A phospho/methyl switch at histone H3 regulates TFIID association with mitotic chromosomes

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Histone methylation patterns are correlated with eukaryotic gene transcription. High-affinity binding of the plant homeodomain (PHD) of TFIID subunit TAF3 to trimethylated lysine-4 of histone H3 (H3K4me3) is involved in promoter recruitment of this basal transcription factor. Here, we show that for transcription activation the PHD of TAF3 can be replaced by PHDs of other high-affinity H3K4me3 binders. Interestingly, H3K4me3 binding of TFIID and the TAF3-PHD is decreased by phosphorylation of the adjacent threonine residue (H3T3), which coincides with mitotic inhibition of transcription. Ectopic expression of the H3T3 kinase haspin repressed TAF3-mediated transcription of endogenous and of reporter genes and decreased TFIID association with chromatin. Conversely, immunofluorescence and live-cell microscopy studies showed an increased association of TFIID with mitotic chromosomes upon haspin knockdown. Based on our observations, we propose that a histone H3 phospho-methyl switch regulates TFIID-mediated transcription during mitotic progression of the cell cycle.

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Introduction

Post-translational modification (PTM) of chromatin has an important function in the regulation of gene expression (Strahl and Allis, 2000; Jenuwein and Allis, 2001). Histone acetylation at promoter regions has been linked to active transcription (Workman and Kingston, 1998). Genome-wide localization studies revealed specific patterns of histone methylation in active and inactive regions of the genome. Most clearly it was found that trimethylation of histone H3 lysine-4 (H3K4me3) marks active RNA polymerase II (pol II)

promoters in human cells in a pattern very similar to H3K9 and H3K14 acetylation (Bernstein *et al*, 2005; Heintzman *et al*, 2007). Biochemical analyses showed that the preinitiation complexes assemble on pol II promoters in a sequential manner with binding of the transcription factor TFIID as the initial step (Orphanides *et al*, 1996; Thomas and Chiang, 2006). Human TFIID is an ~800 kDa protein complex that contains the TATA box-binding protein (TBP) and 13–14 TBP-associated factors (TAFs). Although TBP is the central DNA-binding subunit of TFIID, several TAFs have been implicated in the recognition of promoter DNA around the transcription start site (Verrijzer and Tjian, 1996; Muller *et al*, 2007).

In addition to DNA binding, TAFs can bind to post-translationally modified chromatin. The double bromodomain of TAF1 binds to acetylated lysines in the N-terminal tails of histone H3 and H4 (Jacobson *et al*, 2000). We reported that the plant homeodomain (PHD) finger of TAF3 binds to H3K4me3 (Vermeulen *et al*, 2007; van Ingen *et al*, 2008). Together, this indicates that core promoter binding of TFIID depends on both DNA sequences and the modification status of surrounding nucleosomes. Although the association of TFIID to H3K4me3 through TAF3 revealed a direct link between the basal transcription apparatus and transcriptionally active chromatin, other proteins (or protein complexes) can also bind to H3K4me3. Association with H3K4me3 stimulates chromatin remodelling, gene activation or repression and DNA recombination, which involves the PHD fingers of BPTF, ING4, ING2 and RAG2, respectively (Shi *et al*, 2006; Wysocka *et al*, 2006; Matthews *et al*, 2007; Saksouk *et al*, 2009). In contrast to PHD fingers binding methylated H3K4, the PHD fingers of BHC80 and AIRE selectively bind unmethylated H3K4 (H3K4me0) (Lan *et al*, 2007; Org *et al*, 2008).

The transcription of eukaryotic genes is regulated during the cell cycle. Active transcription during interphase is rapidly silenced as cells enter mitosis (Leresche *et al*, 1996; Heix *et al*, 1998). Models explaining this inhibition involve phosphorylation of basal transcription factors including TFIID and RNA pol II, activation of general repressor proteins, and prevention of factor access to DNA by chromosome condensation (Gottesfeld and Forbes, 1997; Kornberg and Lorch, 1999; Pijnappel *et al*, 2009). Reports about chromatin binding of TFIID during mitosis have been conflicting. Subcellular fractionation of HeLa cells showed TBP and TAF12 (TAFII20/15) in the mitotic cytoplasm though a subpopulation of TBP remains on mitotic chromosomes (Segil *et al*, 1996). Exclusion of TBP and TAF4 from condensed mitotic chromatin was observed by immunofluorescence microscopy of HeLa cells (Segil *et al*, 1996; Kieffer-Kwon *et al*, 2004). These studies indicated that the majority of TFIID is displaced from the disassembling prophase nucleus to the mitotic cytoplasm around the time of nuclear envelope breakdown. In contrast, ChIP studies of nocodazole-blocked cells, which results in arrest of cells at prometaphase, indicated that TAF5 and TFIIB remain associated with active

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promoters (Christova and Oelgeschlager, 2002; Xing *et al.*, 2008). The latter results support the concept of bookmarking genes to facilitate the rapid reactivation of transcription upon exit from mitosis. In this respect, it is interesting to note that the H3K4me3 mark is stably maintained throughout mitosis, and that this also could act in bookmarking chromatin (Kouskouti and Talianidis, 2005).

Histone H3 phosphorylation is a mark enriched in mitosis. In mammalian cells, Aurora B is a potent mitotic H3S10 and H3S28 kinase (Prigent and Dimitrov, 2003). Aurora B-mediated H3S10 phosphorylation has been implicated in dissociation of H3K9me3-binding heterochromatin protein 1 (HP1) from mitotic chromatin (Fischle *et al.*, 2005; Hirota *et al.*, 2005). This H3K9me3/S10ph combinatorial mark and other methyl marks on histones in close proximity to phosphoacceptors have been hypothesized to be components of a binary methylation–phosphorylation switch (Fischle *et al.*, 2003). One of the newly characterized phosphorylation sites is H3T3 (Polioudaki *et al.*, 2004). The mitotic kinase haspin localizes predominantly to chromosomes and phosphorylates histone H3T3 during mitosis (Dai *et al.*, 2005). Very recently, it was found that H3T3ph binds the BIR domain of survivin to attract the chromosome passenger complex to mitotic chromosomes and to activate Aurora B kinase (Kelly *et al.*, 2010; Wang *et al.*, 2010). H3T3 phosphorylation may also inhibit chromatin binding of proteins as *in vitro* binding of chromo-domain helicase DNA-binding protein-1 (CHD1) to H3 methylated at K4 is abrogated by H3T3ph (Flanagan *et al.*, 2005). CHD1 is reported to be released into cytoplasm at the onset of mitosis and reincorporated into chromatin during telophase (Stokes and Perry, 1995). Furthermore, it was found that H3T3ph can interfere with binding of the isolated PHDs of ING2, RAG2 and BHC80 to (modified) histone peptides (Garske *et al.*, 2010). It is possible that H3T3ph serves to displace a number of other histone-binding proteins from chromosomes during mitosis, although this has yet to be demonstrated in cells.

