Histone H3 lysine 4 trimethylation regulates cotranscriptional H2A variant exchange by Tip60 complexes to maximize gene expression

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Histone H3 lysine 4 trimethylation (H3K4me3) and the acetylated H2A variant, H2A.Z/v (H2Avac), are enriched at promoters of highly transcribed loci including the stress response genes. Using the inducible *Drosophila hsp70* loci as a model, we study here the roles of the dSet1 and dTip60 complexes in the generation of these two chromatin modifications. We find that Heat Shock Factor recruits the dTip60 complex to the *hsp70* loci in cells treated with salicylate, which triggers chromatin remodeling at these loci without transcription activation. Under these conditions, H2Avac or H3K4me3 are not enriched at the *hsp70* promoter. By contrast, heat shock-induced *hsp70* transcription induces dSet1-dependent H3K4me3 and H2Avac deposition by the dTip60 complex. The loss of dSet1 or dTip60 abolishes H2Avac incorporation, impairs Pol II release from the *hsp70* promoter, and causes a stalling of mRNA production during phases of transcription maximization. Biochemical assays confirm that nucleosomal H3K4me3 stimulates the histone acetyltransferase and H2Av exchange activities of dTip60 complexes. H2Avac contributes to nucleosome destabilization at promoters, and H3K4me3 restricts its incorporation to phases of acute transcription. The process uncouples cotranscriptional chromatin remodeling by dTip60 complexes from their role in the activation of PARP, which is responsible for the removal of transcription-incompatible or damaged chromatin during the initial stress response. The control of the multifunctional dTip60 complex by H3K4me3 ensures optimal stress response and cell survival by mediating the rapid maximization of *hsp70* expression. Furthermore, this mechanism prevents the accumulation of epigenetic noise caused by random complex-nucleosome collisions.

The genome of eukaryotes is packed into chromatin by histones and other proteins that hinder the access to the DNA for transcription, replication, repair, and recombination. To cope with this obstacle, cells change the structure of chromatin at specific locations within their genomes. These changes mainly occur at the level of the nucleosome core particle consisting of 146 bp of DNA wrapped around an octamer with two of each histones, H2A, H2B, H3, and H4. Chromatin regulation relies on posttranslational modifications (PTMs) of histones and the utilization of histone variants (1, 2). Most PTMs are believed to form docking platforms for enzymes that further restructure chromatin (3). These enzymes associate with "effector" domain proteins that recognize PTMs in nucleosomes. The assembly of enzymes with distinct effectors into complexes allows the introduction of various combinations of PTMs with different roles.

One combination of histone variants and PTMs is found at the transcription start sites (TSSs) of highly expressed genes. Here, H3K4me3 colocalizes with the hyperacetylated H2A variant, H2A.Z (4, 5). The acetylation of H2A.Z at promoters contributes to nucleosome destabilization when combined with the variant H3.3 (6). It is unclear how the cotranscriptional H2A.Z incorporation is regulated in higher eukaryotes. Complexes capable of H2A.Z incorporation function independent of lysine acetyltransferases (KATs) or have repressive roles (7). One candidate for a complex connecting H2A.Z exchange to its acetylation is the *Drosophila* dTip60 complex, which contains the KAT, dTip60, and the ATP-dependent H2A.Z exchange factor, Dom (8). Because H2A.Z is known as H2Av in flies, we will use H2Av in the following. We previously showed that the dTip60 complex replaces the DNA damage marker, phospho-H2Av, with H2Av in an acetylation-dependent reaction (8). We further demonstrated that the histone methyltransferase, dSet1, introduces H3K4me3 at activated stress response genes (9). This enabled us to study the connection between H3K4me3 and H2Avac at the *hsp70* loci. These loci are well suited for the analysis of transcription-related chromatin changes. Pol II is paused at their promoters in unstressed cells, and chromatin changes linked to transcription initiation can be identified. These are erased immediately after stress induction by poly(ADP ribose) polymerase (PARP), which regulates histone eviction at the *hsp70* loci (10). This event can be uncoupled from transcription activation by the treatment of cells with sodium salicylate (SAL), which triggers chromatin remodeling while arresting Pol II at the TSS (11). During the heat shock (HS)-induced transcription, remaining or newly deposited histones are modified in a cotranscriptional manner as exemplified by the accumulation of H2Avac, H3.3, histone chaperones, and chromatin modifiers at these genes (12–15). Also, H3K4me3 becomes detectable at the *hsp70* TSS within 2 min of HS (9).

Here, we report that heat shock factor (HSF) recruits the dTip60 complex to the heat shock elements (HSEs) in SAL-treated cells, but H3K4me3 or H2Avac only accumulate at the *hsp70* TSS during HS. Both the dSet1 and dTip60 complexes are required for H2A/H2Avac exchange at the *hsp70* promoter and for optimal Pol II release into elongation. Biochemical studies

Significance

Histone H3 trimethylated at lysine 4 and the hyperacetylated H2A variant, H2A.Z/v, are found at nucleosomes near promoters of highly expressed loci including the stress response genes. This study uses the inducible *hsp70* loci from *Drosophila* to demonstrate that the dTip60 chromatin remodeling complex incorporates and acetylates H2A.Z/v in a transcription-dependent manner to maximize the rates of Pol II release into elongation. In vivo and in vitro evidence is provided showing that H3 lysine 4 trimethylation regulates both the H2A.Z/v exchange and histone acetyltransferase activities of Tip60 complexes to ensure that nucleosome destabilization at promoters only occurs during transcription.

