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KAT–independent gene regulation by Tip60 promotes ESC self-renewal but not pluripotency

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Summary

Although histone-modifying enzymes are generally assumed to function in a manner dependent on their enzymatic activities, this assumption remains untested for many factors. Here we show the Tip60 (Kat5) lysine acetyltransferase (KAT), which is essential for embryonic stem cell (ESC) self-renewal and pre-implantation development, performs these functions independently of its KAT activity. Unlike ESCs depleted of *Tip60*, KAT–deficient ESCs exhibited minimal alterations in gene expression, chromatin accessibility at Tip60 binding sites, and self-renewal, thus demonstrating a critical KAT–independent role of Tip60 in ESC maintenance. In contrast, KAT–deficient ESCs exhibited impaired differentiation into mesoderm and endoderm, demonstrating a KAT–dependent function in differentiation. Consistent with this phenotype, KAT–deficient mouse embryos exhibited post-implantation developmental defects. These findings establish separable KAT–dependent and KAT–independent functions of Tip60 in ESCs and during differentiation, revealing a complex repertoire of regulatory functions for this essential chromatin remodeling complex.

Graphical abstract

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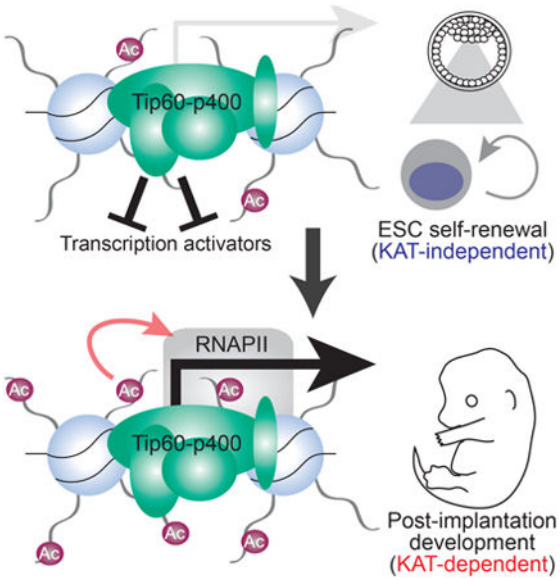

Keywords

chromatin; embryonic stem cells; Tip60; Kat5; acetyltransferase; self-renewal; differentiation; development

Introduction

Embryonic stem cells (ESCs)—cells derived from the inner cell mass of the early blastocyst—have been utilized as an *in vitro* model of differentiation due to their pluripotency and unlimited capacity for self-renewal in culture (Keller, 2005). A complex array of signaling pathways and transcription factors control ESC fate, promoting self-renewal in the presence of either leukemia inhibitory factor (LIF) or inhibitors of differentiation-promoting kinases MEK1/2 and Gsk3β (Ying et al., 2008). In addition to transcription factors, a number of chromatin regulatory proteins help control the expression of pro-self-renewal and pro-differentiation genes (T. Chen and Dent, 2014). Although dozens of chromatin regulators necessary for ESC self-renewal or differentiation have been identified, the specific contributions of many chromatin regulatory proteins to ESC self-renewal and differentiation are poorly understood, due to the redundant and context-dependent contributions of most chromatin modifications to gene expression (Rando and Chang, 2009).

Previously we showed that RNAi-mediated knockdown (KD) of components of the well-conserved Tip60-p400 (also called NuA4) chromatin regulatory complex resulted in multiple defects in ESC pluripotency (Fazzio et al., 2008a). ESCs depleted of Tip60-p400 subunits exhibit cell and colony morphologies indicative of differentiation and reduced expression of some pluripotency markers. However, Tip60-p400-depleted cells are also defective in normal ESC differentiation, forming small, abnormal embryoid bodies under differentiation conditions that fail to upregulate some markers of differentiated cells (P. B. Chen et al., 2013; Fazzio et al., 2008a). Consistent with this self-renewal defect, homozygous knockout of the *Tip60* gene in mouse results in embryonic lethality at approximately the blastocyst

stage (the stage at which ESCs are derived) (Hu et al., 2009). *Tip60*$^{-/-}$ blastocysts are morphologically abnormal and fail to hatch from the zona pellucida when cultured *in vitro*. No post-implantation *Tip60*$^{-/-}$ embryos were observed, demonstrating an absolute requirement for *Tip60* at or before this stage.