In this study, we investigated H3T3ph and haspin involvement in transcription regulation by TFIID. We first performed a structure/function analysis of the H3K4me3-binding subunit TAF3 in transcriptional activation. Next, we observed decreased association of TAF3 and TFIID with H3K4me3 in the presence of H3T3ph. Taken together, our *in vitro* and *in vivo* data strongly support the presence of an H3T3/K4 ‘phospho–methyl switch’ for the regulation of TFIID binding to mitotic chromosomes.

Results

Characterization of transcription activation via TAF3

We found previously that TAF3 can activate transcription stimulated by the H3K4 methylase MLL complex member Ash2L in transient transfection reporter assays (Vermeulen *et al.*, 2007). This activation was dependent on the PHD of TAF3. To further investigate its role in activation, the PHD of TAF3 was replaced by the PHDs from AIRE and BHC80 or by the PHDs from PHF2 and PHF8 (Figure 1A). Binding of AIRE and BHC80 to the histone H3 tails is inhibited by H3K4me3 modification, whereas PHF2 and PHF8 binding is dependent on H3K4me3 (Lan *et al.*, 2007; Org *et al.*, 2008; Horton *et al.*, 2010; Wen *et al.*, 2010). At the primary sequence level, these PHDs display similar conservation to the TAF3 PHD (ranging

Figure 1 Transcriptional activation mediated by PHD of H3K4me3 binders. (A) Schematic representation of TAF3 chimeric constructs with the PHD of TAF3 swapped for the PHD of AIRE, BHC80, PHF2 or PHF8. Binding preferences of the PHDs for histone H3 tail is tabulated. (B) Alignment of the PHDs of TAF3, AIRE, BHC80, PHF2 and PHF8. (C) U2OS cells were transfected in triplicates with 100 ng 5XGal4MLP-luc, 250 ng TK-Renilla-luc, 50 ng Gal4-Ash2L and 500 ng of either pMT2-HA-TAF3 or TAF3 chimeric constructs. Cell lysates were prepared, and relative luciferase activity was determined. The graph represents the fold activation relative to the transfection with Gal4-DBD alone. (D) Expression of the various PHD constructs in (C) is depicted as probed by HA antibody, and GAPDH serves as the loading control.

from 38 to 55%) (Figure 1B). As shown previously, activation by Gal4-Ash2L was greatly enhanced by cotransfection of TAF3 (Figure 1C). Replacement of the TAF3 PHD by the PHDs of the H3K4me0 binders AIRE or BHC80 did not enhance Ash2L-dependent activation. In contrast, PHD replacement

by the H3K4me3 readers PHF2 or PHF8 reconstituted enhancement of Ash2L activation. Immunoblot analysis indicated that the TAF3 chimeric constructs were expressed at similar levels (Figure 1D). Coimmunoprecipitation of endogenous TAF5 protein indicates that (part of) the transfected TAF3 proteins become incorporated into TFIID (Supplementary Figure S1). Interaction with TAF3 or TAF8 is essential for nuclear translocation of TAF10 (Soutoglou *et al*, 2005). All the chimeric TAF3 constructs could induce nuclear localization of YFP-TAF10 (Supplementary Figure S2). Next, we asked whether the transcription activation function by TAF3 was specific for the Gal4-Ash2L system. To this end, Gal4-Ash2L was replaced by Gal4-DBD fusions of transcriptional activators like E2F or ER α (Kaelin *et al*, 1992; Dreijerink *et al*, 2006) or coactivators like CBP or Menin (Kwok *et al*, 1994; Hughes *et al*, 2004; Yokoyama *et al*, 2004) (Supplementary Figure S3). We found that TAF3 can coactivate transcription stimulated by a wide range of activators, which indicates that TAF3 coactivation is not restricted to Ash2L alone. Taken together, these experiments indicate a general transcriptional coactivation function for TAF3 and emphasize that transcriptional coactivation by TAF3 involves recognition of H3K4me3 by PHDs.

Besides a C-terminal PHD metazoan TAF3 contains a N-terminal histone fold domain (HFD), which are separated by a region of ~ 750 residues (Gangloff *et al*, 2001). The HFD of TAF3 is essential for association with its histone fold partner TAF10 (Soutoglou *et al*, 2005), and presumably for incorporation into TFIID, but the role of the linker region is largely unknown. To further characterize the activation function of TAF3, we made several deletion constructs of TAF3, which lacked either the HFD and/or specific regions of the linker region (Figure 2A). Cotransfection of these constructs in the Ash2L-dependent reporter assay indicated that removal of the HFD ($\Delta 100$) strongly reduced the transcription activation of TAF3 (Figure 2B). In addition, deletion of the HFD completely abolished its ability to interact with endogenous TAF5 (Supplementary Figure S4) or to translocate TAF10 to the nucleus (Supplementary Figure S5). This supports the idea that incorporation of TAF3 into TFIID is important for the activation function of TAF3. The linker deletions indicated that removal of 150–300 residues from the linker ($\Delta 400$ –695 only or $\Delta 700$ –850 only) still allowed two- to three-fold activation. In contrast, a larger deletion ($\Delta 400$ –850) or deleting both the linker region and the PHD ($\Delta 251$ –932) completely abrogated TAF3-mediated coactivation (Figure 2B). Immunoblot analysis confirmed the efficient expression of the deletion constructs (Figure 2C), and immunofluorescence analysis showed that all linker region and linker/PHD deletion constructs of TAF3 induce translocation of TAF10 to the nucleus (Supplementary Figure S5). Except the TAF3 construct lacking the linker/PHD ($\Delta 251$ –932), all other deletion constructs could efficiently bind to H3K4me3 (Figure 2D), indicating that the HFD and linker regions are not directly involved in H3K4me3 recognition by the PHD of TAF3. We have previously shown that the mRNA expression of RPL34, RPL31 and RPS10 genes is sensitive to H3K4me3 levels. Hence, we tested the effect of the various TAF3 constructs on the expression of these genes. We found that wild-type TAF3 induced the expression of the RPL/RPS genes (Figure 2E) but a point mutation in the PHD, deletion of the HFD and linker/PHD deletions disrupted the ability of TAF3

to enhance expression (Figure 2E). Immunoblot analysis confirmed the efficient expression of all these TAF3 constructs (Figure 2F). These results point towards the involvement of the HFD and linker region in the PHD-dependent transcription activation both of endogenous and of reporter genes by TAF3.