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confirm that H3K4me3 is sufficient to stimulate the KAT and H2Av exchange activities of dTip60 complexes.

Our data reveal that the dTip60 complex regulates H2Avac incorporation at the TSS of the *hsp70* genes and identify a role for H3K4me3 in this process. The control of the dTip60 complex by cotranscriptional H3K4me3 is an effective mechanism to couple nucleosome destabilization to transcription. Furthermore, the regulation of multifunctional complexes like the dTip60 complex by histone PTMs prevents epigenetic noise that otherwise would be introduced into genomes due to random nucleosome-modifier collisions.

Results

HSF Recruits the dTip60 Complex to the *hsp70* Genes Independent of Transcription. dTip60 is required for PARP-dependent chromatin remodeling, which is uncoupled from *hsp70* activation by HS (10). These data suggest that the dTip60 complex is recruited to the *hsp70* loci before transcription onset. Because Tip60 complexes interact with P53, which—like HSF—has an acidic activation domain (16), we tested whether HSF recruits the dTip60 complex to the *hsp70* loci. HSF is activated by HS or SAL treatment and trimerizes at HSEs in the 5′ region of the *hsp70* loci. Coimmunoprecipitation (co-IP) assays with anti-HSF antisera revealed that activated HSF interacted with dTip60 or the *Drosophila* Inhibitor of Growth 3 (dIng3), a subunit of the dTip60 complex (8), after both treatments (Fig. 1*A*). We next confirmed by chromatin IP/quantitative PCR (ChIP/qPCR) that HSF recruits the dTip60 complex to the HSEs upstream of the *hsp70* TSS. The experiments were combined with the RNAi-mediated knock down (KD) of HSF (*HSFi*). As shown in Fig. 1*B*, *HSFi* led to more than a 90% reduction of HSF protein levels within 72 h. The cells were then exposed to HS or SAL followed by ChIP with antibodies against HSF or dTip60 and qPCR with primer pairs covering the 5′ and 3′ regions of the *hsp70* loci (Fig. 1*C*). Both treatments caused the accumulation of HSF and dTip60 at HSEs in controls (*Escherichia coli lacZi*), whereas *HSFi* caused a significant reduction of dTip60 signals ($P < 0.01$). These data reveal that activated HSF tethers the dTip60 complex to the *hsp70* HSEs. The accumulation of dTip60 at the activated *hsp70* loci was also observed on polytene chromosome squashes from third instar larvae (Fig. S1).

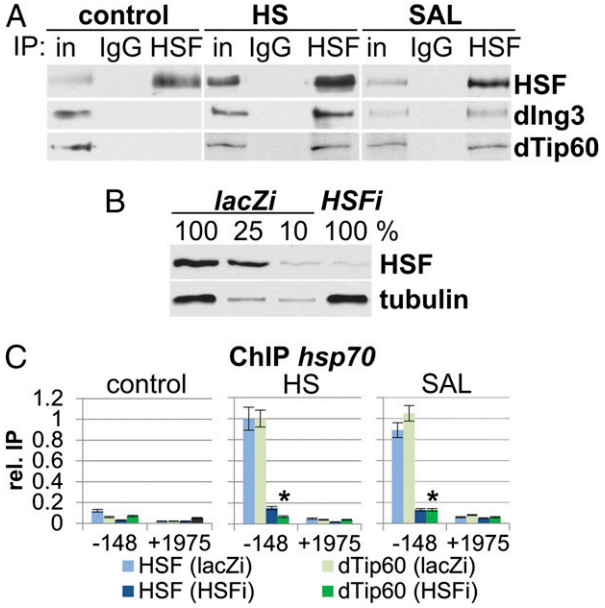

Fig. 1. HSF recruits the dTip60 complex to the *hsp70* loci independent of transcription activation. (*A*) IPs with IgG or anti-HSF antibodies on cells exposed to 10 min of HS or to 10 mM SAL. Blots were probed with antibodies to proteins indicated at the right. In, input 10% of nuclear extract used in IP. (*B*) Immunoblot of nuclear extracts from mock-treated (*lacZi*) versus *HSFi*-treated cells probed with anti-HSF antibodies. Tubulin served as loading control. (*C*) ChIP/qPCR values from *hsp70* loci expressed as relative IP (rel. IP) from *HSFi* versus *lacZi* under normal conditions (ctrl), HS, or SAL (HS *lacZi* = 1). Antibodies against HSF or dTip60 were used in ChIP. Numbers correspond to amplicon centers relative to the *hsp70* TSS. Error bars: SEM of three independent RNAi treatments (*$P < 0.01$).

