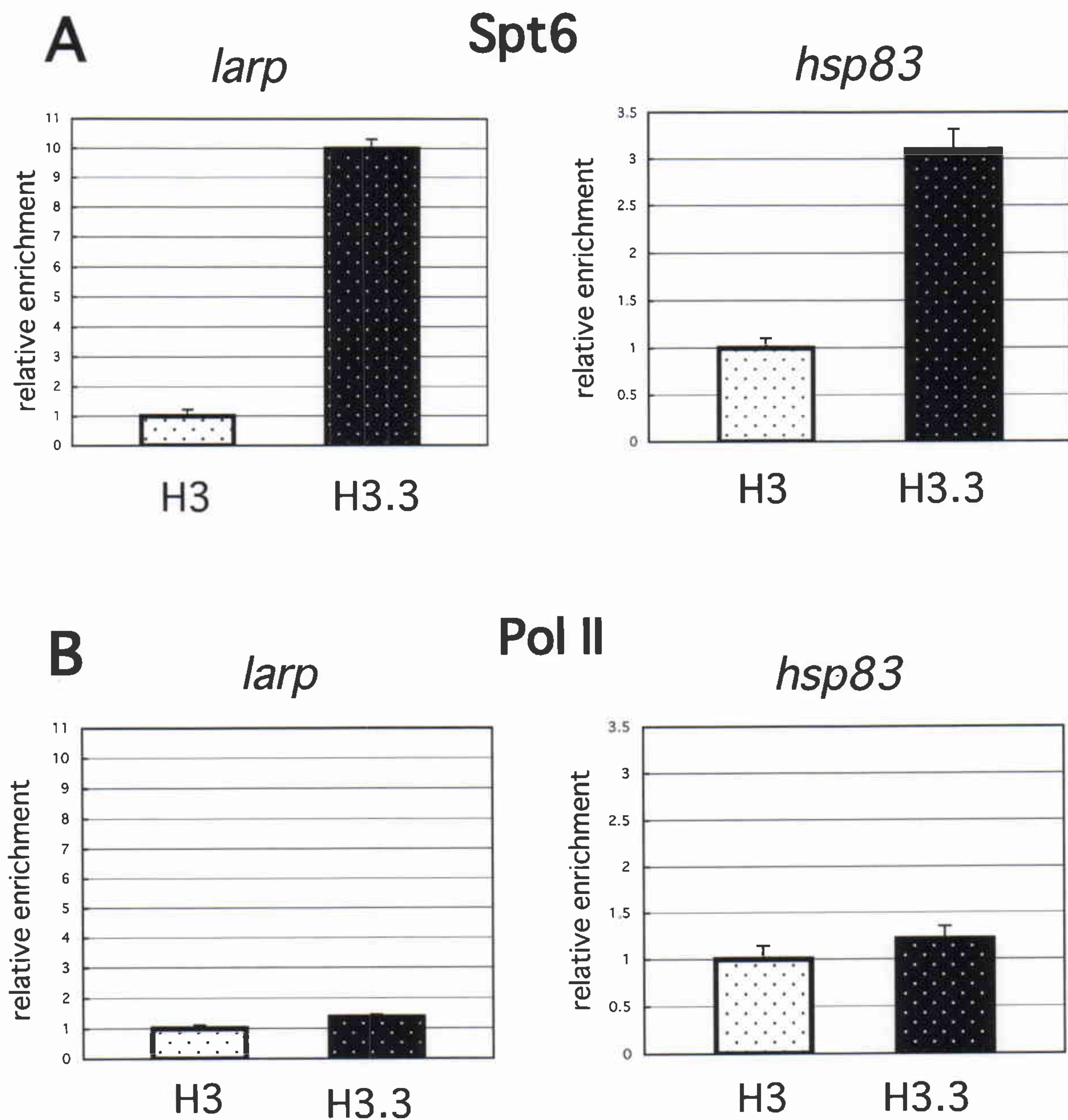


Figure 11 Spt6 localizes on nucleosomes containing H3.3 rather than H3

A; Re-ChIP assay with Spt6 antibodies.

B; Re-ChIP assay with Pol II antibodies (8WG16).