H3T3ph antagonizes binding of TAF3-PHD to H3K4me3 *in vitro*

Crosstalk between different histone H3 modifications has been demonstrated previously for the TFIID complex (Vermeulen *et al*, 2007). Acetylation of H3K9 and H3K14 residues in combination with H3K4me3 enhanced TAF3-PHD-mediated TFIID association to H3K4me3 whereas asymmetric dimethylation of the adjacent arginine residue (H3R2me2a) reduced association of TFIID (Vermeulen *et al*, 2007). To extend this, we decided to analyse the effects of histone H3 phosphorylation on the association of TFIID to H3K4me3. H3 peptide-binding assays were used to assess the effects of H3T3ph and H3S10ph on H3K4me3 binding of TFIID present in nuclear extracts. TAF5 and TAF6, subunits of TFIID, showed decreased association with H3K4me3 in the presence of H3T3ph, whereas H3S10ph had no effect (Figure 3A). Unfortunately, the lack of suitable TAF3 antibodies precluded a direct analysis of this subunit. Hence, we analysed the binding of overexpressed wild type, HFD and the linker/PHD deletions of TAF3 to various H3 peptides (Figure 3B). H3T3 phosphorylation reduced binding of both wild type and the HFD deletion of TAF3 to H3K4me3. We further checked if the decreased binding of TFIID to H3K4me3 in the presence of H3T3ph was dependent on TAF3-PHD using a GST fusion of TAF3-PHD. Whereas TAF3-PHD exhibited a robust and specific binding to H3K4me3, this was completely abolished by H3T3ph modification (Figure 3C). A similar analysis of H3S10ph showed that this modification has no effect on K4me3 binding (data not shown). The affinity of TAF3-PHD for H3K4me3 versus H3T3ph/K4me3 was determined by equilibrium-binding assays using tryptophan fluorescence. This showed that the adjacent H3T3ph reduces the TAF3-PHD affinity for H3K4me3 by ~ 80 -fold, whereas H3S10 phosphorylation had no effect (Figure 3D). These results are consistent with the structure of the TAF3-PHD/H3K4me3 complex, which showed that H3T3 is buried in the PHD and that H3S10 does contact this domain (van Ingen *et al*, 2008). Taken together, TFIID and TAF3-PHD binding to H3K4me3 is decreased by the presence of H3T3ph. We analysed whether the inhibitory effect of H3T3ph was specific to the PHD of TAF3. Using GST-fusions of BPTF, ING2 and ING4 in H3 peptide-binding assays, we found that K4me3 binding of these PHDs was also inhibited by the presence of H3T3ph (Figure 3E), as was H3-tail binding of the H3K4me0-dependent PHD of BHC80 (Supplementary Figure S6). This indicates that the inhibitory effect of H3T3ph is a general phenomenon for H3K4-binding PHD proteins.

The histone H3 threonine 3-specific haspin kinase represses TAF3-mediated transcription activation

The haspin kinase is directly involved in the phosphorylation of H3T3 (Dai *et al*, 2005), and we tested the effects of haspin on the PHD-dependent transcription activation function of TAF3. Figure 4A shows that Ash2L-stimulated TAF3 activation was reduced upon overexpression of haspin. This is

Figure 2 Effect of HFD and linker regions on TAF3 function. (A) Domain representation of the TAF3 deletion constructs. (B) The experiment was done as in Figure 1C except that cells were transfected with 100 ng 5XGal4MLP-luc, 250 ng TK-Renilla-luc and different TAF3 constructs at various concentrations to obtain similar expression levels either in the presence or absence of 50 ng Gal4-Ash2L. (C) Expression of the various TAF3 constructs in (B) is depicted as probed by HA antibody. The asterisk indicates detection of a background band. (D) Lysates from transfected cells overexpressing wild type or TAF3 deletion constructs were incubated with streptavidin beads coated with the indicated H3 peptides. Bound proteins were analysed by SDS-PAGE separation followed by immunoblotting. For comparison, 10% of the input was analysed in parallel. (E) U2OS cells were transfected in triplicates with pcDNA, wild-type TAF3 and the mutant and deletion constructs. RNA was extracted from these cells and quantitative RT-PCR analysis was performed. The graph represents levels of different mRNA normalized to β -actin and then further expressed as expression relative to the lane with pcDNA control transfection. (F) Expression of the various TAF3 constructs in (E) is depicted as probed by HA antibody. The asterisk indicates detection of a background band.

dependent on the haspin phosphorylation function as overexpression of a kinase-dead mutant of haspin had little effect on TAF3 activation of Ash2L-activated transcription (Figure 4A). Haspin inhibition of TAF3 function did not result from a general mitotic repression of transcription as haspin overexpression did not force interphase cells into mitosis (Dai *et al*, 2005; Supplementary Figure S7A and see below). As expected, immunofluorescence analysis of transfected cells showed costaining of haspin and H3T3ph

(Supplementary Figure S7A). In addition, immunoblot analysis revealed a strong increase of H3T3ph in wild-type haspin transfected cells (Figure 4B). Next, the effect of haspin overexpression on other transcriptional activators was tested. This showed that similar to Ash2L, the activation by ER α or E2F was also inhibited by the haspin kinase (Figure 4C and D). Next, we tested the effect of haspin on TAF3-dependent transcription of endogenous genes (Figure 5A and B; Supplementary Figure S7B). This analysis showed that