Transcription Is Required for H2Av Acetylation Downstream of the *hsp70* Promoter. We next tested whether there was any difference in the distribution and activities of the dTip60 complex in SAL versus HS samples. For this purpose, we performed HS/time course experiments combined with ChIP/qPCR by using amplicons spanning *hsp70* loci. ChIP with anti-dTip60 antibodies revealed that the protein accumulated at the HSEs within less than 30 s of HS. Additional signals downstream of the TSS became detectable within 1 min (Fig. 2*A*). By contrast, SAL treatment only led to an increase of dTip60 signals at the HSEs. Hypoacetylated H2Av is present in the 5′ region of the *hsp70* loci in unstressed cells but was expected to become evicted by PARP after stress induction (10, 15). ChIP/qPCR with antibodies against H2Av showed a strong reduction within 30 s of HS or SAL treatment (Fig. 2*B*). Although H2Av levels remained low in SAL-treated cells, a substantial signal increase downstream of the TSS was observed after 1 min of HS. ChIP with antibodies against H2Avac confirmed that the newly deposited H2Av was hyperacetylated (Fig. 2*C*). We observed a similar distribution pattern for dSet1 and H3K4me3 (9), but the dynamics of their turnover was never compared with the one of H2Avac. To investigate this possibility, we performed ChIP with anti-dSet1 antisera (Fig. 2*D*). As expected, dSet1 was not recruited to the *hsp70* loci upon SAL treatment. Under thermal stress, however, the distribution of dSet1 closely matched the one of H2Avac with a peak in the nucleosome ∼300 bp downstream of the TSS. In conclusion, these data demonstrate that the dTip60 complex is recruited by HSF to the HSEs by SAL treatment or HS. From there, it spreads into chromatin downstream of the TSS after HS-induced transcription activation. This event coincides with the appearance of H2Avac and dSet1 in the same regions. In SAL samples, dSet1, H2Avac, or dTip60 are not found downstream of the *hsp70* promoter.

dSet1 Stimulates Histone Acetylation and H2Av Exchange by the dTip60 Complex. We next tested by KD experiments whether the dTip60 complex is responsible for H2Avac at the activated *hsp70* loci, and which role dSet1 plays in this process. Immunoblotting experiments confirmed that a 72-h treatment of cells with ds-RNA for dSet1 or dTip60 led to a more than 90% reduction for each protein (Fig. S2*A*). Because anti-Dom antibodies were not available, we confirmed by reverse transcription/qPCR (RT/qPCR) that *Dom* mRNA levels were reduced by more than 95% in *Domi* samples (Fig. S2*B*). We then compared the levels of dSet1 or H3K4me3 in the 5′ region of the *hsp70* gene in the KD cells exposed to SAL versus 10 min of HS. Consistent with our previous studies, only HS-dependent transcription activation led to a significant increase of both dSet1 and H3K4me3 at the *hsp70* TSS (Fig. 3 *A* and *B*; ref. 9). KDs confirmed that H3K4me3 depended on dSet1 ($P < 0.01$). The KD of dTip60 ($P < 0.02$) or Dom ($P < 0.01$) only caused a slight reduction of H3K4me3 levels. This phenomenon could be explained by the impaired PARP activation, which diminishes *hsp70* transcription (10).

dIng3 contains a plant homeodomain (PHD)-type zinc finger that has been implicated in H3K4me3 binding for other Ings (17). dIng3 protein levels were unaffected by *dSet1i* or *dTip60i*, making it possible to test whether dIng3 might be recruited to

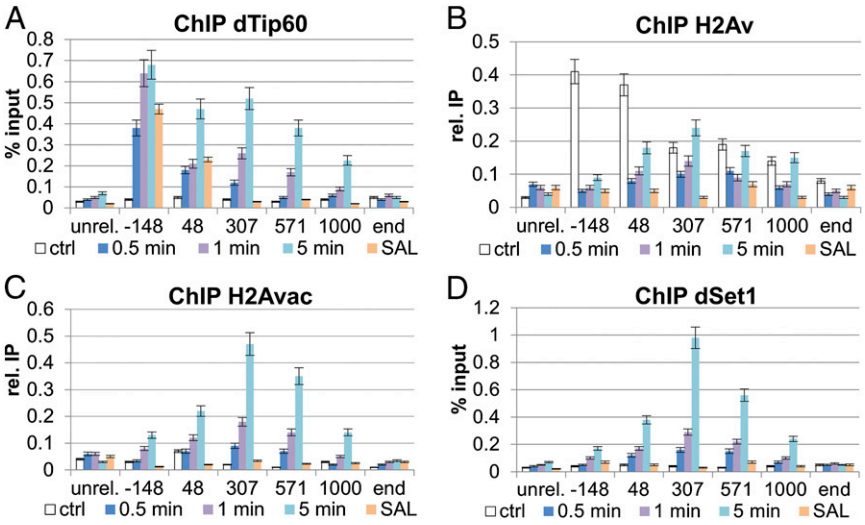

Fig. 2. dTip60, H2Avac, and dSet1 accumulate downstream of the *hsp70* promoter upon transcription activation. (*A–D*) ChIP/qPCR data from cells under normal conditions (ctrl), at 0.5, 1, and 5 min at 37 °C, or in 10 mM SAL (see legends below *x* axis). Numbers on the *x* axis correspond to amplicon centers relative to the *hsp70* TSS (9). Unrel, unrelated intergenic amplicon 32 kb downstream of *hsp70* locus (9, 10). Values on *y* axis correspond to percent input for ChIP with anti-dTip60 or anti-dSet1 antisera, and to relative IPs for H2Av or H2Avac after normalization against anti-H3 ChIP. Error bars correspond to the SEM of three independent replicates.

