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Spt6 enhances the elongation rate of RNA polymerase II *in vivo*

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Several eukaryotic transcription factors have been shown to modulate the elongation rate of RNA polymerase II (Pol II) on naked or chromatin-reconstituted templates *in vitro*. However, none of the tested factors have been shown to directly affect the elongation rate of Pol II *in vivo*. We performed a directed RNAi knock-down (KD) screen targeting 141 candidate transcription factors and identified multiple factors, including Spt6, that alter the induced *Hsp70* transcript levels in *Drosophila* S2 cells. Spt6 is known to interact with both nucleosome structure and Pol II, and it has properties consistent with having a role in elongation. Here, ChIP assays of the first wave of Pol II after heat shock in S2 cells show that KD of Spt6 reduces the rate of Pol II elongation. Also, fluorescence recovery after photobleaching assays of GFP-Pol II in salivary gland cells show that this Spt6-dependent effect on elongation rate persists during steady-state-induced transcription, reducing the elongation rate from ~1100 to 500 bp/min. Furthermore, RNAi depletion of Spt6 reveals its broad requirement during different stages of development.

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Introduction

In eukaryotic organisms, production of mature mRNA by RNA polymerase II (Pol II) is an orchestrated, multistep process facilitated by a plethora of factors. Transcription by Pol II can be categorized into three main stages: initiation, elongation and termination (Svejstrup, 2004). Transcription elongation is increasingly emerging as a stage that contains rate-limiting steps in transcription of many metazoan genes (Guenther *et al*, 2007; Muse *et al*, 2007). During elongation, Pol II must contend with nucleosomes, the repeating units of chromatin, which act as a barrier to elongation (Izban and Luse, 1992; Workman, 2006). To facilitate the processivity and elongation rate of Pol II for transcription through nucleosomes, factors broadly known as elongation factors interact with Pol II and track through the body of the gene along with the Pol II complex (Saunders *et al*, 2006).

The *Drosophila Hsp70* heat shock (HS) gene is a well-suited gene model for studying inducible genes that are regulated at the elongation step (Lis, 2007). Prior to induction, the gene is prepared for activation with a Pol II molecule that is paused 20–40 bp downstream of the transcription start site (Gilmour and Lis, 1986; Rougvie and Lis, 1988; Rasmussen and Lis, 1993). Thermal induction results in recruitment of the P-TEFb kinase, which releases the Pol II molecule from the pause site to begin its progression into productive elongation (Lis *et al*, 2000; Boehm *et al*, 2003; Ni *et al*, 2004, 2007). Nuclear run on and *in vivo* cross-linking assays revealed that these pioneering Pol II molecules that leave the pause site transcribe at a rate of ~1.2 kb/min (O'Brien and Lis, 1993), consistent with what has been reported for other genes in other systems (Ucker and Yamamoto, 1984; Thummel *et al*, 1990). In addition, recent multiphoton microscopy (MPM) imaging data has confirmed the earlier reported elongation rate at the *Hsp70* gene (Yao *et al*, 2007).

The transcription elongation factor Spt6 has been shown to be present on the body of genes in various model systems in a transcription-dependent manner (Saunders *et al*, 2003; Kim *et al*, 2004; Kaplan *et al*, 2005; Ni *et al*, 2007). Further evidence for its role in transcription elongation come from observations that yeast Spt6 predominantly interacts with the hyperphosphorylated form of Pol II (Hartzog *et al*, 1998; Lindstrom *et al*, 2003), and that human Spt6 physically interacts with Pol II by binding to the Ser-2 phosphorylated form of CTD through its SH2 domain (Yoh *et al*, 2007). Recent results suggest that Spt6 also provides a link to processing, termination, export and RNA surveillance by physically associating with Iws1 and the components of the exosome complex (Andrulis *et al*, 2002; Krogan *et al*, 2002; Kaplan *et al*, 2005; Yoh *et al*, 2007). Spt6 also physically interacts with histones, predominantly with histone H3 in both yeast and human and possesses nucleosome assembly activity *in vitro* (Bortvin and Winston, 1996; Winkler *et al*, 2000). In yeast, a mutation in Spt6 results in the loss of normal chromatin structure and the emergence of cryptic transcription from within the open reading frame (ORF) of tested genes (Kaplan *et al*, 2003). In support of this finding, it was also shown that Spt6 facilitates reassembly of nucleosomes at inducible genes upon repression of transcription (Adkins and Tyler, 2006). Therefore, based on the available data, the *in vivo* function of Spt6 in transcription could be divided into two categories: first, reassembly of nucleosomes on the gene behind the transcribing Pol II enzyme; second, providing a link to concurrent and down-stream processing and export

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events. However, the contribution of this factor in directly facilitating the transcription elongation by Pol II *in vivo* remains largely unexplored.

To address whether a transcription factor facilitates the normal elongation rate of Pol II *in vivo*, past studies have tracked the dissociation kinetics of the 'last wave' of Pol II molecules at an engineered galactose-inducible gene after cells were shifted to a glucose-rich medium (Mason and Struhl, 2005). Surprisingly, none of the mutations in any of the elongation factors led to detectable elongation rate defect (Mason and Struhl, 2005; Schwabish and Struhl, 2006, 2007).

Using complementary molecular and imaging techniques, in this study we report that the *Drosophila* Spt6 is a *bona fide* transcription elongation factor, which positively stimulates the elongation rate of Pol II *in vivo*. We show that Spt6 is critical for normal transcription elongation at the *Hsp70* gene both shortly after HS induction, as well as later into the HS after establishment of a steady state HS response. We also note that depletion of Spt6 by RNAi results in no change or a small decrease in trimethylation of H3-K36 on the body of *Drosophila Hsp70* gene. However, as we show here, addition of this methylation mark is not critical for normal elongation rate of Pol II at *Hsp70*. We also show that Spt6 is required for maximal recruitment of two other elongation factors, Spt5 and Paf1, to the induced *Hsp70*. Furthermore, the effects of Spt6 RNAi expression during development indicate that Spt6 is critical for normal development and morphogenesis throughout the life cycle of *Drosophila*.

Results

Spt6 is required for optimal induction of HS genes in vivo

To identify factors that are required for transcription of the induced *Hsp70* gene, we carried out a functional RNAi screen on a subset of known and suspected *Drosophila* transcription factors in S2 cells. These factors were selected based on the present literature on transcription in all eukaryotic organisms. In total, we knocked down 141 potential targets implicated in different aspects of transcription and assessed the level of *Hsp70* mRNA after a 20-min HS induction (Supplementary Table I). Knock-down (KD) of 33 of these factors resulted in 33% or more decrease in the accumulation of Hsp70 transcript (Table I). One of the factors that emerged from this screen was Spt6 (Table I). Spt6 KD resulted in a 43% reduction in the level of induced *Hsp70* RNA when Spt6 protein levels were decreased 70% in comparison to that of the control RNAi-treated (bacterial β-galactosidase LacZ RNAi) cells (Figure 1A).

In earlier studies, we have reported recruitment of Spt6 to nucleosome-containing regions of *Hsp70* upon induction (Andrulis *et al*, 2000; Saunders *et al*, 2003), as well as its physical association with the components of the exosome complex (Andrulis *et al*, 2002). However, the exact mechanism through which Spt6 participates in transcription from HS genes *in vivo* remained unanswered.