Figure 3 Phosphorylation of histone H3T3 antagonizes binding of H3K4me3 readers. (A) Nuclear extract was incubated with streptavidin beads coated with the indicated H3 peptides. Bound proteins were analysed by SDS-PAGE separation followed by immunoblotting. For comparison, 10% of the input was analysed in parallel. (B) Same as in (A) except that lysates from transfected cells overexpressing TAF3 WT, $\Delta 100$ or $\Delta 251-932$ were used for binding. (C) Bacterial lysates containing GST-TAF3 were incubated in increasing amounts with streptavidin beads coated with the indicated H3 peptides. Bound proteins were analysed by Coomassie staining. Arrows indicate position of the GST-fusion protein. Amount in the input lane corresponds to the midpoint in the titration of GST-TAF3 lysate. (D) Dissociation constants of TAF3-PHD binding to either only K4me3 modified or a K4me3 and T3ph doubly modified H3 peptide (residues 1–17) as determined by tryptophan fluorescence. (E) Pull downs were performed as in (A) using bacterial lysates containing GST-TAF3, GST-BPTF, GST-ING2 or GST-ING4.

Figure 4 Histone H3T3 kinase haspin represses TAF3-mediated transcription activation. (A) U2OS cells were transfected in triplicates with 100 ng 5XGal4MLP-luc, 100 ng TK-Renilla-luc, 50 ng Gal4-Ash2L, 500 ng pMT2-HA-TAF3 and 50 ng of either wild type (WT) or kinase-dead mutant (KD) of myc-haspin. Cell lysates were prepared, and relative luciferase activity was determined. The graph represents the fold activation relative to the transfection with Gal4-Ash2L. (B) Protein expression of transfected HA-TAF3 and haspin constructs in (A) were analysed by SDS-PAGE followed by immunoblotting. H3T3ph levels were probed as a read out of haspin kinase function and histone H4 served as a loading control. (C) 293T cells were seeded in DMEM without phenol red containing 5% dextran-coated, charcoal-treated serum and transfected with 250 ng 5XGal4TK-Luc, 25 ng CMV Renilla-luc, 50 ng Gal4-ER α , 500 ng pMT2-HA-TAF3 and 50 ng of either wild type or kinase-dead mutant of haspin. At 24 h after transfection, the medium was changed to medium containing either the ligand 10 nmol/17 h estradiol (E2; Sigma), or the vehicle-ethanol. The graph represents the fold activation relative to the transfection with Gal4-ER α in the presence of E2. (D) Transient transfection luciferase assay was performed as in Figure 4A except that Gal4-Ash2L was replaced by Gal4-E2F. The graph represents the fold activation relative to the transfection with Gal4-E2F.

Figure 5 Haspin represses TAF3 activation of endogenous genes. (A, B) U2OS cells were transfected in triplicates with pcDNA, TAF3 and wild type or kinase-dead haspin. RNA was extracted from these cells and quantitative RT-PCR analysis was performed. The graph represents the levels of different mRNA normalized to β -actin and then further expressed as expression relative to the lane with pcDNA control transfection. (C) Protein expression of transfected HA-TAF3 and haspin constructs were analysed by SDS-PAGE followed by immunoblotting. H3T3ph levels were probed as a read out of haspin kinase function and α -tubulin served as a loading control.

Figure 6 TFIIID dissociates from chromatin upon haspin expression. (A) Schematic representation of chromatin fractionation. (B) SE, W1, W2 and Chr fractions obtained from either haspin expression induced or uninduced cells were analysed by immunoblotting to detect transcription factors, haspin or histone modifications in the various fractions.

haspin expression inhibits the TAF3 activation of RPL34, RPL31 and RPS10, which is dependent on the H3T3 phosphorylation (Figure 5C). Together, these analyses indicated that haspin-mediated phosphorylation inhibits coactivation of transcription by TAF3.

Loss of TFIIID association with chromatin upon haspin expression

Having established that H3T3ph can inhibit TFIIID and TAF3 binding to K4me3-modified histone H3 peptides, we investigated the effect of haspin phosphorylation on chromatin binding of TFIIID *in vivo*. To this end, we used HeLa cells with inducible expression of haspin (Dai *et al*, 2005) for isolation of chromatin. Different fractions from induced and non-induced cells were obtained by salt extraction and micrococcal nuclease (MNase) digestion of the nuclear pellet (Figure 6A), and these fractions were analysed by immunoblotting. TFIIID was detected by antibodies against the TBP-binding subunit, TAF1 and the structural subunit, TAF5 (Bhattacharya *et al*, 2007). Figure 6B shows that haspin overexpression results in a loss of TFIIID from the

chromatin fraction. TBP was also decreased significantly from the chromatin fraction upon haspin expression. The remaining TBP could be part of other complexes like B-TFIID, SL1 or TFIIB (Thomas and Chiang, 2006). As negative control, we analysed chromatin association of NC2, which is known to interact with free TBP on DNA (Thomas and Chiang, 2006). As expected, haspin overexpression induces H3T3 phosphorylation, but has no effect on H3S10ph levels which is in line with the specificity of the haspin kinase for H3T3 and the observation that haspin overexpression does not strongly increase the mitotic population of cells (Dai *et al*, 2005). Analysis of H3K4me3 and H4 levels indicate equal chromatin content in extractions from uninduced and induced cells. Taken together, these results indicate that the haspin kinase can influence chromatin association of TFIIID.