the *hsp70* loci upon *dTip60i* (Fig. S2*C*). Anti-dIng3 ChIP/qPCR revealed that dIng3 levels were lower in SAL-treated wild-type cells compared with HS (Fig. 3*C*). These data were consistent with ChIP data showing that less dTip60 was in the 5′ region of the *hsp70* loci in SAL-treated cells (Fig. 2*A*). *dTip60i* diminished dIng3 ChIP signals after HS or SAL treatment by 90%, whereas *Domi* only had a moderate effect. This result supports that dTip60, but not Dom, is required for complex stability and/or interaction with HSF. *dSet1i* caused a reduction of dIng3 levels in HS samples to values comparable to the ones found in SAL-treated *lacZi* cells, indicating that the cotranscriptional recruitment of dSet1 to regions downstream of the *hsp70* TSS leads to increased dTip60 complex levels in this region.

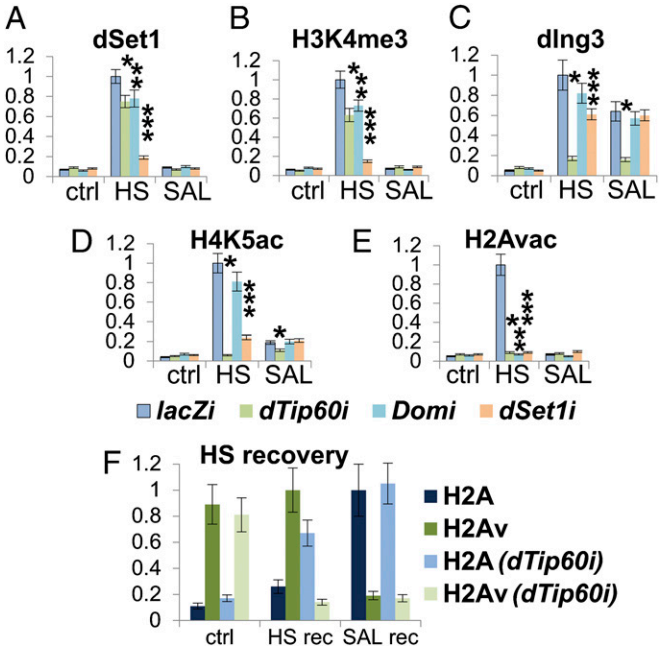

Fig. 3. dTip60, Dom, and dSet1 are required for H2Avac deposition at the TSS of the transcribed *hsp70* loci. (*A–E*) ChIP/qPCR data from *lacZi*, *dTip60i* (*$P < 0.02$), *Domi* (**$P < 0.01$), or *dSet1i* (***$P < 0.01$) using antibodies against proteins indicated on top. Primers spanning the *hsp70* promoter were used. Ctrl, control; HS, 20-min heat shock; SAL, 20 min of 10 mM salicylate. (*F*) ChIP/qPCR data for H2A or H2Av at the *hsp70* TSS after 20 min HS or 10 mM SAL followed by a 5-h recovery at 25 °C. *dTip60i*, cells were treated for 72 h with dTip60 dsRNA. Values were normalized to anti-H3 signals, and maximal levels were set as 1. Error bars represent the SEM of three experimental replicates.

Because additional H2Avac was found downstream of the body of the transcriptionally engaged *hsp70* loci, we next tested whether *dSet1i* or *Domi* would affect its accumulation and dTip60-dependent H4 acetylation. Tip60 complexes acetylate H2Av, H2A, and H4 at lysines 5, 8, 12, and 16 in vitro (18). In flies, H4K12ac and H4K16ac are regulated by different KATs, whereas H4K5ac and H4K8ac were not studied in detail (19, 20). We therefore determined the in vivo acetylation targets of the dTip60 complex by using embryos lacking its subunit, dMrg15 (8). $dMrg15^{-/-}$ embryos show gastrulation defects, indicating that early developmental gene regulation is impaired in these animals (Fig. S3). Immunolabeling experiments revealed that H4K5ac signals were strongly reduced in $dMrg15^{-/-}$ mutants, whereas H4K8ac levels were only moderately affected (Fig. S3 *A* and *B*). No noticeable changes in H4K12ac were detectable (Fig. S3*C*). The results were confirmed by immunoblotting experiments with histone extracts from these embryos (Fig. S3*D*).

Because H4K5ac is found at promoters and enhancers in higher eukaryotes (21), we next tested by RNAi-ChIP/qPCR whether dTip60 was responsible for this acetylation at the *hsp70* loci. The work confirmed that H4K5ac was elevated at the *hsp70* TSS upon both SAL and HS with approximately fivefold higher values in HS samples (Fig. 3*D*). *dTip60i* caused a reduction of H4K5ac under both experimental conditions, whereas *Domi* only had a moderate effect. *dSet1i* did not affect H4K5ac in SAL-treated cells, but the higher H4K5ac levels in HS samples were reduced to levels found in SAL assays. The results indicate that dSet1 is responsible for the cotranscriptional stimulation of H4K5ac by dTip60, while Dom is mostly dispensable for this process.