Tip60-p400 has two biochemical activities that contribute to its functions within the nucleus. The Tip60 subunit is a lysine acetyltransferase (KAT) that targets histones H4, H2A, H2A variants, and non-histone proteins (Ikura et al., 2000; Keogh et al., 2006; Squatrito et al., 2006; Xu and Price, 2011). Histone acetylation near gene promoters or enhancers is strongly associated with gene expression, consistent with Tip60's known function as a co-activator that collaborates with numerous transcription factors (Squatrito et al., 2006). In addition to its role as a co-activator, Tip60 also directly regulates the activities of numerous transcription factors through acetylation of lysine residues (Farria et al., 2015). Finally, besides regulation of transcription, Tip60 plays important roles in DNA damage repair, senescence, and apoptosis (Doyon et al., 2004; Ikura et al., 2000; Kusch et al., 2004; Xu and Price, 2011) (Jiang et al., 2011; Sykes et al., 2006; Tang et al., 2006; Van Den Broeck et al., 2012). Importantly, the KAT activity of Tip60 has been shown to be essential for its role in each of these processes.

The second chromatin remodeling activity found within Tip60-p400 complex is catalyzed by the p400 subunit (gene name: *Ep400*). The p400 protein, like its homologs in other eukaryotes, catalyzes ATP-dependent incorporation of histone H2A variant H2A.Z into chromatin via exchange of H2A-H2B dimers within nucleosomes for free H2A.Z-H2B dimers (Gévry et al., 2007; Mizuguchi et al., 2004). Interestingly, p400 was recently shown to incorporate histone H3 variant H3.3 into chromatin (Pradhan et al., 2016). H2A.Z and H3.3 are often enriched near gene regulatory regions, consistent with a role for p400 (like Tip60) as a co-activator of transcription (Melters et al., 2015). However, p400 also appears to repress transcription in some contexts, as well as promote DNA repair in concert with Tip60 (Gévry et al., 2007; Papamichos-Chronakis et al., 2011; Xu et al., 2012).

How does Tip60-p400 promote ESC self-renewal and pre-implantation development? Tip60-p400 binds near the promoters of both active genes and lowly expressed developmental genes in ESCs, and acetylates the promoter-proximal histones of both groups (P. B. Chen et al., 2013; 2015; Fazzio et al., 2008a; Ravens et al., 2015). Given the well-established activating roles of histone acetylation, these data imply that Tip60-p400 may drive expression of highly expressed housekeeping and pluripotency genes, but that its developmental targets are resistant to this activation, possibly due to the repressive activities of Polycomb complexes or other factors (Aloia et al., 2013; Simon and Kingston, 2013). However, this model is unlikely to be correct, since Tip60-p400 is largely dispensable for transcriptional activation in ESCs, and instead functions mainly to repress its developmental targets (P. B. Chen et al., 2013; Fazzio et al., 2008a; 2008b). Therefore, the Tip60 KAT activity must either inhibit transcription of developmental genes in ESCs, or repression of these genes by Tip60-p400 is KAT–independent.