Table I RNAi screen results showing factors that affect HS-induced *Hsp70* mRNA levels

Targeted factor (RNAi)	CG number	% Hsp70 mRNA (+/− error[a])	Function
Art4/CARM1	*CG5358*	65 (4)	Histone modification
Cdk7	*CG3319*	49 (1)	Kinase, initiation, elongation
Cdk8	*CG10572*	60 (1)	Coactivator
CKIIa	*CG17520*	54 (4)	Kinase
Cpsf30	*CG3642*	64 (12)	Processing, termination
CstF50	*CG2261*	56 (10)	Processing, termination
CycT	*CG6292*	20 (5)	Kinase, elongation
Elf1	*CG40228*	66 (7)	Elongation
ElonginA	*CG6755*	28 (6)	Elongation
ElonginC	*CG9291*	52 (7)	Elongation
Ell	*CG32217*	65 (16)	Elongation
ERCC3	*CG8019*	61 (3)	Initiation and elongation
Fcp1	*CG12252*	48 (3)	CTD phosphatase
GAF	*CG33261*	58 (6)	Core transcription
Gcn5	*CG4107*	66 (3)	Coactivator
HDAC3	*CG2128*	67 (2)	Histone modification
HSF	*CG5748*	5 (3)	Activator
Med15	*CG4184*	54 (8)	Coactivator
Med31	*CG1057*	60 (9)	Coactivator
NELF-D	*CG9984*	67 (16)	Elongation
Paf1	*CG2503*	66 (8)	Elongation
PARP	*CG40411*	43 (11)	Chromatin modification
pk92B	*CG4720*	61 (9)	Upstream signaling
Pros45	*CG1489*	54 (5)	Proteosome
Rrp6	*CG7292*	59 (4)	Exosome
Spt2	*CG5815*	61 (3)	Elongation, processing
Spt4	*CG12372*	61 (12)	Elongation
Spt6	*CG12225*	57 (1)	Elongation
TFIIB	*CG5193*	44 (3)	General transcription, initiation
TFIIFa	*CG10281*	39 (4)	General transcription, initiation
UAP56	*CG7269*	62 (6)	Export
UbcD6	*CG2013*	44 (8)	Histone modification
Upb8 (not)	*CG4166*	49 (1)	Coactivator

Table shows relative *Hsp70* mRNA levels after a 20-min heat shock (HS) induction.
mRNA levels are shown as percentage of untreated or control (LacZ).
[a]Error denotes the range of at least two independent experiments.

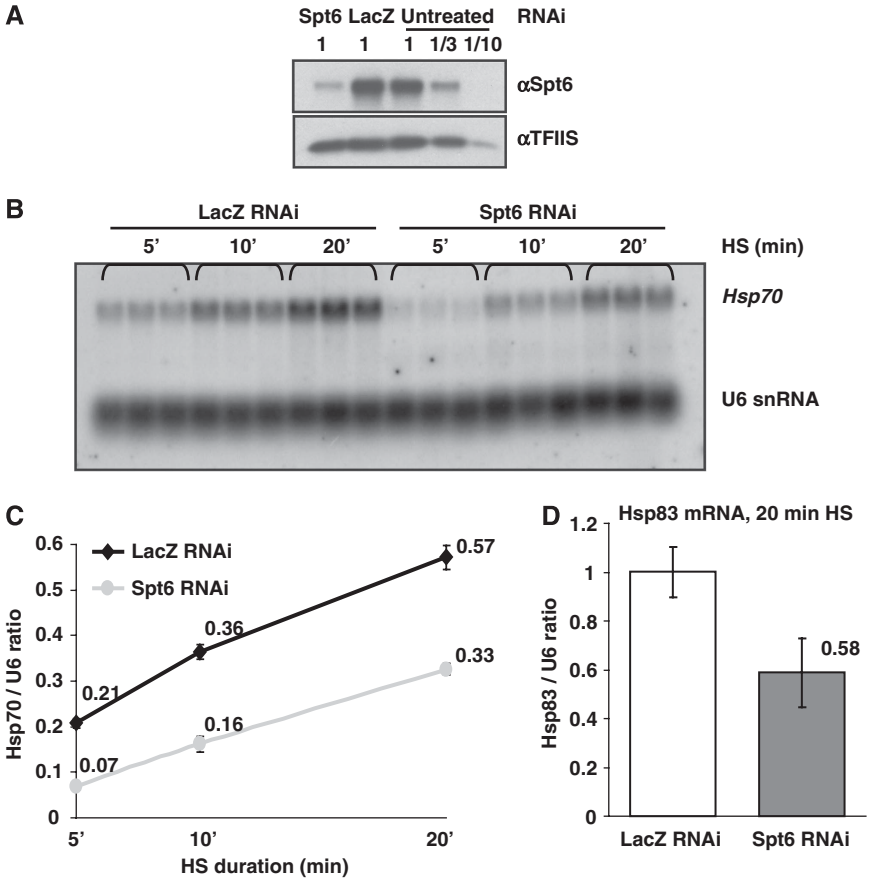

Figure 1 Spt6 RNAi decreases the accumulation of *Hsp70* and *Hsp83* mRNA upon induction. (**A**) Immunoblot on lysates from LacZ (mock treatment) and Spt6 RNAi-treated cells with Spt6 and TFIIS (loading control) antibodies. 1 corresponds to 4.0×10^5 cells. Serial dilution of the untreated extract was also loaded to quantify the efficiency of RNAi KD. (**B**) Time course analysis of *Hsp70* mRNA accumulation in RNAi-treated samples. Cells were heat shocked for the indicated time. The level of *Hsp70* transcript was analysed by northern blot. Pol III-transcribed U6 snRNA was used as an internal standard for normalization (each set represents three biological replicates). (**C**) Graph depicts the normalized values of *Hsp70* transcripts (error bars represent s.e.m.). (**D**) Levels of *Hsp83* mRNA accumulation after a 20-min HS induction as examined by northern blot analysis (error bars show standard deviation).

To gain a more mechanistic insight into how Spt6 facilitates transcription, we examined *Hsp70* transcript accumulation following a 5, 10 and 20 min instantaneous HS. The results from northern blot analysis revealed that Spt6 KD decreases the induction and accumulation of *Hsp70* mRNA (Figure 1B and C). This defect in mRNA accumulation is more prominent at earlier stages of the thermal stress (three-fold defect at 5 min compared with a two-fold decrease by 20 min). Moreover, as shown in Figure 1D, Spt6 KD also results in almost a two-fold decrease in the levels of *Hsp83* mRNA 20 min after HS induction. These results suggest that Spt6 is necessary for the optimal induction of HS genes *in vivo*. It is worth pointing out that we did not observe any cryptic transcription emanating from upstream of our probe hybridization site ($\sim +1700$) in Spt6 KD cells. This allowed us to investigate the different aspects of transcription elongation at this rapidly inducible HS gene.