Studies of H2Avac revealed that only HS led to measurable increases of this acetyl-mark (Fig. 3*E*). The signal was significantly lower in all three KD samples. The data indicate that the KAT and ATPase modules of the dTip60 complex cooperate in the acetylation-dependent deposition of H2Av. Furthermore, dSet1 recruitment is pivotal for H2Avac accumulation downstream of the activated *hsp70* TSS.

H2A Accumulates at the *hsp70* TSS upon Loss of dTip60. Tip60 complexes cannot exchange nucleosomal H2Av with H2A or H2Av, suggesting that H2A must be transiently deposited at the *hsp70* TSS (8). These findings are supported by studies reporting that the H2A/H2B deposition factor, FACT, accumulates at

the activated *hsp70* loci (12). Furthermore, H2AK5ac, a dTip60-dependent acetylation, transiently occurs at the activated *hsp70* genes (8, 10). Unfortunately, anti-H2A antisera provided variable results in ChIP experiments during early HS and SAL treatment, presumably due to the high levels of H2A poly-ADP ribosylation in these samples (22). Because unmodified H2Av is enriched at the *hsp70* TSS in unstressed cells, and nucleosome reassembly occurs during recovery from stress (14), we compared nucleosomal H2A or H2Av content between both treatments after a 5-h recovery phase at 25 °C (Fig. 3*F*). The data revealed that the *hsp70* TSS became enriched for H2A in SAL samples, whereas H2Av was present after HS. Upon the KD of dTip60, H2A accumulated at the *hsp70* TSS after HS. These results support that H2A is initially deposited, but then exchanged with H2Av by the dTip60 complex during ongoing transcription. These data are also consistent with our and others' results showing that dTip60 acetylates both H2A and H2Av at the activated *hsp70* loci (Fig. 2*C* and 3*F*; ref. 10).

dTip60 Is Required for Pol II Release into Elongation at the Activated *hsp70* Gene. *dSet1i* causes the stalling of *hsp70* mRNA production during phases of maximal transcription up-regulation (9). To test whether *dTip60i* had a similar effect, we determined the *hsp70* mRNA accumulation kinetics in HS/time course experiments. After the preparation of total RNA at different HS time points, the *hsp70* mRNA levels were determined in RT/qPCR (Fig. 4*A*). Consistent with our previous results, the mRNA accumulation was normal in *dSet1i* samples during the first 5 min of HS, but then stalled. The KD of *dTip60* caused a reduction of *hsp70* mRNA production from early HS on; however, a nearly complete stalling of mRNA accumulation was also observed from 5 min of HS on. A likely explanation for the early transcription defects in *Tip60i* samples could be the failed PARP activation, because a similar early *hsp70* expression defect was reported in *PARPi* samples (10). Because PARP dissociates from the *hsp70* loci upon HS, the observed plateau effects in *dTip60i* samples are the result of impaired later transcription cycles after the introduction of H3K4me3 and H2Avac.

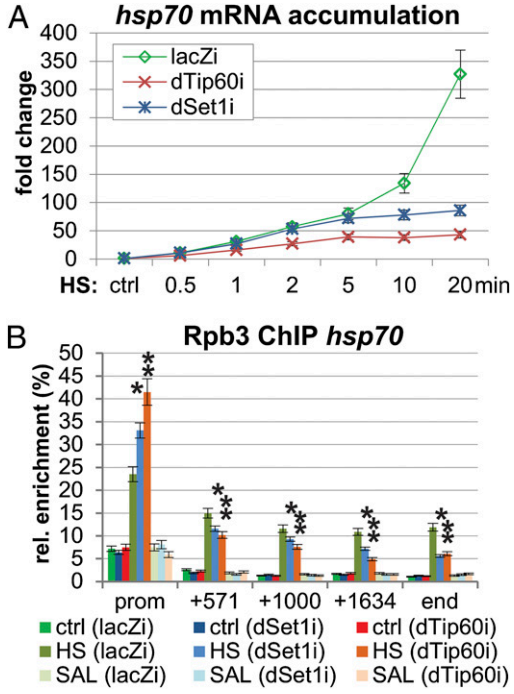

Fig. 4. Release of Pol II into elongation and *hsp70* mRNA production are affected by the loss of dSet1 or dTip60. (*A*) RT/qPCR data with *hsp70*-specific amplicons from *dSet1i* or *dTip60i* versus *lacZi* cells at 0.5, 1, 2, 5, 10, and 20 min of HS. Fold activation, signals were plotted relative to values from unstressed cells at 25 °C (ctrl). (*B*) ChIP/qPCR with anti-Rpb3 antibodies on cells depleted for dSet1 or dTip60 after 20 min of HS. $*P < 0.01$; $**P < 0.02$. Error bars represent the SEM of three experimental replicates.