Here we show that Tip60 functions independently of its KAT activity to repress differentiation genes in ESCs and promote ESC self-renewal. Consistent with this repressive

function, Tip60 limits promoter-proximal chromatin accessibility at many Tip60 target genes, and this function is similarly KAT–independent. By contrast, KAT–deficient ESCs are impaired for differentiation, revealing a critical role for the Tip60 KAT activity in pluripotency. Upon induction of differentiation, KAT mutant ESCs exhibit defects in production of mesoderm and endoderm cell types, due to reduced induction of numerous key drivers of differentiation. Unlike *Tip60* null mice (Hu et al., 2009), KAT–deficient mutant mice proceed past the blastocyst stage, consistent with the ability of KAT mutant ESCs to self-renew. However, KAT mutant mice exhibit post-implantation developmental defects beginning around the start of gastrulation, analogous to the ESC differentiation defect observed *in vitro*. Together, these findings establish separable KAT–independent and KAT–dependent roles of Tip60 in pluripotency and embryonic development that are both essential, but which act at different stages.

Results

Tip60 KAT activity is dispensable for gene regulation and self-renewal in ESCs

Tip60 is one of several HATs that acetylate the N-terminal tails of histones H4 and H2A, whereas p400 is one of two SWI/SNF family ATPases that mediate H2A.Z deposition (Altaf et al., 2009; Lalonde et al., 2014). To test the importance of these activities in ESCs, we generated independent ESC lines with homozygous mutations encoding amino acid substitutions in the acetyl CoA binding site of Tip60 ($Tip60^{ci/ci}$) or the ATP-binding pocket of p400 ($Ep400^{ci/ci}$; Figure S1A-B), both of which were previously shown to block enzymatic activity (Ikura et al., 2000; Xu et al., 2010). We confirmed that these mutations broadly reduced H4 acetylation and H2A.Z deposition, respectively, in ESCs (Figure S1C-D). Since *Tip60* or *Ep400* depletion in ESCs causes loss of self-renewal (Fazzio et al., 2008a), we utilized previously validated shRNAs (P. B. Chen et al., 2013) to perform acute KD of *Tip60* or *Ep400*, along with an *Ep400* hypomorphic mutant ($Ep400^{hypo}$) that exhibits reduced levels of p400 protein (Figure S1E), for comparison. Surprisingly, $Tip60^{ci/ci}$ and $Ep400^{ci/ci}$ lines had normal ESC morphology and maintained expression of pluripotency markers such as alkaline phosphatase (AP; Figure 1A), and SSEA-1 (Figure S1F), whereas *Tip60* KD or $Ep400^{hypo}$ cells exhibited reduced AP and SSEA-1 staining and flattened colony morphologies, as observed previously (P. B. Chen et al., 2015; Fazzio et al., 2008a). $Tip60^{ci/ci}$ and $Ep400^{ci/ci}$ cells proliferated more rapidly than *Tip60* KD and $Ep400^{hypo}$ cells (Figure 1B), although $Tip60^{ci/ci}$ cells proliferated slightly less rapidly than wild type controls. Finally, to test for functional redundancy, we constructed double homozygous mutant $Tip60^{ci/ci}$ $Ep400^{ci/ci}$ lines. As with the single mutants, these lines expressed markers of pluripotent stem cells and normal ESC colony morphology, similar to that of $Tip60^{ci/ci}$ single mutants (Figure S1F-G). These data suggest loss of Tip60 KAT activity and p400 ATP-dependent nucleosome remodeling activity have minimal effects on ESC maintenance.

To test whether gene expression is altered in $Tip60^{ci/ci}$ and $Ep400^{ci/ci}$ mutant ESCs, in spite of their normal self-renewal, we performed RNA-seq on biological replicates of $Tip60^{ci/ci}$ and $Ep400^{ci/ci}$ mutants, along with positive and negative controls. Consistent with previous findings (P. B. Chen et al., 2013; Fazzio et al., 2008a), *Tip60* KD and $Ep400^{hypo}$ cells each exhibited up-regulation of numerous genes enriched for developmental factors, and down-

regulation of a smaller group of genes (Figure 1C-F, Figure S2A-B). In contrast, few genes were significantly altered in *Tip60*$^{ci/ci}$, *Ep400*$^{ci/ci}$, or *Tip60*$^{ci/ci}$ *Ep400*$^{ci/ci}$ double mutants (Figure 1C-F, Figure S2C-F). These data demonstrate that while Tip60 and p400 are necessary for gene regulation and self-renewal in ESCs, their catalytic activities are dispensable for these processes.