The left panels show the results on *larp.* The right panels show the results on *hsp83*. The relative enrichment of H3 was set to 1. Spt6 was efficiently immunoprecipitated with the nucleosomes containing H3.3 rather than H3. Pol II was immunoprecipitated with the nucleosomes containing H3.3 and H3 at the same extent.

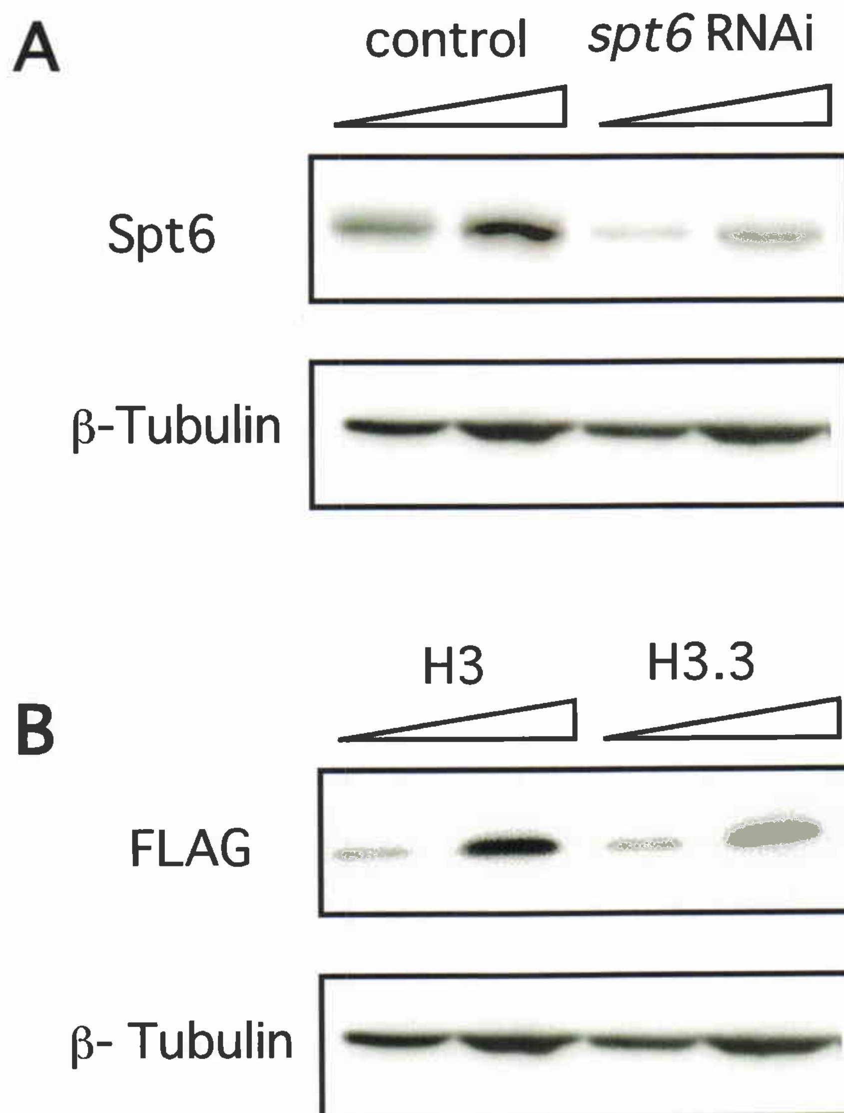


Figure 12 Western blot analyses of Spt6 and FLAG-histone

A; Spt6 was reduced by the *spt6* RNAi.

Western blot analysis with Spt6 antibodies showed that the level of Spt6 in the pupal stage was reduced by the *spt6* RNAi. β-Tubulin is a control.

B; The expression levels of FLAG-H3 and FLAG-H3.3 are the same.

Western blot analysis with FLAG antibodies showed that the expression levels of exogenous FLAG-H3 and FLAG-H3.3 were almost the same in these transgenic flies.