Spt6 positively affects the rate of productive elongation of the 'first wave' of Pol II molecules after HS induction

Both cell growth of *spt6* mutants in the presence of the cellular nucleotide-depleting drug, 6-azauracil in yeast (Hartzog *et al*, 1998), and *in vitro* transcription assays on naked templates (Endoh *et al*, 2004; Yoh *et al*, 2007) have implicated Spt6 in transcription elongation; however, no direct analysis has been carried out to address the requirement for this factor in the transcription elongation *in vivo*. Therefore, we sought to examine the effect of Spt6 KD on the elongation rate and processivity of Pol II at transcriptionally active HS genes. This was carried out in a kinetic analysis by taking 'snapshots' of the density and distribution of Pol II, on HS genes at different time points after HS induction. To see whether Spt6 levels were reduced on the body of *Hsp70* in Spt6 RNAi cells, we performed ChIP with antibody against Spt6. Figure 2A shows that in addition to the global reduction in the levels of Spt6 (Figure 1A), Spt6 KD also decreases association of Spt6 with the body of induced *Hsp70* 18 min after HS induction. Upon transcription induction, HS genes in both yeast and Drosophila undergo rapid nucleosome disassembly (Zhao *et al*, 2005; Petesch and Lis, 2008), making the chromatin architecture of the gene more permissive for passage of Pol II. Given that Spt6 physically interacts with histones and has histone chaperone activity (Bortvin and Winston, 1996; Winkler *et al*, 2000; Adkins and Tyler, 2006), we sought to investigate the role of this factor in the transcription elongation of the first wave of RNA Pol II molecules that have to negotiate with the nucleosomal barrier during the early stages of HS activation. As shown in Figure 2B, Spt6

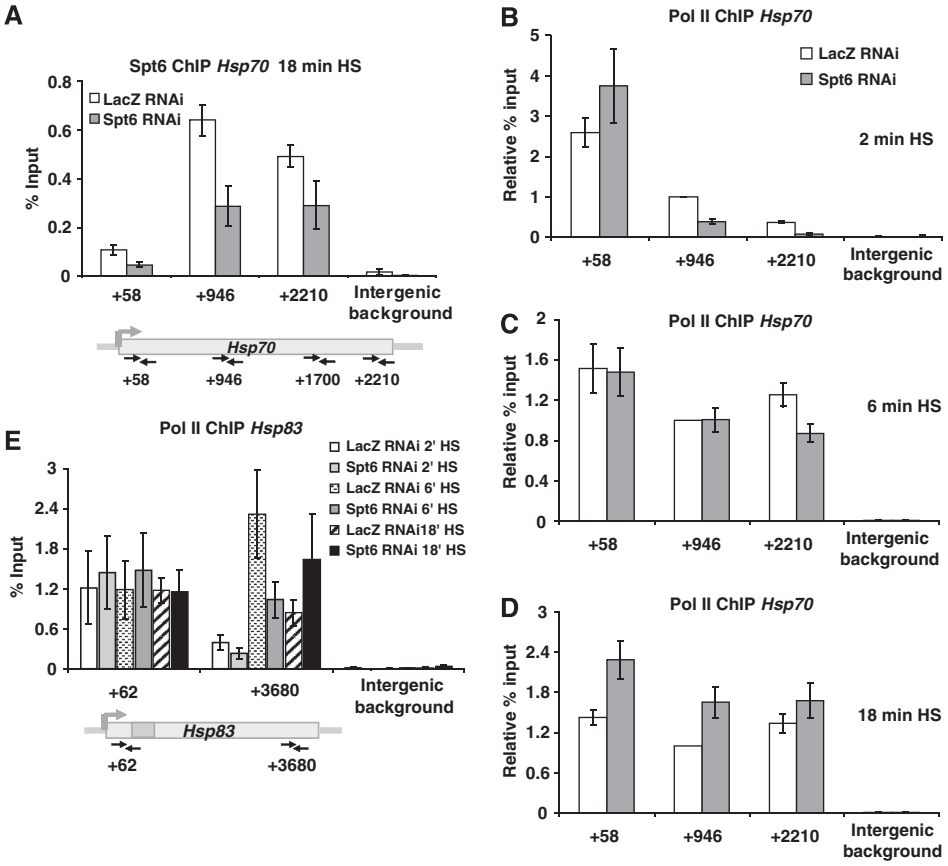

Figure 2 Depletion of Spt6 reduces the elongation rate of RNA polymerase II immediately after HS induction. ChIP results showing association of Spt6 (**A**) with different regions of the *Hsp70* transcript in both Spt6 and LacZ RNAi-treated samples. Time course analysis of Pol II (α-Rpb3) density and distribution at *Hsp70* by 2 min (**B**), 6 min (**C**), 18 min (**D**) after HS induction in Spt6 and LacZ RNAi samples. Numbers below each bar represents the position of real-time PCR primers relative to the *Hsp70* transcription start site, as depicted at the bottom of panel A. For each RNAi treatment and time point, percent inputs were normalized to the respective +946 region. (**E**) Pol II occupancy on the body of *Hsp83* at different time points after HS induction. The grey box downstream of the +62 primer denotes the relative position of *Hsp83* intron. For all experiments error bars denote s.e.m. of at least three biological replicates. The intergenic background primer pair targets a region 32 kb downstream of the last Hsp70 gene at the 87C genomic loci.

KD, followed by a short 2-min HS induction, does not negatively affect the density of Rpb3 (a subunit of Pol II) at the 5′-proximal region of *Hsp70*, a region where Spt6 is not strongly recruited after induction (Andrulis *et al*, 2000; Saunders *et al*, 2003). However, we noticed that the density of Pol II molecules gradually decreases as we survey the middle (+946) and 3′-end (+2210) of the gene relative to the LacZ KD (Figure 2B). This loss of signal suggests that in the context of chromatin *in vivo*, KD of Spt6 reduces the elongation rate of the initial Pol II molecules that enter the productive elongation mode. We also included an intermediate 6-min HS time point in our analysis when the density of Pol II and transcription factors on the body of *Hsp70* are near the peak level (Boehm *et al*, 2003). At this time point in the Spt6 RNAi cells, the density of Pol II molecules at the 5′ and middle region of the gene is comparable to that of the LacZ RNAi sample. However, the fact that Pol II molecules are not transcribing at the normal rate is noticeable, as the density of Pol II at the 3′-end (+2210) of the gene was still not restored to levels similar to that of the control cells (Figure 2C).

We next analysed the status of Rpb3 after an 18-min HS induction, when steady state transcription from the *Hsp70* gene has been established (Boehm *et al*, 2003) and did not observe any drop in Spt6 RNAi cells throughout the body of the gene (Figure 2D). In fact, we even observed higher levels of Pol II on the body of *Hsp70* with the Spt6 KD samples in comparison to LacZ dsRNA-treated cells (Figure 2D), a result expected if the transcription elongation rate decreased relative to the rate of initiation and entry of Pol II molecules into elongation.