We showed that the transcription defects in *dSet1i* are due to the retention of Pol II at promoters (9). To test whether *dTip60i* had a similar effect, we performed ChIP/qPCR with antibodies against the Pol II subunit, Rpb3 (Fig. 4*B*). After the KD, the cells were subjected to HS or SAL treatment for 20 min. The levels of promoter-bound Rpb3 were unchanged under non-HS conditions and in SAL-treated cells, confirming that Pol II occupancy before stress induction did not require dSet1 or dTip60. Upon HS, Rpb3 levels increased drastically along the axis of the *hsp70* gene in controls, which was consistent with our previous data (9). By contrast, approximately 40% and 85% higher Pol II levels were found at the promoter of *dSet1i* ($P < 0.01$) and *dTip60i* ($P < 0.02$) samples, respectively. At the same time, a significant decrease in the body of the gene occurred, supporting that Pol II is retained at the promoter. These data reveal that dTip60 and dSet1 are critical for the optimal release of Pol II from the *hsp70* promoter.

H3K4me3 Stimulates the KAT and H2Av Exchange Activities of the dTip60 Complex in Vitro. We reported (8) that the dTip60 complex only weakly acetylates recombinant nucleosomes, but has strong KAT activity toward native nucleosomes. Because dSet1 was responsible for the stimulation of H4K5ac and H2Avac at the *hsp70* loci, we speculated that H3K4me3 could have a direct role in this stimulation. A candidate H3K4me3-binder within the complex is the PHD of dIng3, which is similar to PHDs from other Ings capable of binding to H3K4me3 in peptide binding assays (17). To test whether this also is the case for dIng3, we immobilized H3 peptides containing H3K4me0, H3K4me2, or H3K4me3 onto magnetic beads and added a GST-dIng3PHD fusion protein. As controls, peptides with methyl-K9 or methyl-K36 were used. As shown in Fig. 5*A*, the dIng3PHD had high affinity to H3K4me2/3-containing H3 peptides, supporting that dIng3 could mediate the stimulation of the dTip60 complex by H3K4me3. To test this hypothesis, we performed KAT assays with purified dTip60 complexes (Fig. S4). For this purpose, we generated recombinant H3 chemically trimethylated at K4 by using the methyl-lysine analog (MLA) technique (23), which was then reconstituted into nucleosomal arrays (Fig. 5*B*). Immunoblotting experiments with anti-H3K4me3 antisera confirmed that the methylation and reconstitutions were successful. We next tested in radiolabeling assays with tritiated acetyl-CoA (acCoA) whether the KAT activity of the dTip60 complex would be stimulated by these arrays (Fig. 5*B*). Recombinant nucleosomes were only weakly acetylated at H2A and H4, whereas native nucleosomes showed higher levels of acetylation. Quantitative measurements revealed that the acetylation levels were approximately fivefold higher in the latter (Fig. S5). By contrast, the acetylation of H3K4-MLA arrays was approximately 35-fold higher. These results indicate that nucleosomal H3K4me3 is sufficient to stimulate the KAT activity of the dTip60 complex.

We next tested whether H3K4me3 would also have a stimulatory effect on arrays containing H2Av. Because H2Av is similar in size to H3, we used anti-H2Aavac antibodies instead of acCoA. As shown in Fig. 5*C*, the acetylation of H2Av/H3K4me3 analog-containing arrays was much higher compared with the ones containing H2Av/H3. Quantitative measurements revealed that the stimulation was approximately 13-fold (Fig. S5). We previously observed that phospho-H2Av interfered with H4 acetylation (8), which could explain that H2A-containing arrays are acetylated stronger.

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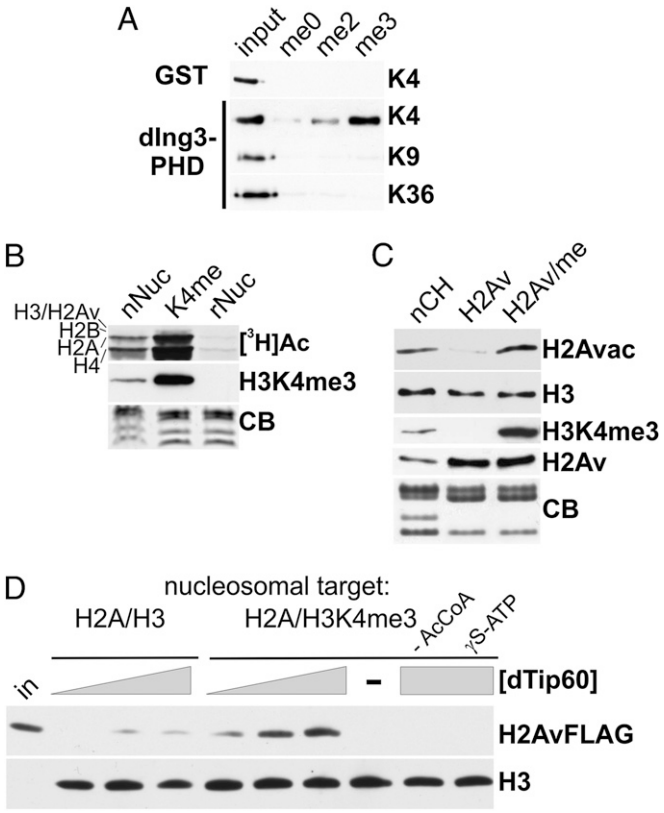