Knockdown of haspin increases TFIIID association with mitotic chromosomes

H3T3 phosphorylation by haspin occurs during mitosis and coincides with a general inhibition of transcription. Previous

immunofluorescence studies showed that the majority of TFIID dissociates from mitotic chromatin (Kieffer-Kwon *et al*, 2004). We investigated the involvement of phosphorylation of H3T3 by haspin in TFIID dissociation from mitotic chromosomes by immunofluorescence and live-cell imaging. To this end, we used human U2OS osteosarcoma cells stably expressing GFP-tagged TAF5. The GFP-TAF5 protein in these cells is stably integrated into the TFIID complex and is expressed to 25% of the endogenous TAF5 levels (de Graaf *et al*, 2010). GFP-TAF5 cells were subjected to siRNA-mediated knockdown of haspin and the decrease in haspin expression was analysed by immunoblotting (Supplementary Figure S8A). Histone H2B tagged with dsRed was cotransfected to visualize chromatin. Imaging of U2OS-GFP-TAF5 cells by video microscopy indicated that TAF5 was excluded from mitotic chromosomes after nuclear envelope breakdown and the exclusion was best visible when the chromosomes aligned at the metaphase plate (Figure 7A, left panels; Supplementary Movie 1). TAF5 swiftly relocated on the chromosomes at late anaphase when the cells progressed into cytokinesis. This coincides with the dephosphorylation of H3T3 in late anaphase (Dai *et al*, 2005). Analysis of haspin knockdown cells progressing through metaphase showed chromosomal retention of TAF5 from prometaphase until late anaphase (Figure 7A, right panels; Supplementary Movie 2). Imaging at a lower magnification confirmed this observation in several cells within the same field (Supplementary Movies 3 and 4). Our results are in agreement with previous studies reporting TFIID dissociation from mitotic chromatin (Segil *et al*, 1996; Kieffer-Kwon *et al*, 2004) and suggest that the mitotic kinase haspin has an important function in this process.

To substantiate that the accumulation of TAF5 in haspin-depleted cells correlated with the status of H3T3ph, we performed immunofluorescence of GFP-TAF5 coupled with immunolabelling of endogenous H3T3ph upon control or haspin siRNA knockdown. Condensed chromosomes were identified by DAPI staining of DNA, which was corroborated by the presence of H3T3ph on the mitotic chromosomes in control cells. As shown in Figure 7B (left panel), exclusion of TAF5 was observed during prometaphase, metaphase and anaphase, which coincided with H3T3ph. Cells treated with haspin siRNA (identified by reduced H3T3ph) retained TAF5 on the mitotic chromosomes (Figure 7B, right panel). Different siRNAs against haspin gave very similar results in immunofluorescence and video microscopy (data not shown). High levels of chromosome-associated TAF5 correlated with depletion of cytoplasmic TAF5. To test whether accumulation of TAF5 on mitotic chromosomes was unique to the absence of H3T3ph, the effect of inhibitors of the H3S10 kinase, Aurora B, were analysed. Treatment by either ZM447439 (Ditchfield *et al*, 2003; Figure 7C or Hesperadin (Hauf *et al*, 2003; data not shown) did not affect TAF5 exclusion from mitotic chromosomes. Further, we confirmed that siRNA knockdown of haspin did not eliminate H3S10ph and that Aurora B kinase inhibitors did not eliminate H3T3ph by costaining of mitotic chromosomes with H3T3ph and H3S10ph antibodies (Supplementary Figure S8B). Quantification of GFP levels on chromosomes versus cytoplasm showed a substantial enrichment of GFP TAF5 on chromosomes only upon haspin knockdown (Figure 7D). In conclusion, our observations support the involvement of

the haspin-mediated H3T3ph modification in evicting TFIID from the chromatin during mitosis.

Discussion

In this study, we characterize transcription activation by the TFIID subunit TAF3 and emphasize the significance of the PHD of H3K4me3 readers in stimulating Ash2L-dependent transactivation. Further, we identify the role of the HFD and linker region of TAF3 in modulating its transcription activation function. We investigated the crosstalk of H3K4me3 with adjacent H3T3ph and provide evidence for the model that phosphorylation of the histone H3 tail is part of a 'phospho-methyl' switch to dissociate TFIID from mitotic chromosomes.

Functional analysis of TAF3

TAF3, an integral subunit of the basal transcription factor TFIID, contains a N-terminal HFD, which is essential for the association of TAF3 with its histone fold partner TAF10 (Soutoglou *et al*, 2005). Metazoan, but not protozoan, TAF3 contains a C-terminally located PHD finger, which binds with high affinity and specificity to H3K4me3. Our previous studies suggested that TAF3-mediated binding of TFIID to H3K4me3-marked promoters can serve to anchor TFIID to chromatin during promoter activation (Vermeulen *et al*, 2007). This established a direct link between H3K4me3 and the basal RNA polymerase II transcription machinery. The PHD of TAF3 is separated from the HFD by means of a largely uncharacterized linker region of ~750 residues (Gangloff *et al*, 2001), and our results indicated that the linker is relevant for TAF3 activity. This region may be involved in direct regulation of transcription by means of functional elements within the linker sequence or it could serve to span TFIID binding at the core promoter to H3K4me3 nucleosomes, which peaks generally at ~300 bp downstream of transcription start site (Guenther *et al*, 2007). TAF3 was also reported to associate with TRF3 to form a heterodimeric complex required for myogenic differentiation of myoblasts to myotubes (Deato and Tjian, 2007). Whereas myoblasts contain the canonical TBP-containing TFIID complex, their terminal differentiation into myotubes coincides with replacement of the TFIID complex by a TRF3-TAF3 complex. The PHD of TAF3 is not involved in transcriptional activation of myogenin, whereas the HFD of TAF3 is essential (Deato *et al*, 2008). TAF3 has also been identified as a negative regulator of p53 activation (Bereczki *et al*, 2008). In this case, the PHD was also not required. In contrast, we observe that TAF3 can enhance the activity of several transcriptional activators, and that this depends strongly on both the HFD and the PHD domains and to a lesser extent on the linker region. HFD dependence of TAF3-mediated transcription indicates involvement of its HFD dimerization partner TAF10 and our results indicate that incorporation into TFIID is important for coactivation by TAF3 of endogenous and reporter genes. Replacement of its PHD by H3K4me0-binding PHDs drastically reduced TAF3 activation function, whereas replacing with H3K4me3-binding PHDs supported transcription activation. Taken together, the results with the TAF3 mutant proteins support the model that transcriptional activation via TAF3 depends on a stable incorporation of TAF3 into TFIID and specific recognition of H3K4me3-marked promoters.