Hsp83 is another HS gene that also recruits Spt6 (Andrulis *et al*, 2000; Kaplan *et al*, 2000). This gene is transcribed at relatively high levels before HS induction, and its expression only increases a few fold upon thermal stress (Xiao and Lis, 1989). We also observed a slower transcription elongation rate at this gene in Spt6-depleted S2 cells. Six minutes after HS induction, the time at which we expect to detect the induced Pol II molecules at the 3′-end of this gene, the Rpb3 density is about two-fold less at the +3680 region of the gene in Spt6 RNAi samples compared with LacZ RNAi-treated control (Figure 2E). Therefore, Spt6 not only facilitates elongation when a repressed gene becomes active (*Hsp70*), but it is also essential for efficient elongation rate of genes that are constitutively active and are slightly upregulated upon induction (Hsp83). The kinetics of Pol II elongation at a constitutively active *Hsp83* gene in Spt6 KD cells also suggests that this factor is critical for elongation during the steady state of transcription. These results indicate that Spt6 is required for

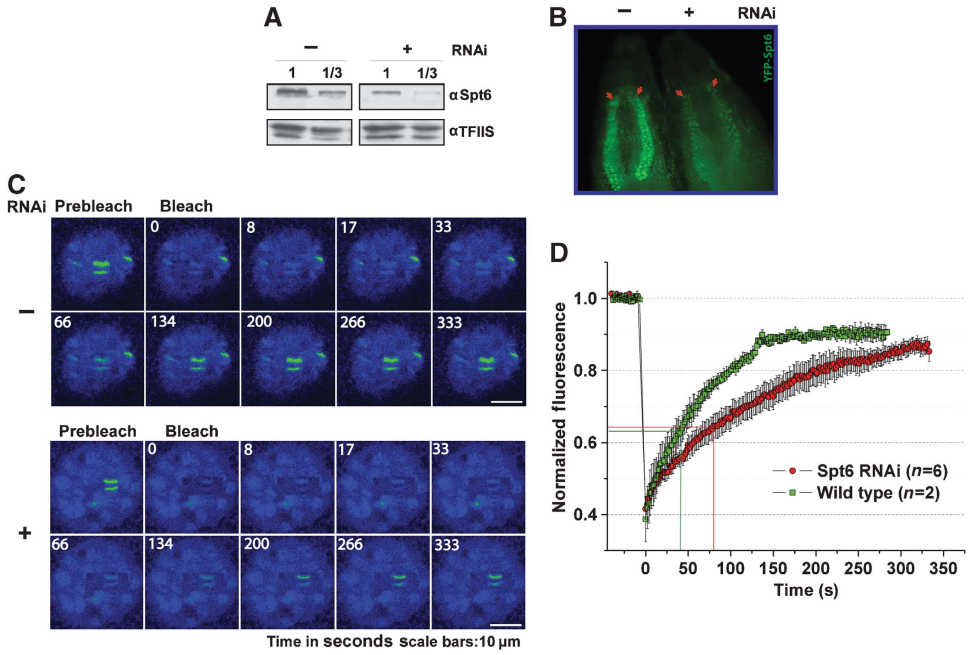

Figure 3 FRAP analysis of Pol II at 87A; 87C HS loci upon full HS activation in Spt6 RNAi and control animals. (**A**) Western blot analysis showing general KD of Spt6 in the third instar larvae of actin5C-GAL4/UAS-Spt6^{RNAi} (RNAi+) and TM6/UAS-Spt6^{RNAi} (RNAi−). TFIIS staining served as a loading control. Dilution of the lysates was also loaded to quantify the KD efficiency. (**B**) KD of Spt6 in the salivary glands of Spt6^{RNAi} animals was assessed by examining the fluorescent intensity, resulting from expression of UAS-YFP-Spt6 insert in YFP-Spt6/+; 6983-GAL4/+ (RNAi−) or YFP-Spt6/+; 6983-GAL4/UAS-Spt6^{RNAi} (RNAi+) lines. (**C**) Images of EGFP-Rpb3 at HS loci after full gene activation in control (upper panel) or RNAi+ glands (bottom panel) before and after photobleaching. Time after the start of photobleaching is shown on the upper corner of each image in seconds. Genotypes are UAS-EGFP-Rpb3/CyO; 6983-GAL4/Ubx (RNAi−) and UAS-EGFP-Rpb3/+; 6983-GAL4/UAS-Spt6^{RNAi} (RNAi+). (**D**) Normalized fluorescence intensity plots of the FRAP analysis for the 87A and 87C loci in control (green) and Spt6 RNAi (red) samples. Green and red lines denote the points from which $t_{1/2}$ is derived.

efficient transcription elongation at both the *Hsp70* and *Hsp83* genes, which each have distinct transcription regulatory profiles.

Spt6 is required for transcription elongation at normal rates during the steady state of Hsp70 transcription

After an 18-min HS, the density of Pol II molecules on the body of *Hsp70* is slightly higher all across the gene in the Spt6 RNAi cells when compared with the LacZ RNAi samples (Figure 2D). It is tempting to speculate that slower Pol II elongation rates at this stage lead to an increase in Pol II density on the body of *Hsp70*. However, as a result of saturation of the Pol II density after prolonged gene inductions, it is not possible to address whether these polymerase molecules are travelling at normal rates during the steady state of HS induction by ChIP assay.

To overcome this technical obstacle and to determine the kinetics of Pol II elongation during the steady state of transcription in the absence of Spt6, we took advantage of our recently described MPM-based fluorescence recovery after photobleaching (FRAP) technique (Yao *et al*, 2006, 2007). This technique allows us to monitor the recovery rate of fluorescent-tagged transcription factors at the distinctive HS loci 87A and 87C of living salivary gland cells. The recovery depends on the time it takes for the photobleached elongating Pol II to complete transcription and be replaced with unbleached GFP-Pol II. We generated a KD UAS-Spt6^{RNAi} fly line that transcribes a 670 bp Spt6-dsRNA and compared the recovery rate (elongation rate) of EGFP-Rpb3 molecules at the HS loci in the salivary gland of these larvae with that of the control animals. We tested the effectiveness of the Spt6 RNAi construct in the third instar larvae by crossing the UAS-Spt6^{RNAi} line to a line that ubiquitously and constitutively expresses GAL4 under the control of the Actin5C promoter (Figure 3A). Moreover, Spt6 KD in the salivary glands of these flies was also confirmed by comparing the fluorescence intensity of transgenically expressed YFP-Spt6 molecules in control and Spt6^{RNAi} animals (Figure 3B).

By 10 min after HS induction, high levels of EGFP-Rpb3 molecules localize to the two adjacent 87A and 87C loci, making the two HS puffs easily distinguishable from other genomic loci (Yao *et al*, 2006) (Figure 3C, prebleach). In addition, 10 min after HS activation, the native *Hsp70* genes are at the steady state level of transcription (Yao *et al*, 2007). We photobleached these loci and monitored the recovery of EGFP-Rpb3 fluorescent signal for >5 min in both wild-type and Spt6^{RNAi} salivary glands (Figure 3C). Interestingly, FRAP results revealed that the recovery rate of EGFP-Rpb3 in the Spt6^{RNAi} glands is almost twice as slow as wild-type glands ($t_{1/2}$ of ~80 s compared with $t_{1/2}$ of ~41 s, Figure 3D). The EGFP-Rpb3 signal reaches plateau in Spt6 RNAi glands almost ~300 s after photobleaching (Figure 3D). Based on this, we estimate the rate of transcription by Pol II at the 2.4 kb *Hsp70* to be about 480 bp/min in the Spt6-depleted glands, which is more than two-fold slower than the elongation rate in control glands (~1100 bp/min).