Fig. 5. Nucleosomal H3K4me3 stimulates the KAT and H2Av exchange activities of the dTip60 complex. (*A*) Immunoblots of methyl-H3 peptide pull-down assays with GST or GST-dIng3PHD labeled with anti-GST antibodies. The labels at the top are the number of methyl groups (me0, me2, me3) at given lysine positions (right). In; 10% input. (*B, Top*) Fluorogram of histones radiolabeled with tritiated acetyl-coA ([^{3}H]Ac) by FH-dTip60 complexes. (*B, Middle*) Immunoblot of nucleosomes stained with anti-H3K4me3 antibodies. (*B, Bottom*) CB, Coomassie blue-stained gel with 1-μg nucleosomes. K4me, recombinant nucleosomes with H3K4me3-MLA; nNuc, native; rNuc, recombinant nucleosomes. (*C*) Immunoblots of nucleosomes acetylated by dTip60 complexes probed with antisera against factors indicated to the right. H2Av, recombinant nucleosomes containing H2Av; H2Av/me, recombinant nucleosomes with H2Av and H3K4me3-MLA H3; nCH, 1 μg of native core histones. (*D*) Immunological analysis of H2AvFlag/H2B heterodimer exchange by the dTip60 complex with anti-Flag antibodies. Nucleosomes with H2A/H3 or H2A/H3K4me3-MLAs were used. In, 10% of H2AvFLAG/H2B heterodimers. (*D, Bottom*) Five percent array probed with anti-H3 antibodies. Gray bars indicate increasing amounts of dTip60 complex. (-), reaction with mock-purified material; -AcCoA, minus acetyl-CoA; γS-ATP, with nonhydrolysable ATP analog.

We next assessed whether H3K4me3 also stimulates the H2Av exchange activity of dTip60 complexes. For this purpose, we generated arrays containing H2A/H3 or H2A/H3K4me3-analogs and bound them to paramagnetic beads. The nucleosomes were then incubated with increasing concentrations of dTip60 complex, H2Av-Flag/H2B heterodimers, ATP, and acCoA. Unincorporated heterodimers were then removed, and the levels of incorporated H2Av-Flag were determined by immunoblotting experiments (Fig. 5*D*). Although unmethylated arrays showed a moderate incorporation of H2Av-Flag/H2B, the variant levels were in the H3K4me3-MLA arrays ninefold higher. The omission of acCoA and the use of a nonhydrolysable ATP analog confirmed that H2Av exchange depended on both cofactors, supporting that the dTip60 complex was responsible for the reaction. In conclusion, the data indicate that nucleosomal H3K4me3 is sufficient to stimulate the KAT and H2Av exchange activities of the dTip60 complex.

Discussion

H3K4me3 Connects Promoter-Proximal H2Avac to Transcription Up-Regulation. The work presented here reveals that the dTip60 complex is responsible for the acetylation-dependent incorporation of H2Av into nucleosomes immediately downstream of the promoter of the transcribed *hsp70* gene to facilitate Pol II release into elongation (for a model, see Fig. 6). This process requires H3K4me3, which is cotranscriptionally introduced by dSet1. In mammals, promoters of highly expressed genes are occupied by unstable nucleosomes containing hyperacetylated H2Avac and H3.3 (6). Several lines of evidence support that a constant disassembly and reassembly of nucleosomes at the activated *hsp70* loci also occurs in flies. H3.3 and dSet1 are continuously turned over at the *hsp70* loci, and H3.3 is the major target for dSet1-dependent K4me3 in *Drosophila* (9, 14, 24). Furthermore, the accumulation of H2A and its transient acetylation by dTip60 support that H2Avac loss is followed by a transient deposition of H2A, which then becomes rapidly exchanged by H2Avac again (Fig. 3*F*; refs. 10 and 12). The highly expressed stress response genes likely depend on a coordinated nucleosome destabilization for rapid transcription up-regulation far more than moderately active genes. This scenario might also apply to highly expressed developmental genes, which also require H3K4me3 for their full expression (9, 25). Interestingly, mitotically transmitted H3K4me3 is primarily required for the reestablishment of transcription amplitudes at developmental genes in the daughter cells (26). Our data support that H3K4me3 regulates this process by stimulating H2Avac-dependent nucleosome destabilization. The coordinated mechanism is of particular importance for the reactivation of gene expression cascades after cell division in rapidly developing organisms like *Drosophila*.