A Over-expression of histone in control

	OR	*H3.1*		*H3.3*		*GFP*
	+; *+*	*+;* *H3.1①*	*+;* *H3.1②*	*+;* *H3.3①*	*+;* *H3.3②*	*+;* *GFP*
Ay-GAL4	348	358	322	389	358	323
TM6B	166	167	155	185	170	150
viability	2.10	2.14	2.08	2.10	2.11	2.15

Viability = Ay-GAL4(Tb+)/TM6B(Tb-)

B Over-expression of histone in *spt6* RNAi

	RNAi	*RNAi/H3.1*		*RNAi/H3.3*		*RNAi/GFP*
	+; *+*	*+;* *H3.1①*	*+;* *H3.1②*	*+;* *H3.3①*	*+;* *H3.3②*	*+;* *GFP*
Ay-GAL4	32	57	40	149	130	31
TM6B	415	455	325	320	390	361
viability	0.0771	0.125	0.123	**0.476**	**0.333**	0.0859

Viability = Ay-GAL4(Tb+)/TM6B(Tb-)

Table 2 The lethal phenotype of *spt6* knockdown was suppressed by the over-expression of FLAG-H3.3.

A; Over-expression of histone in control.

B; Over-expression of histone in the *spt6* RNAi.

TM6B is a control. The number of pupae was counted separating Ay-GAL4 from TM6B using a *Tb* marker. Over expression of histones in control did not affect the viability of flies. The viability was significantly increased by the over-expression of FLAG-H3.3 in the *spt6* RNAi.