The results from this FRAP experiment led us to draw two major conclusions: first, in addition to having an important function in facilitating productive transcription elongation during the initial stage of HS induction, Spt6 is also required

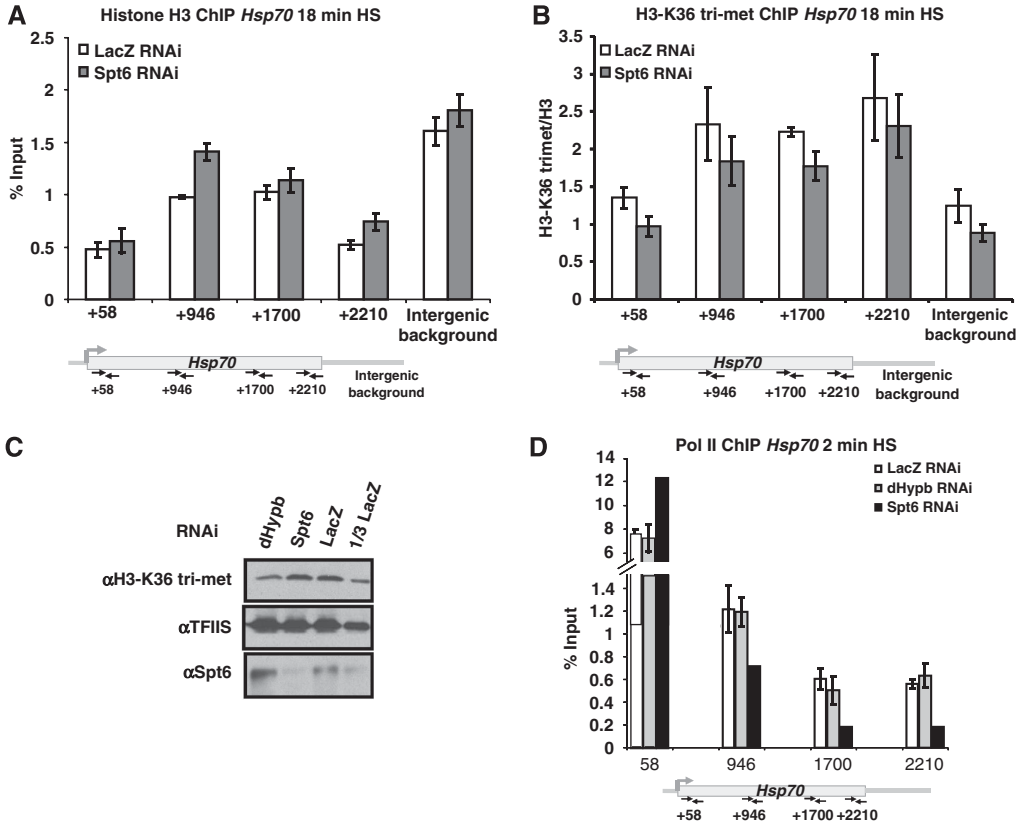

Figure 4 H3-K36 trimethylation does not positively stimulate the elongation rate of Pol II at *Hsp70*. (**A**) ChIP experiment showing the density of histone H3 throughout the body of *Hsp70* 18 min after HS induction in LacZ (white) and Spt6 (gray) RNAi-treated cells ($n \geqslant 3$, error bars denote s.e.m.). (**B**) H3-K36 trimethylation levels at different regions of *Hsp70* 18 min after HS were normalized to histone H3 density at the respective regions in LacZ (white) and Spt6 (gray) RNAi samples ($n = 3$, error bars denote s.e.m.). (**C**) Western blot analysis on lysates from LacZ, Spt6 and dHypb RNAi-treated cells. Samples were probed for H3-K36 trimethylation mark. TFIIS antibody was used as a loading control. (**D**) ChIP analysis showing the first wave of Pol II molecules traversing through *Hsp70* 2 min after HS induction in LacZ (white), dHypb (gray) and Spt6 (dark gray) RNAi-treated cells ($n = 2$ for LacZ and dHypb, error bars denote range; $n = 1$ for Spt6). Spt6 RNAi was included as a positive control for elongation rate defects. Results from this single experiment are consistent with what we have described in Figure 2B.

for the transcription elongation at normal rates during the steady state of transcription from *Hsp70* at which time the nucleosome architecture of *Hsp70* is in a more permissive transcriptional state (Petesch and Lis, 2008). Second, the role of Spt6 in stimulating the transcription elongation could be shown beyond cultured cells in living animal tissue.

Histone H3-K36 trimethylation does not detectably stimulate Pol II elongation rate

To provide a possible mechanistic explanation for how Spt6 stimulates the elongation rate of transcription, we first checked the density of histone H3 over *Hsp70* in S2 cells. In yeast, mutation in Spt6 results in a decrease in the density of histones on the body of transcriptionally active genes, exposing the cryptic transcription initiation sites within the gene (Kaplan *et al*, 2003). Interestingly, and in contrast to what has been reported in yeast, we noted that Spt6 RNAi treatments produced no change or a small increase in the density of histone H3 on segments from the body of the induced *Hsp70* (Figure 4A).

The body of actively transcribed genes show enrichment of histone H3-K36 trimethylation, and the extent of this modification correlates with the level of transcription (Li *et al*, 2007). Earlier studies have shown that Spt6 is required for H3-K36 methylation of nucleosomes at actively transcribed genes as well as H3-K36 trimethylation of chromatin in *in vitro* assays (Carrozza *et al*, 2005; Youdell *et al*, 2008). We examined the status of H3-K36 trimethylation at *Hsp70* and observed no change or a small decrease in the density of H3-K36 trimethylation on the body of *Hsp70* during the steady state of HS induction in Spt6 KD cells (Figure 4B). Nevertheless, to see whether trimethylation of H3-K36, a mark that is associated with the elongation step at actively transcribed genes (Li *et al*, 2007), is essential for normal elongation, we knocked down the *Drosophila* H3-K36 trimethyltransferase, Hypb (dHypb) (Bell *et al*, 2007), and measured the elongation rate of Pol II early after HS induction at *Hsp70*. The KD of dHypb results in 60% or more decrease in the cellular level of H3-K36 trimethylation (Figure 4C), as well as ~70% decrease in the H3-K36 trimethylation mark on the body of *Hsp70* (Supplementary Figure 1). However, we did not observe any decrease in the elongation rate of Pol II molecules at *Hsp70* as detected by ChIP 2 min after HS induction (Figure 4D). These results suggest that stimulation of elongation rate at *Hsp70* by Spt6 is not mediated through H3-K36 methylation and that trimethylation of H3-K36 by dHypb is not critical for efficient transcription elongation at *Hsp70*.