Figure 7 TFIID association with mitotic chromosomes increases upon haspin knockdown. **(A)** U2OS-GFP-TAF5 cells transfected with either non-targeting control siRNA (left panels) or haspin siRNA (right panels) and cotransfected with H2B-dsRed were released from an 18-h thymidine synchronization and subjected to overnight video microscopy at 37°C. Images were collected every 2 min. Selected images are shown as combined panels of GFP fluorescence (green) on the left, and on the right, the GFP signal is shown superimposed on corresponding H2B-dsRed images. The entire series is available in the Supplementary Movie section. From different experiments, 129 out of 151 cells that divided during the filming showed an exclusion of TAF5 from mitotic chromatin, and 79 out of 124 cells retained TAF5 on chromatin upon haspin knockdown. Different siRNAs against haspin gave similar results. **(B)** U2OS-GFP-TAF5 cells with either a non-targeting control siRNA transfection (left panel) or haspin siRNA-mediated knockdown (right panel) were labelled with indicated antibodies and viewed under the confocal microscope to look for association or exclusion of TAF5 from the mitotic chromosomes. DAPI stained condensed DNA and H3T3ph stained chromosomes served as indicators for cells in various stages of mitosis. At least 70% of cells that were formaldehyde fixed during various mitotic phases exhibited a similarity in the pattern of mitotic exclusion or non-exclusion of TAF5. In all, 99 of the 129 mitotic cells showed an exclusion of TAF5 from mitotic chromatin and 78 out of 125 mitotic cells retained TAF5 on chromatin upon haspin knockdown. Different siRNAs against haspin gave similar results. Scale bar is 10 μ m. **(C)** U2OS-GFP-TAF5 cells treated with Aurora B inhibitor ZM447439 or hesperadin (data not shown) for 2 h prior to fixation were screened for any effects on TAF5 localization due to a decrease in H3S10ph status. In all, 23 out of 27 mitotic cells showed exclusion of TAF5 from mitotic chromatin upon inhibition of Aurora B kinase. Scale bar is 10 μ m. **(D)** The ratio of chromosomal versus cytoplasmic-specific staining of TAF5 in **(B, C)** is quantified for 10 cells each and represented as a graph.

Crosstalk between H3 phosphorylation and methylation

Recognition of the H3K4me3 mark by TAF3 and TFIID is subjected to crosstalk at the histone H3 tail. We reported previously that asymmetric dimethylation of H3R2 selectively inhibits TFIID binding to H3K4me3 and that acetylation of H3K9 and H3K14 potentiates TFIID interaction, which most likely depends on the double bromo-domain of TAF1 (Vermeulen *et al*, 2007). H3R2me2a has also been shown to reduce the affinity of CHD1 and ING4 for H3K4me3 (Flanagan *et al*, 2005; Palacios *et al*, 2008). It is important to note that previous experiments indicated a mutual exclusion of H3K4me3 and H3R2me2a in promoter regions (Guccione *et al*, 2007; Kirmizis *et al*, 2007). In contrast to most H3K4me3-binding PHD domains, RAG2-PHD binding to H3K4me3 is enhanced rather than inhibited by dimethylation of H3R2 (Ramon-Maiques *et al*, 2007). Similar to the effects of H3R2me2a, we tested whether H3T3ph adjacent to H3K4me3 modulates effector proteins for H3K4me3. Using nuclear extracts, we found that TFIID binding to H3K4me3 is inhibited by H3T3ph (Figure 3A). Our H3 peptide-binding assays indicate the H3T3ph effect occurs at the level of the PHD of TAF3 (Figure 3C). H3T3ph also inhibits H3K4me3 binding of the PHDs of BPTF, ING2 and ING4 (Figure 3E) and H3 tail binding of the PHD of BHC80 (Supplementary Figure S6). A recently reported screen using a PTM-randomized combinatorial H3 peptide library found that phosphorylation of H3T3 resulted in a considerable attenuation of H3 interaction of several PHD modules (ING2, RAG2, BHC80 and AIRE) (Garske *et al*, 2010). Together, this indicates a general inhibitory role of H3T3ph and emphasizes the importance of H3T3ph in regulating the interaction of proteins with chromatin.

An important issue for crosstalk between different histone modifications is whether these marks actually cooccur *in vivo*. Several observations support cooccurrence of H3T3ph and H3K4me3 during mitosis. First of all, it is clear that the H3K4me3 mark persists throughout mitosis (Kouskouti and Talianidis, 2005). H3T3ph is a very abundant mark during mitosis and H3T3 phosphorylation is dependent on haspin (Polioudaki *et al*, 2004; Dai *et al*, 2005). Structural and functional characterization of the haspin kinase indicate that H3K4me3 reduces substrate recognition and kinase activity, suggesting a regulatory role of H3K4 methylation in deposition of H3T3ph by haspin (Eswaran *et al*, 2009). However, even though *in vitro* studies revealed that H3K9 methylation antagonizes H3S10 phosphorylation by Aurora B kinase (Rea *et al*, 2000), H3K9me3 and H3S10ph marks have been observed together *in vivo* using both mass spectrometry and dual modification H3K9me3S10ph-specific antibodies. Indeed, phosphorylation of H3S10 in mitosis displaces HP1 proteins bound to the adjacent H3K9me3 (Fischle *et al*, 2005; Garcia *et al*, 2005; Hirota *et al*, 2005). Similarly, cooccurrence of H3T3ph and H3K4me3 is supported by a recent study, which used combinatorial modification-specific antibodies to show that phosphorylated H3T3 is *in cis* to H3K4me3 (Markaki *et al*, 2009). These findings indicate that H3K4me3 and H3T3ph modifications can coexist on a single H3 molecule during mitosis.

TFIID and mitosis

TFIID association with mitotic chromatin has been addressed previously by cell biological and biochemical experiments.