KAT–independent regulation of promoter-proximal chromatin accessibility by Tip60

Since KATs function mainly as co-activators of gene expression, we next focused on how Tip60 functions independently of its KAT activity to repress transcription in ESCs. We confirmed normal expression of Tip60 and p400 in *Tip60*$^{ci/ci}$ *Ep400*$^{ci/ci}$ ESCs (Figure S3A), and found that *Tip60*$^{ci/ci}$ and *Ep400*$^{ci/ci}$ ESCs assemble intact Tip60-p400 complexes with compositions similar to that of wild type cells, in contrast to *p400*^{hypo} mutant ESCs (Figure S3B).

Given its size (~1.5 MDa; 17 core subunits), we considered the possibility binding of Tip60-p400 complex reduces the accessibility of underlying chromatin, regardless of its enzymatic functions. To test this possibility, we performed ATAC-seq (Buenrostro et al., 2013) to quantify changes in chromatin accessibility at Tip60 binding sites. In *Tip60*$^{fl/+}$ control ESCs (expressing wild type *Tip60*), chromatin accessibility is higher at Tip60 binding sites than flanking regions (Figure 2A-B), consistent with the observed enrichment of Tip60 near gene regulatory elements such as promoters and enhancers (P. B. Chen et al., 2013; Fazzio et al., 2008a; Ravens et al., 2015). Interestingly, we observed significantly increased chromatin accessibility upon *Tip60* KD, but minimal changes in accessibility in KAT–deficient ESCs (Figure 2A-B). Clustering of these data identified two prominent patterns of chromatin accessibility, segregated primarily by whether the Tip60–binding sites were promoter-proximal or -distal (Figure 2C). Examination of promoter-proximal regions of Tip60 target genes revealed that *Tip60* KD increased chromatin accessibility within a several hundred base pair window extending from the promoter into the gene body, corresponding to Tip60-p400 binding sites on chromatin (Figure 2D) (P. B. Chen et al., 2015; 2013; Ravens et al., 2015). In contrast, KAT-deficient ESCs were minimally affected. Unlike promoter-proximal regions, chromatin accessibility at gene-distal Tip60–binding sites was relatively unaltered by *Tip60* KD or KAT mutation (Figure 2E). Consistent with these findings, KAT–deficient Tip60 bound to Tip60-p400–target genes at levels similar to wild type (Figure S3C). These data demonstrate Tip60 functions independently of its KAT activity to regulate promoter-proximal chromatin accessibility in ESCs.

Tip60 KAT activity is necessary for differentiation and post-implantation development

Consistent with the self-renewal defect of *Tip60* KD ESCs (Fazzio et al., 2008a), *Tip60* homozygous null (*Tip60*$^{-/-}$) mice die at the peri-implantation stage: *Tip60*$^{-/-}$ blastocysts fail to hatch and survive in culture, and no post-implantation *Tip60*$^{-/-}$ embryos can be recovered (Hu et al., 2009). Since *Tip60*$^{ci/ci}$ ESCs self-renew normally, we next tested whether the Tip60 KAT activity is also dispensable for mouse development. To this end, we generated and intercrossed *Tip60*$^{ci/+}$ heterozygous mice to produce *Tip60*$^{ci/ci}$ homozygotes (see Experimental Procedures for details). However, we recovered no *Tip60*$^{ci/ci}$ pups at birth (χ^2= 40.45; P < 0.001), suggesting the Tip60 KAT activity is essential for development