Spt6 KD leads to accelerated termination at transcriptionally active HS genes

Although the density of Pol II throughout the body of the HS genes in the Spt6 RNAi cells is comparable to or even

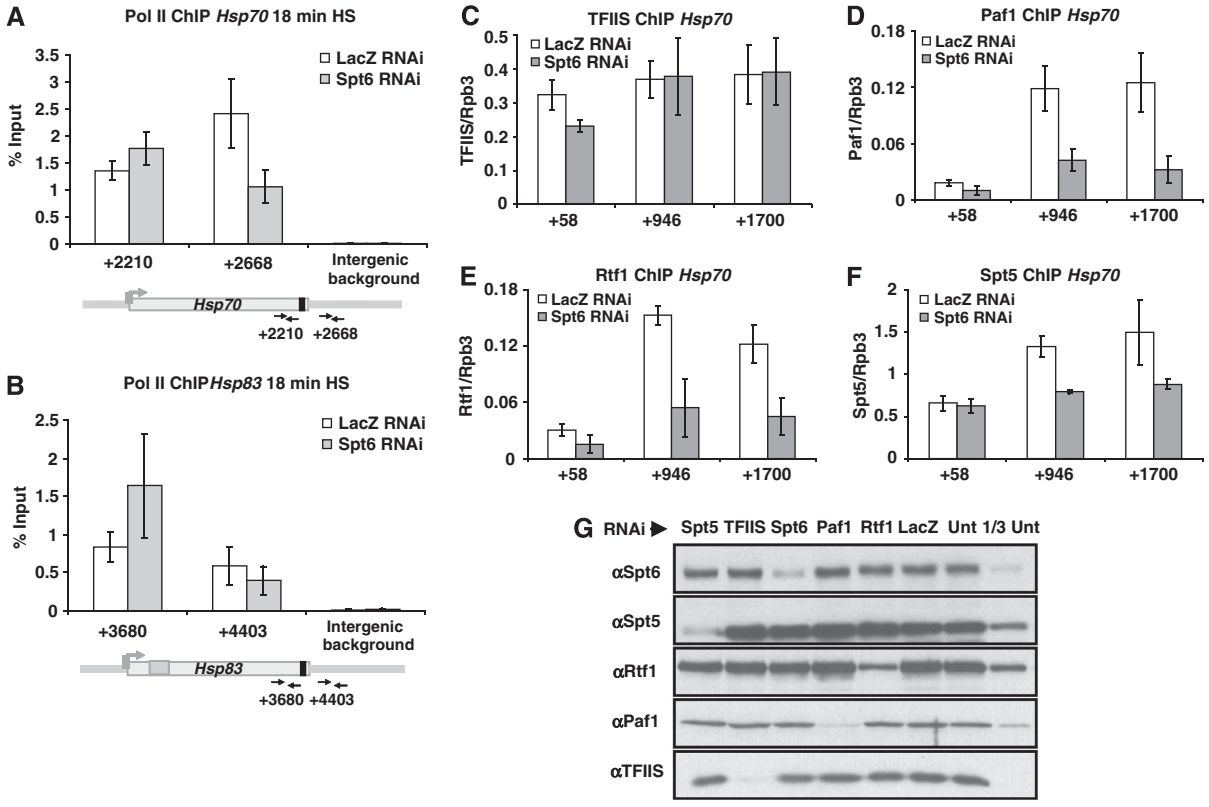

Figure 5 Spt6 is critical for normal transcription termination and maximal recruitment of Paf1, Rtf1 and Spt5 to the Hsp70 gene. (**A**) Rpb3 (Pol II) density upstream (+2210) and downstream (+2668) of the polyadenylation signal in Spt6 and LacZ RNAi samples 18 min after HS induction ($n=4$, error bars denote s.e.m.). The black box between the two primer pair regions represents the relative position of the polyadenylation signal. (**B**) Same experiment as in A showing the Hsp83 gene ($n=3$, error bars denote s.e.m.). (**C**–**F**) Association of TFIIS (C), Paf1 (D), Rtf1 (E) and Spt5 (F) with different regions of the *Hsp70* gene 18 min after full HS activation. The values on *x*-axis show the centre of primer pairs used in the real-time PCR experiment. The *y*-axis refers to the percent inputs values for each factor normalized to the level of Pol II (Rpb3) present at the same region (error bars denote s.e.m. of three independent experiments). (**G**) Cellular levels of each of the indicated proteins in Spt6, LacZ RNAi treated and untreated samples. Other RNAi treatments were included to show the specificity of antibody for each experiment.

higher than LacZ RNAi cells during the steady state of transcription (Figure 2D and E), we noticed that the density of Pol II molecules sharply drops immediately after the polyadenylation signal (poly(A)) in the Spt6 RNAi-treated cells 18 min after HS activation (Figure 5A and B). This reduction in the density of Pol II downstream of the poly(A) site during the steady state of HS induction was observed at both *Hsp70* and *Hsp83* genes and is reminiscent of recently described results in which Spt6 mutation or depletion leads to decreased read-through transcription at two tandem genes in yeast and decreased utilization of downstream polyadenylation sites (Kaplan *et al*, 2005; Yoh *et al*, 2007).

Stimulation of Pol II elongation rate by Spt6 is not mediated through TFIIS

Recent work has revealed that depletion of *Drosophila* TFIIS leads to reduction in recruitment of Pol II and defects in the elongation rate of polymerase, plausibly through Pol II backtracking and arrest early after HS induction (Adelman *et al*, 2005). We sought to test whether the elongation rate defect that we observe immediately after HS induction in Spt6 KD cells is due to a defect in recruitment of TFIIS. Apart from a 25% decrease in the density of TFIIS at the +58 region of *Hsp70* in the Spt6 depleted cells, we observed no difference in the density of TFIIS on the body of *Hsp70* in Spt6 RNAi-treated cells in comparison to LacZ RNAi cells (Figure 5C). Although TFIIS KD decreases the density of paused Pol II before induction, it also results in a larger drop in the density of Pol II at the 3′-end of the gene 2 min after HS induction (Adelman *et al*, 2005). Here we show that this gradual drop in the density of Pol II on the body of the gene 2 min after HS induction in Spt6 RNAi cells is not an indirect consequence of the deficiency in recruitment of TFIIS (Figure 5C).

Spt6 is required for maximal recruitment of Paf1 and Spt5 to transcriptionally active Hsp70

In yeast, Spt6 is required for association of Ctr9, a component of the Paf1 complex, with the body of transcriptionally active galactose-inducible genes (Kaplan *et al*, 2005). We have tested the requirement of Spt6 for association of Paf1 with transcriptionally active genes in a metazoan organism. Spt6 RNAi results in a similar reduction in association of Paf1 with the nucleosome-containing regions (+946 and +1700) of activated *Hsp70* (Figure 5D). The *Drosophila* Rtf1 is another transcription elongation factor, which unlike the yeast ortholog, does not appear to be a stable component of the Paf1 complex (Adelman *et al*, 2006). However, this factor requires Paf1 for association with transcriptionally active HS genes

(Mueller *et al*, 2004; Adelman *et al*, 2006). We find that Spt6 RNAi also leads to a significant decrease in association of this factor with the nucleosome-occupied regions (+946 and +1700) of *Hsp70* (Figure 5E). This defect in recruitment of Rtf1 is possibly an indirect consequence of Paf1 absence from the coding region of the *Hsp70* gene.