Immunofluorescence staining and a limited ChIP study indicated that majority of TFIID leaves the condensed mitotic chromatin (Segil *et al*, 1996; Kieffer-Kwon *et al*, 2004; Blobel *et al*, 2009), although chromatin fractionation and other ChIP experiments indicated that at least some TFIID remains associated with chromatin during mitosis (Christova and Oelgeschlager, 2002; Xing *et al*, 2008). It is interesting to note that the promoters analysed by ChIP in these studies all contained the canonical TATA sequence. In this context, it is also interesting that MLL association with mitotic chromatin and persistence of the H3K4me3 mark (Kouskouti and Talianidis, 2005) has been proposed to facilitate inheritance of active gene expression states during cell division (Blobel *et al*, 2009). In our experiments, exclusion of TAF5 from the metaphase plate and anaphase chromosomes is clearly evident both in immunofluorescence of fixed cells and by live-cell imaging of GFP-tagged TAF5, but it is possible that a minor population of TFIID remains bound to mitotic chromatin. We speculate that H3T3 phosphorylation is delayed by the H3K4me3 mark, consistent with the reduced haspin activity observed on H3K4me3-containing peptide (Eswaran *et al*, 2009). This suggests a model in which the methylation state of the adjacent H3K4 residue has a function in the timing of depletion of PHD proteins from mitotic chromosomes. H3T3ph may be involved in displacement of a variety of proteins in mitosis (Garske *et al*, 2010; Higgins, 2010). Perhaps the graded inhibitory effect of H3K4 methylation regulates H3T3ph deposition on chromatin so that it will first displace proteins that do not require H3K4me3 for binding (like BHC80), before dislodging H3K4me3-binding proteins (like TAF3, ING2 etc.). High densities of H3K4me3 or strong core promoter binding may allow retention of TFIID at specific promoters. Such a mechanism could also serve to ensure that haspin (which is also present in interphase nuclei) displaces TFIID only in mitosis when its activity is high, and not in interphase. This principle could be relevant to regulation of other lysine-methylation switches.

Our combined results show that H3T3 phosphorylation by the mitotic haspin kinase has a negative effect on the transcription function of TAF3 and on the association of TFIID to mitotic chromosomes. This suggests that a 'phospho-methyl' switch involving H3T3 and H3K4 is relevant for the interaction of TFIID to chromatin. In case of TFIID, this switch may serve to allow a rapid reassociation of TFIID to H3K4me3-marked chromatin after chromosome segregation. Indeed, we observe a rapid association of TFIID during late anaphase, which occurs well before chromosome decondensation. We propose that this correlates with removal of the H3T3ph mark by the action of yet-to-be identified protein phosphatases.

Taken together, our experiments identify a new 'phospho-methyl' switch involving the haspin kinase and the PHD of TAF3 in regulating TFIID activity and chromatin association. Our results also highlight the importance of crosstalk between histone modifications during mitotic progression of the cell cycle.

Materials and methods**Plasmids, cell lines and recombinant protein expression**

The pMT2HA-mTAF3, pMT2-HA-mTAF3-M882A, GST-TAF3, GST-ING2, GST-BPTF, Gal4-Ash2L, 5X Gal4-MLP-luc and Renilla

TK-luc plasmids have been described previously (Vermeulen *et al*, 2007). For PHD-swaps of AIRE, BHC80, PHF2 and PHF8, chimeric constructs were obtained by overlap extension PCR of residues 1–850 of TAF3 and either residues 287–349 of AIRE, 437–497 of BHC80, 1–66 of PHF2 or 1–66 of PHF8 and further cloning into pDON201 by Gateway Cloning. The PCR templates for PHF2 (Clone ID: IRAKp961D08139Q) and PHF8 (Clone ID: DKFZp686E0868Q) were obtained from ImaGENES. For Gal4-Menin, the entry clone for N-terminal tagging of the *MEN1* cDNA, pENTRY-Menin-N, was generated by PCR amplification of the *MEN1* cDNA from vector pCDNA3.1M+ (a kind gift from Dr G Weber, Karolinska Hospital, Sweden) and recombination of the PCR product into vector pDONR (Invitrogen). The mammalian expression vector pBIND (Promega) for N-terminal Gal4 tagging was made compatible to the Gateway system by introducing the RFB recombination cassette, by ligation after *EcoR* V digestion. Subsequently, the *MEN1* cDNA was recombined from pENTRY-Menin-N into the pBIND (GW)-N vector. The details of primers used, cloning of TAF3-HFD, linker region deletion constructs, and sequences are available upon request. YFP-TAF10 and GST-ING4 were kind gifts from Dr L Tora (IGBMC, Strasbourg, France) and Dr O Gozani (Stanford University, CA), respectively. Gal4-ER α and Gal4-CBP were kindly provided by Dr E Kalkhoven (WKZ, Utrecht). Gal4-E2F, pcDNA-AIRE and BHC80 were kindly provided by Dr R Bernards (NKI, Amsterdam), Dr M Matsumoto (University of Tokushima, Tokushima) and Dr Y Shi (Harvard Medical School, Boston), respectively. Wild type and mutant myc-tagged haspin constructs and HeLa cells with inducible expression of EGFP-Haspin have been reported (Dai *et al*, 2005). Construction and characterization of U2OS GFP-TAF5 cells has been described (de Graaf *et al*, 2010).

Expression of GST-fusion proteins was induced, and soluble lysates were prepared essentially as described in Dominguez *et al* (2004).

Transient transfection, reporter assays and immunoblotting

Cells were cultured in DMEM containing 10% FBS (unless mentioned otherwise) and transfected in triplicates using FuGENE 6 (Roche Applied Science). Firefly reporter luciferase construct was cotransfected with TK or CMV promoter-driven Renilla luciferase plasmid as a control for transfection efficiency. Gal4-activator or coactivator fusions, TAF3 constructs and Myc-haspin WT or kinase-dead mutant constructs were also cotransfected. Cell lysates were prepared 40 h after transfection, and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). To analyse protein expression, whole cell lysates or protein fractions were analysed by immunoblotting using HA (3F10, Roche or 12CA5), Gal4-DBD (RK5C1, Santa Cruz, CA), α -tubulin (CP06, Calbiochem), haspin (ab21686, Abcam), TAF1 (6B3, Upstate), TAF5 (Christova and Oelgeschlager, 2002), TAF6 (25TA-2G7, Euromedex), TBP (20C7; van der Knaap *et al*, 1997), NC2 α (4G7; Gilfillan *et al*, 2005), GFP (11814460001, Roche), H3T3ph (07–424, Upstate), H3S10ph (14955, Abcam), H3K4me3 (ab8580, Abcam) and H4 (ab 31827, Abcam) antibodies and enhanced chemiluminescence for detection (ECL, Amersham Biosciences).