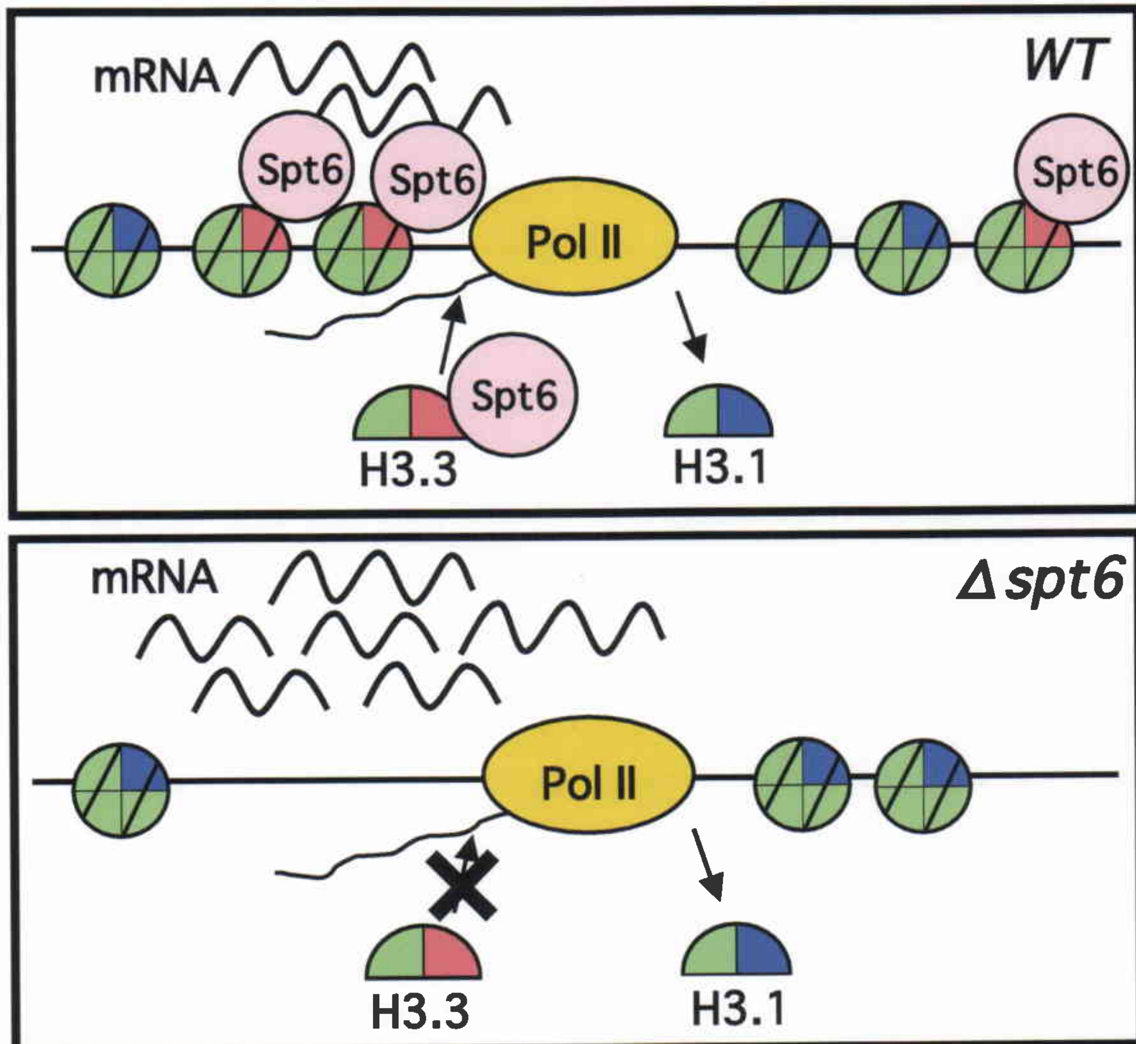


Figure 13 The possible model for the function of Spt6 in vivo
Spt6 might maintain chromatin structure during transcription through the deposition of H3.3 to keep the proper gene expression. In the *spt6* mutant histone loss and disruption of chromatin structure may cause an increase of aberrant transcripts.

Acknowledgements

I express my gratitude to S. Hirose for his supports, guidance and valuable discussions during the course of this study. I thank Y. Hiromi, R. Ueda, H. Sasaki and T. Kakutani for advice and discussions. I also thank all the members of Hirose's laboratory and Hiromi's laboratory for helpful discussions. I am grateful to R. Ueda for gifts of *spt6* RNAi lines, to T. Nakayama for gifts of FLAG-H3 and FLAG-H3.3 transgenic flies and to A. Vincent for gifts of *spt6* mutant (*PL83*).

References

Ahmad, K. and Henikoff, S. 2002a The histone variant H3.3 markes active chromatin by replication-independent nucleosome assembly. *Mol. Cell* 9: 1191-1200

Ahmad, K. and Henikoff, S. 2002b Histone H3 variants specify modes of chromatin assembly. *Proc. Natl. Acad. Sci.* 99: 16477-16484

Andrulis, E.D., Guzman, E., Doring, P., Werner, J., and Lis, J.T. 2000. High-resolution localization of *Drosophila* Spt5 and Spt6 at heat shock genes in vivo: roles in promoter proximal pausing and transcription. *Genes & Dev.* 14: 2635-2649

Andrulis, E.D., Werner, J., Nazarian, A., Erdjument-Bromage, H., Tempst, P. and Lis, J.T. 2002. The RNA processing exosome is linked to elongating RNA polymerase II in Drosophila. *Nature* 420: 837-841

Baxevanis,A.D., Godfrey, J.E. and Moudrianakis, E.N. 1991. Associative behavior of the histone (H3-H4)2 tetramer: dependence on ionic environment. *Biochemistry* 30: 8817-8823

Bentley, D.L. 2005. Rules of engagement: co-transcriptional recruitment of pre-mRNA processing factors. *Curr. Opin. Cell Biol.* 17: 251-256

Bonnefoy, E., Orsi, G.A., Couble, P. and Loppin, B. 2007. The essential role of Drosophila HIRA for de novo assembly of paternal chromatin at fertilization. *PLoS Genet.* 3: 1991-2006

Bortvin, A. and Winston, F. 1996. Evidence that Spt6p controls chromatin structure by direct interaction with histones. *Science* 272: 1473-1476

Bourbon, H.M., Gonzy-Treboul, G., Peronnet, F., Alin, M.F., Ardourel, C., Benassayag, C., Cribbs, D., Dutsch, J., Ferrer, P., Haenlin, M., Lepesant, J.A., Noselli, S., and Vincent, A. 2002. A P-insertion screen identifying novel X-linked essential genes in Drosophila. *Mech. Dev.* 110: 71-83

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., ZuckerJ.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., Gifford, D.K., Melton, D.A., Jaenisch, R., and Young, R.A. 2005. Core transcriptional regulatory circuitry in human Embryonic Stem cells. *Cell* 122: 947-956

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118: 401-415

Chang, C.H. and Luse, D.S. 1997. The H3/H4 tetramer blocks transcript elongation by RNA polymerase II in vitro. *J. Biol. Chem.* 272: 23427-23434