We have observed that Spt5 KD by RNAi results in accelerated dissociation of Pol II molecules from downstream of the poly(A) signal site at *Hsp70* (unpublished data). Similar observations have been reported in yeast, in which yeast strains mutant for Spt5 preferentially use the upstream poly(A) signals (Cui and Denis, 2003), or that Spt5 prevents premature RNA dissociation from the transcription machinery at the terminator sequences *in vitro* (Bourgeois *et al*, 2002). To test whether the accelerated dissociation of Pol II downstream of the poly(A) signal site that we detect at *Hsp70* in Spt6 RNAi cells is a consequence of defect in association of Spt5 with active *Hsp70*, we also quantified the levels of Spt5 by ChIP during the steady state of HS induction. Unlike Spt6, Spt5 is strongly present at the 5′-proximal region of *Hsp70* ORF both before and after HS activation (Saunders *et al*, 2003). Interestingly, although the density of Spt5 is comparable in both Spt6 and LacZ RNAi samples at the 5′-proximal region of the *Hsp70* gene during the steady state of HS activation, a modest decrease in association of Spt5 with the middle and 3′-end (+946 and +1700) of the *Hsp70* gene was observed in Spt6 RNAi samples (Figure 5F). Therefore, it is possible that the accelerated dissociation of Pol II downstream of the Poly(A) signal that we observe in Spt6 RNAi is partly due to a decrease in the association of Spt5 with the Pol II transcription machinery. We also note that Spt6 RNAi does not lead to a noticeable global decrease in the cellular levels of the tested elongation factors (Figure 5G), suggesting that the lower levels of Paf1, Rtf1 and Spt5 on the body of *Hsp70* are due to recruitment defects.

Spt6 is critical for normal fly development throughout the life cycle

Generation of Spt6 mutant flies by P-element transformation results in lethality at the embryonic stage (Peter *et al*, 2002), highlighting the requirement of this transcription factor for proper development during this stage. Here, we report that requirement for Spt6 during *Drosophila* development is not limited to the embryonic stage (Figure 6A). We observed that constitutively and ubiquitously driven expression of the Spt6 RNAi by the tubulin GAL4-driver line causes lethality during the larval stage. Moreover, transcription of Spt6 dsRNA from UAS-Spt6^{RNAi} under the control of the 6983 GAL4-driver line, which has a broad expression pattern during the pupal stage, leads to defects in abdominal cuticle deposition and trichome (hair) formation during metamorphosis (Figure 6B). The majority of these flies also fail to eclose and die as pharate adults (98.3% lethality in Spt6 RNAi compared with 1.2% lethality in parental line, $n>1000$). These data summarized in a flow chart (Figure 6A) show the critical role that Spt6 plays during different developmental stages of *Drosophila* life cycle, presumably as a consequence of Spt6 being required for regulation of transcription from many genes throughout development.

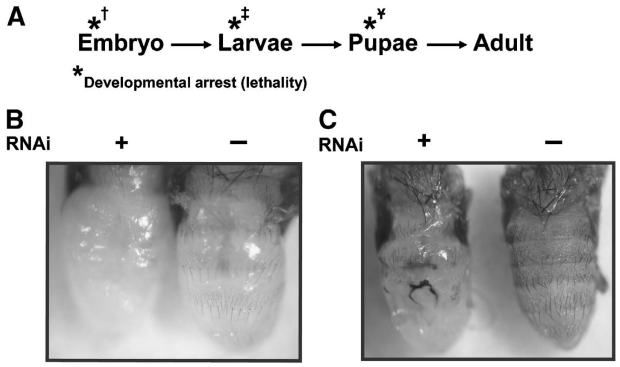

Figure 6 Spt6 is critical for normal fly development throughout the lifecycle. (**A**) A chart depicting requirement of Spt6 during different stages of development. †Results from Peter *et al* (2002) revealed that P-element insertion at the Spt6 gene leads to lethality at the embryonic stage. ‡Determined by driving expression of UAS-Spt6^{RNAi} with the robustly and ubiquitously expressed Tubulin-GAL4 driver line. ¥Crossing the UAS-Spt6^{RNAi} to the 6983-GAL4 line, which has a broad expression pattern during the pupal stage causes lethality as pharate adults. (**B**) Expression of UAS-Spt6^{RNAi} dsRNA during the pupal stage by the 6983-GAL4 driver results in lethality as pharate adults with aberrant abdominal development. Notably defects in cuticle deposition, hair development at around 80 h after puparium formation (APF). (**C**) Same as in B, but at later developmental stage before eclosion (about 96 APF). What appear to be melanotic lesions were also visible in the abdominal region of the Spt6 RNAi animals at a high frequency.

Discussion

Many factors including the FACT, DSIF, Paf1 and THO/TREX complexes as well as TFIIS, IWS1 and Spt6 have been shown to be present on the body of transcriptionally active genes during the elongation stage of transcription in different model systems (Saunders *et al*, 2003; Kim *et al*, 2004; Sims *et al*, 2004). Although many of these elongation factors have been shown to stimulate the catalytic activity of Pol II *in vitro* (Wada *et al*, 1998; Gerber *et al*, 2001, 2004; Rondon *et al*, 2003, 2004; Endoh *et al*, 2004), surprisingly mutation in none of these factors has resulted in detectable elongation rate defects at an inducible gene *in vivo* (Mason and Struhl, 2005). This lack of observed defect in elongation could be due to redundancy among the transcription elongation factors or that these factors have a role only for certain genes or under particular conditions. In some cases, the effects may not be detectable with an assay that examines the rate of clearance of the last wave of Pol II after glucose repression. Using the native major *Drosophila* HS gene, *Hsp70*, in this report we show that Spt6 is a genuine transcription elongation factor that is critical for normal transcription elongation rate by Pol II *in vivo*. A combination of biochemical (ChIP) and imaging techniques (MPM-based FRAP) allows for rigorous testing of the kinetics of the transcription elongation both early after HS induction as well as during the steady state of transcription. Therefore, here we also promote the use of native *Hsp70* gene as an attractive gene system for studying the elongation properties of Pol II *in vivo*, in different elongation factor-mutant/RNAi backgrounds.

Immediately after HS induction, the chromatin landscape of the *Hsp70* gene undergoes dramatic modifications by disassembly of the canonical nucleosome structure that lead to an ~3-fold decrease in the nucleosome (micrococcal

nuclease protection) and a corresponding depletion in core histone H3 (ChIP) (Wu *et al*, 1979; Wu, 1980; Petesch and Lis, 2008). Although the initial phase of nucleosome disassembly is rapid and independent of transcription, a second phase of the nucleosome disruption appears to be contingent on the elongating Pol II machinery and, interestingly, does not lead to further loss of H3 (Petesch and Lis, 2008). We show that Spt6 is critical for Pol II to elongate at its normal rate during this first burst of transcription immediately after HS induction (Figure 2). A 'snapshot' of Pol II at the 3′-end of the *Hsp70* gene indicates that the density of Pol II is at least five-fold less in the Spt6-depleted cells at this short time point after induction. This deficiency in the transcription elongation during the early stages of HS in Spt6-depleted cells correlates nicely with the inability of these cells to efficiently perform the second stage of more complete nucleosome disruption from the body of *hsp70* as observed by MNase protection assay (Petesch and Lis, 2008). This suggests that Spt6 may be critical for this second transition in disrupting the nucleosome architecture, which does not require additional loss of histone (Petesch and Lis, 2008). In support of this idea, we observed that even during the steady state of HS induction, histone H3 density is slightly higher on the body of *Hsp70* in Spt6 KD cells (Figure 4A).