Immunoprecipitations, RNA isolation and RT-qPCR analysis

For RNA preparations, U2OS cells were seeded in six-well plates and transfected as described above in triplicates with wild type and mutant TAF3 constructs and pcDNA as a control. Cells were harvested 48 h post-transfection by trypsinization, washed with PBS and stored at -80°C . One tenth of the cells were kept separately and analysed by western blot with an anti-HA antibody. Total RNA was extracted from the rest of the cells using RNeasy Mini Kit (Qiagen) and quantified using NanoDrop (Thermo scientific). cDNA was synthesized using SuperScript III reverse transcriptase, oligonucleotide (dT) 12–18 (Invitrogen) and 500 ng of total RNA according to the manufacturer's protocol. mRNA levels were analysed by quantitative PCR on a Chromo4-equipped PCR cyclor (Bio-Rad) and normalized against a standard reference cDNA. For immunoprecipitations, transient transfection of plasmid DNA was performed in 293T cells using Polyethylenimine. At 40 h post-transfection, cells were harvested and lysed in IP buffer (50 mM Tris-HCl pH 8.0, 420 mM KCl, 5 mM MgCl $_2$, 0.5 mM EDTA, 0.1% NP40 and protease inhibitors). Cleared lysate was added to Anti-HA Affinity Matrix (Roche). Precipitated proteins were separated on 10% SDS-PAGE and blotted onto PVDF membranes. The membrane was developed with the appropriate antibodies and ECL (Pierce).

Peptide-binding assays and H3 peptide affinity measurements

H3 peptides immobilized on streptavidin-conjugated dynabeads (M280, Dynal) were incubated with HeLa nuclear extracts (prepared as described in Dignam *et al* (1983)), extracts from transfected U2OS cells overexpressing wild type or deletion constructs of TAF3 or crude bacterial lysates expressing GST-TAF3-PHD, GST-ING2-PHD, GST-BPTF-PHD, GST-ING4-PHD or GST-BHC-80 in binding buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP40, 10 mM ZnCl $_2$, 1 mM DTT, and protease and phosphatase inhibitors) for 2 h at 4°C . Following extensive washing, bound proteins were eluted and analysed by SDS-PAGE and Coomassie brilliant blue R-250 (Bio-Rad) and for experiments with GST-BHC80 and nuclear extracts or transfected lysates by subsequent immunoblotting. Peptide affinity measurements were performed by tryptophan fluorescence essentially as described in Vermeulen *et al* (2007).

Chromatin fractionation

Haspin expression in HeLa EGFP-Haspin cells was induced with 1 $\mu\text{g}/\text{ml}$ doxycycline treatment for 24 h and uninduced cells were left untreated. Before collecting by centrifugation, the cells were subjected to 10-min incubation with 1 μM Okadaic acid to maintain phosphorylation. Cell pellets were resuspended in hypotonic lysis buffer (10 mM Hepes pH 8.0, 1.5 mM MgCl $_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF and phosphatase inhibitors: 100 μM sodium (ortho)vanadate, 5 mM sodium pyrophosphate and 1 μM okadaic acid) and lysed using 27G syringe. One volume of $2\times$ extraction buffer (40 mM Hepes-KOH pH 8.0, 1.5 mM MgCl $_2$, 0.4 mM EDTA, 50% glycerol, 840 mM NaCl and protease and phosphatase inhibitors) was added dropwise and lysates were tumbled for 30 min at 4°C followed by centrifugation for 45 min at 225 000 g and 4°C . The supernatant was set aside as the soluble extract (SE), and the pellet was washed twice in wash buffer (20 mM Hepes-KOH pH 7.9, 420 mM NaCl, 340 mM sucrose, 0.1% Triton X-100, 1 mM EDTA, 1 mM β -mercaptoethanol and protease and phosphatase inhibitors) by homogenizing and centrifugation at 4°C for 30 min at 225 000 g, and the resulting supernatants were collected as wash 1 (W1) and wash 2 (W2), respectively. The chromatin pellet was homogenized in a nucleosome isolation buffer (20 mM Hepes-KOH pH 7.9, 250 mM sucrose, 10 mM MgCl $_2$, 0.1% Triton X-100, 3 mM CaCl $_2$, 5 mM β -mercaptoethanol and protease and phosphatase inhibitors), and the samples were incubated at 37°C for 5 min, prior to incubation with 10 U/ml micrococcal nuclease (Sigma) at 37°C . The reaction was stopped by the addition of 10 mM EGTA and cooling on ice. The soluble chromatin fraction (Chr) was obtained by centrifugation at 10 000 g for 10 min at 4°C .

siRNA knockdown, immunofluorescence and live-cell imaging

U2OS-GFP-TAF5 cells were grown on coverslips and transfected with either siRNA oligos only or siRNA oligos along with H2B-dsRed DNA by using the Dharmafect 1 reagent or Dharmafect Duo, respectively. Haspin siRNAs (D-005327-01 and D-005327-02), non-targeting control siRNA (D-001810-10) and siGlo Red transfection indicator (D-001630-02) oligos were purchased from Dharmacon. Haspin siRNA knockdown with both of the oligos gave comparable results. For immunolabelling, 40 h after transfection, cells were fixed with 4% formaldehyde/PBS for 20 min, permeabilized using 0.2% Triton X-100, blocked in 5% FBS/PBS for over 30 min; then incubated with anti-GFP, anti-H3T3ph, anti-H3S10ph in 5% FBS/PBS for 1 h at room temperature followed by a 30-min incubation in goat anti-mouse IgG-Alexa488 or goat anti-rabbit IgG-Alexa568 (Invitrogen). To detect DNA, 10 $\mu\text{g}/\text{ml}$ DAPI was used. Fluorescence microscopy was performed using a Zeiss 510 Meta confocal microscope with a $\times 63$ 1.4NA Plan-ApoChromat objective.

For live-cell imaging, transfected cells in LabTekII four-well chamber slides were released from an 18-h thymidine block to obtain as many cells in the same phase of the cell cycle. Live-cell imaging was done at the DeltaVision RT system (Applied Precision) in a heated chamber (37°C) with a $\times 40$ or $\times 60/1.42\text{NA}$ PlanApoN oil objective (Olympus) using Softworx software. Green fluorescent (200 ms exposure) and red fluorescent (175 ms exposure) images were acquired every 2 min using a CoolSnap HQ/ICX285. Images were processed using ImageJ software. Images are maximum intensity projections of all Z planes.

Supplementary data

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

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Conflict of interest

The authors declare that they have no conflict of interest.

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