Endoh, M., Zhu, W., Hasegawa, J., Watanabe, H., Kim, D.K., Aida, M., Inukai, N., Narita, T., Yamada, T., Furuya, A. et al. 2004. Human Spt6 stimulates transcription elongation by RNA polymerase II in vitro. *Mol. Cell. Biol.* 24: 3324-3336

Fuchs, T.M., Deppisch, H., Scarlato, V. and Gross, R. 1996. A new gene locus of *Bordetella pertussis* defines a novel family of prokaryotic transcriptional accessory proteins. *J. Bacteriol.* 178: 4445-4452

Hake, S.B., Garcia, B.A., Duncan, E.M., Kauer, M., Dellaire, G., Shabanowitz, J., Bazett-Jones, D.P., Allis, C.D. and Hunt, D.F. 2006. *J. Biol. Chem.* 281: 559-568

Hughes, T.R., Marton, M.J., Jones, A.R., Roberts, C.J., Stoughton, R., Armour, C.D.,

Bennett, H.A., Coffey, E., Dai, H., and He, Y.D., et al. 2000. Functional discovery via a compendium of expression profiles. *Cell* 102: 109-126

Izban, M.G. and Luse, D.S. 1991. Transcription on nucleosomal templates by RNA polymerase II in vitro: Inhibition of elongation with enhancement of sequence-specific pausing. *Genes & Dev.* 5: 683-696

Jin, C. and Felsenfeld, G. 2007. Nucleosome stability mediated by histone variants H3.3 and H2A.Z. *Genes & Dev.* 21: 1519-1529

Kaplan, C.D., Morris, J.R., Wu, C., and Winston, F. 2000. Spt5 and spt6 are associated with active transcription and have characteristics of general elongation factors in *D. melanogaster. Genes & Dev.* 14: 2623-2634

Kaplan, C.D., Laprede, L. and Winston, F. 2003. Transcription elongation factors repress transcription from cryptic sites. *Science* 301: 1096-1099

Lee, C.K., Shibata, Y., Rao, B., Strahl, B.D. and Lieb, J.D. 2004. Evidence for nucleosome depletion at active regulatory regions genome-wide. *Nat. Genet.* 36: 900-905

Loppin, B., Bonnefoy, E., Anselme, C., Laurencon, A., Karr, T.L. and Couble, P. 2005. The histone H3.3 chaperone HIRA is essential for chromatin assembly in the male pronucleus. *Nature* 437: 1386-1390

Lori, A.P. and Wassarman, D.A. 2002. Localizing transcription factors on chromatin by immunofluorecence. *Methods* 26: 3-9

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F. and Richmond, T.J. 1997. Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* 389: 251-260

McKittrick, E., Gafken, P.R., Ahmad, K. and Henikoff, S. 2004. Histone H3.3 is enriched in covalent modifications associated with active chromatin. *Proc. Natl. Acad. Sci. USA* 101: 1525-1530

Nakayama, T., Nishioka, K., Dong, Y.X., Shimojima, T., and Hirose, S. 2007. Drosophila GAGA factor directs histone H3.3 replacement that prevents the heterochromatin spreading. *Genes & Dev.* 21: 552-561

Pirrotta, V. 1988. Vectors for P-mediated transformation in Drosophila. *Biotechnology* 10; 437-456

Tagami, H., Ray-Gallet, D., Almouzni, G. and Nakatani, Y. 2004. Histon H3 and H3.3 complexes mediate nucleosome assembly pathways dependent or independent of DNA synthesis. *Cell* 116: 51-61

Winston, F., Chaleff, D.T., Valent, B. and Fink, G.R. 1984. Mutations affecting Ty-mediated expression of the *HIS4* gene in *Saccharomyces cerevisiae*. *Genetics* 107: 179-197

Wirbelauer, C., Bell, O. and Schubeler, D. 2005. Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active histone modifications show a promoter-proximal bias. *Genes & Dev.* 19: 1761-1766

Yoh, S.M., Cho, H., Pickle, L., Evans, R.M. and Jones, K.A. 2007. The Spt6 SH2 domain binds Ser2-P RNAPII to direct Iws1-dependent mRNA splicing and export. *Genes & Dev.* 21: 160-174