Similar to what has been observed at other genes and in other systems (Pokholok *et al*, 2005; Bell *et al*, 2007; Li *et al*, 2007), we note that the middle and 3′-end of the *Hsp70* gene are the main sites of H3-K36 trimethylation (Figure 4B) and that depletion of Spt6 results in a small decrease in the density of H3-K36 trimethylation over the *Hsp70* gene (Figure 4B). However, depletion of dHypb, the H3-K36 trimethyltransferase, does not cause any defect in the elongation rate of first wave of Pol II after HS induction. This allowed us to tease apart two different functions of Spt6: its role as a stimulator of the transcription elongation rate and its function as a factor contributing to the trimethylation of H3-K36 at actively transcribed genes.

After establishment of the HS response, the density of Pol II slightly increases within the body of *Hsp70* gene in the Spt6 KD samples (Figure 2), similar to an increase reported for Spt6 and FACT mutants in yeast (Mason and Struhl, 2003, 2005). As we have not detected any apparent cryptic transcription from upstream of the +1700 region (the region that our northern blot probe hybridizes to), it is unlikely that this increase in Pol II density emanates from cryptic initiation sites within the gene. One plausible explanation for this Pol II density increase in the Spt6 KD cells is a slower elongation rate even during the steady state of HS transcription (18 min HS). Thus, it is possible that slower movement of the transcription machinery leads to an increase in the density of Pol II at this gene, resulting in a higher density for Pol II as detected by ChIP assay 18 min after HS induction. Indeed, results showing slower movement of Pol II at steady state in the FRAP assay at the HS loci (Figure 3) support this hypothesis.

The accelerated dissociation of Pol II that we observe downstream of the polyadenylation signal at both *Hsp70* and *Hsp83* genes in Spt6 KD cells is similar to other reports in organisms as diverse as yeast and human (Kaplan *et al*, 2005; Yoh *et al*, 2007). In yeast, Spt6 mutation diminishes read-through transcription and formation of a bicistronic transcript at the tandemly arrayed GAL10 and GAL7 genes (Kaplan *et al*, 2005). Furthermore, Spt6-depleted human HeLa cells preferentially use the premature upstream polyadenylation site at an engineered HIV:LacZ reporter construct (Yoh *et al*, 2007). A slower elongation rate by Pol II might simply give the termination factors a larger window of opportunity to dismantle the transcription machinery at the 3′-end of the gene (Zhang and Gilmour, 2006). Another nonmutually exclusive explanation for this Pol II density drop at the termination site is loss of factors such as Spt5 (Figure 5F) that could potentially enhance the stability of transcribing polymerase. Spt5 has a high degree of homology to the prokaryotic elongation factor NusG, a factor that has been implicated in the stabilization of elongating Pol II (Greenblatt *et al*, 1993).

In yeast, a mutation in Spt6 decreases association of Ctr9, a component of the Paf1 complex, with transcriptionally active genes (Kaplan *et al*, 2005). Likewise, we have also found that Spt6 KD decreases recruitment of both Paf1 and Rtf1 to *Hsp70*. However, Paf1 KD did not lead to any noticeable defect in the elongation rate of the 'first wave' of Pol II molecules 2 min after HS induction (Supplementary Figure 2A and B). We also do not detect any decrease in recruitment of Spt6 to the body of *Hsp70* gene 2 min after HS induction in Paf1 depleted cells (Supplementary Figure 2C), in contrast to what occurs 10 min after HS induction (Adelman *et al*, 2006). It remains possible that Paf1 helps stabilize Spt6 association with the hsp70 gene during steady state of HS induction.

In summary, the *Drosophila Hsp70* gene provides a convenient model system for analysing the role of suspected transcription elongation factors in transcription elongation. First, the rapid and relatively synchronous induction of *Hsp70* allows the rate of progression of the first wave of induced Pol II to be tracked by simple kinetic ChIP assays. Additionally, the steady state transcription rate of these highly induced genes can be examined optically by FRAP studies at *Hsp70* polytene loci in cultured salivary glands. Our results reveal that in addition to the earlier reported functions for Spt6 (Andrulis *et al*, 2002; Kaplan *et al*, 2003; Yoh *et al*, 2007), this factor is also a *bona fide* transcription elongation factor that positively stimulates the elongation rate of the elongating Pol II. This requirement for Spt6 is not limited to when Pol II has to negotiate with the nucleosome barrier during the early stage of HS but also applies to the steady state of transcription when the chromatin landscape of the gene is in a more transcriptionally permissive state (Petesch and Lis, 2008).

Materials and methods

RNAi

Production of dsRNA targeting LacZ (β-galactosidase), Spt6 and dHypb genes was done as described earlier (Adelman *et al*, 2005) (primer sequences provided in the Supplementary data). For RNAi treatment, 1 ml of exponentially growing *Drosophila* S2 cells were split to a density of 1×10^6 with serum free medium and treated with 10 μg of dsRNA. After 45 min, the volume was raised to 3 ml with 2 ml of 20% FBS-containing M3 + BPYE (Sigma) medium. After 84 h, the cells were harvested and instantaneously heat shocked for the indicated period at 36.5°C.

Transgenic Spt6^{RNAi} fly line

A 670-bp region within the sixth exon of *Drosophila* Spt6 was amplified using the following primers: Spt6 F (5′)-CTAGTCTAGA GGTGGGTCTGGACATT-3′. Spt6 R (5′)-AGCATTCGACTGAGAACC GGTCAA-3′ and cloned in a head-to-head orientation in the pWIZ vector (Sik Lee and Carthew, 2003) (kind gift of Dr R Carthew).

The Spt6^{RNAi} pWIZ construct was introduced into the germline by P-element transformation (Genetic Services, Inc.). Homozygous transgenic lines were obtained after a series of crosses and, Spt6$^{RNAi\ 41\text{-}3}$, which harbours the construct on the third chromosome, was used for the experiments.

FRAP analysis

Imaging analysis was performed on the animals with the following genotype: Control: UAS-Rpb3-EGFP/CyO; 6983-GAL4/Ubx. Spt6^{RNAi}: UAS-Rpb3-EGFP/+; 6983-GAL4/UAS-Spt6$^{RNAi\text{-}41\text{-}3}$. FRAP analysis was done as described earlier (Yao *et al*, 2006, 2007).

Northern blot and tandem RNAi-ChIP experiments are described in the Supplementary data section.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contribution

MBA, JTL and KA conceptualized and designed the experiments. KA, NF, SJP and MBA carried out the RNAi screen. JY obtained and analysed the data in Figure 3C and D. All other data were obtained by MBA. MBA and JTL wrote the manuscript. WWW provided support and advice for the experiment in Figure 3C and D.

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