Regulation of the Multifunctional Tip60 Complexes. The dTip60 complex plays two distinct roles at stress genes. First, it activates PARP to remove damaged and transcription-incompatible chromatin signals before transcription activation (10). Key for the

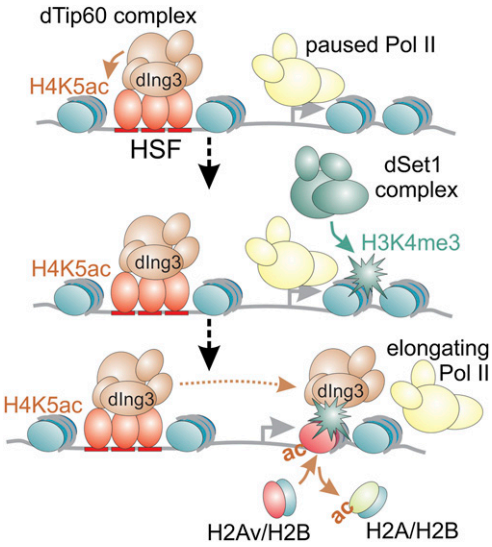

Fig. 6. H3K4me3 stimulates H2Avac exchange by the dTip60 complex at the *hsp70* promoter to facilitate Pol II release into elongation. Upon stress induction, HSF recruits the dTip60 complex to the HSEs. The dTip60 complex moderately acetylates H4K5 in the absence of H3K4me3. Upon transcription onset, the dSet1 complex introduces H3K4me3 at the *hsp70* TSS, which is then bound by dTip60 complex subunit, dIng3, via its PHD. This event stimulates the acetylation-dependent exchange of H2A/H2B with H2Av/H2B by the complex. H2Avac destabilizes these nucleosomes, which facilitates Pol II release from the TSS.

recruitment is the interaction between the complex and activated HSF (Fig. 1). During this phase, only moderate H4K5ac is introduced at the HSEs (Fig. 3*D*). H4K5ac was also found at enhancers in mammalian cells (21). The second, transcription- and H3K4me3-linked function involves both the KAT and ATPase within the dTip60 complex to ensure that H2Avac is incorporated downstream of the *hsp70* TSS (Figs. 2 and 3). Tip60 complexes are multifunctional and acetylate transcription factors and other nonhistone proteins (16), which might explain why their activities are controlled by their chromatin environment. In particular the modulation of the KAT activity is crucial for the subsequent H2Av exchange by Dom (Fig. 5; ref. 8). This initial reaction step is likely to be mediated by H3K4me3 binding of dIng3. In vitro studies showed that Ing3 directly contacts Tip60, raising the possibility that H3K4me3 binding by its PHD allosterically regulates the KAT (27). The ATP-dependent H2Av exchange is likely to be controlled by acetyl-lysine binding by a conserved bromodomain effector subunit of Tip60 complexes. In yeast, the KAT and ATPase modules are separated; however, only one of two ATPase subcomplexes contains this bromodomain factor and is stimulated in H2Av exchange by acetylation (28). Combined with our data, these findings support that at least two effectors coordinate the acetylation-dependent H2Av exchange by Tip60/Dom complexes.

Currently, effector domains are believed to mainly function in the tethering of complexes to histone PTMs, but a regulatory role for these factors is highly likely (3). In fact, many chromatin modifying complexes from higher eukaryotes interact with transcription factors or other proteins that recruit them to their genomic targets. Such complexes often contain multiple catalytic subunits and effectors, suggesting that the regulation of their enzymatic modules is controlled by their chromatin environment. A combination of the same enzymes with distinct effectors also broadens the spectrum of PTM combinations that can be introduced by a finite number of enzymes. Finally, the control of chromatin modifiers by their environment also suppresses epigenetic noise caused by random modifier-nucleosomes collisions. Such a control is of particular importance for nondividing tissues in higher eukaryotes, in which nucleosomes are not replenished during S phase.

Materials and Methods

RNAi, RT/qPCR, and ChIP/qPCR. Co-IPs were performed as described (8). The HS, SAL, fixation, and staining protocols were published (9, 13). RNAi, reverse transcription, and qPCR were performed as described (8, 9). HS, SAL treatment, and ChIP experiments were executed as described (9, 11). ChIP was performed by using chromatin from 0.5 to 1 $\times$ 10^7 S2 cells, which was dissolved in 3 M urea in ChIP buffer and dialyzed against buffer. For anti-H2A.Zac, anti-dIng3, anti-H4K5ac, and anti-dTip60 ChIP experiments, the cross-linking was performed with 2 mM EGS for 20 min followed by 1% formaldehyde for 15 min. Antibodies and primers are listed in *SI Materials and Methods*. Significance of the data was calculated using Student's paired two-tailed *t* test under the assumption of equal variance.

Biochemical Assays. Histone peptide pulldowns were performed as described (17). In brief, 1.5 μL of Dynabeads T1 (Invitrogen) were saturated with biotinylated H3 peptides (a gift of M. Vermeulen, UMC Utrecht, Utrecht, The Netherlands). The beads were then washed with binding buffer (50 mM Tris·Cl at pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, and 0.5 mM PMSF) before the incubation with 0.5 μg of fusion proteins.

dTip60 complex purifications and KAT assays were performed as described (8, 9, 13). For H2Av exchange reactions, 250 ng of nucleosomal arrays were mixed with 2.8 pmol of H2AvFlag/H2B heterodimers, 5, 10, or 15 fmol of dTip60 complex, 1 mM ATP/γ-S ATP, 1 μM AcCoA in 50 μL of 25 mM Hepes at pH 7.6, 70 mM KCl, 0.02% Nonidet-P40, 5% (vol/vol) glycerol, 1 mM DTT, 0.1 mM EDTA, 1.75 mM $MgCl_2$, and Complete protease inhibitor mixture (Roche). Band intensities were determined by using ImageJ (National Institutes of Health).

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