(Figure 3A). To elucidate the developmental defect of *Tip60*$^{ci/ci}$ animals, we examined the morphology of embryos at multiple stages. *Tip60*$^{ci/ci}$ embryos were recovered as late as 10.5 days post fertilization (E10.5; Figure 3A), but were much smaller than *Tip60*$^{+/+}$ or *Tip60*$^{ci/+}$ littermates (Figure 3B), and exhibited morphological abnormalities as early as E6.5 (Figure S4A-C). The contrasting phenotypes between *Tip60*$^{-/-}$ and *Tip60*$^{ci/ci}$ mice reveal an essential KAT–independent role for Tip60 in pre-implantation development, as well as an essential KAT–dependent role in early post-implantation development.

The phenotypes of *Tip60*$^{ci/ci}$ embryos are evident at or just before gastrulation, where the three primary germ layers are established, suggesting that although *Tip60*$^{ci/ci}$ ESCs self-renew normally, they may not differentiate properly. We tested this possibility using embryoid body differentiations of control, *Tip60* KD, and *Tip60*$^{ci/ci}$ ESCs. Previously, we showed that KD of *Tip60*, *Ep400*, or (Tip60-p400 subunit) *Dmap1* resulted in defects in EB formation (P. B. Chen et al., 2013; Fazzio et al., 2008a), suggesting Tip60-p400 is required for this initial step of differentiation. In contrast, *Tip60*$^{ci/ci}$ ESCs efficiently formed EBs, which expanded in culture at near wild type levels, although modest differences in EB morphology were observed relative to *Tip60*$^{fl/+}$cells (Figure 3C-D). However, induction of mesodermal and endodermal markers was delayed and/or reduced in *Tip60*$^{ci/ci}$ EBs (Figure 3E) compared to *Tip60*$^{fl/+}$ controls. These data suggest that the Tip60 KAT activity is important for specification of mesodermal and endodermal cell types *in vitro*.

To test whether the ESC differentiation defects were recapitulated *in vivo*, we stained post-implantation *Tip60*$^{ci/ci}$ embryos for *T* (also known as *Brachyury*), a marker of cells migrating through the primitive streak to become mesodermal or endodermal cell types (Herrmann, 1991; Rivera-Pérez and Magnuson, 2005). Although *T* staining of *Tip60*$^{+/+}$ and *Tip60*$^{ci/+}$ embryos was evident at E6.5 and strong at E7.5, *Tip60*$^{ci/ci}$ embryos exhibited reduced staining at both stages (Figure 3F-G). These data show that gastrulation is delayed or impaired in *Tip60*$^{ci/ci}$ embryos. This phenotype could result from impaired lineage commitment, poor migration of cells through the primitive streak, or other factors. Regardless, this developmental defect is consistent with the impaired induction of early mesodermal and endodermal markers observed for KAT-defective ESCs *in vitro*.

Impaired expression of multiple drivers of differentiation in KAT–deficient ESCs

What is the molecular basis for the *in vivo* and *in vitro* developmental defects of *Tip60*$^{ci/ci}$ mutants? These phenotypes could result from failure to upregulate key lineage-specific transcription factors and/or a disruption in signaling pathways that promote lineage commitment. To address these possibilities, we compared the changes in gene expression during a time course of differentiation of control (*Tip60*$^{fl/+}$) and *Tip60*$^{ci/ci}$ ESCs using RNA-seq on biological replicate samples. We observed differences in both the timing and levels of markers of mesoderm and endoderm (Figure 4A; e.g. *FoxA2*, *Gata4*, *Sox17*, *T*, *Hand1*, *Flk1*), expanding on our preliminary analyses (Figure 3E). Next we used k-means clustering to identify groups of genes induced early or late during differentiation in *Tip60*$^{fl/+}$ control cells and characterized the effects of the KAT mutation on their induction. We observed 1,338 genes of this type that mainly fall into three clusters based on the timing of their expression peak (Figure 4B). In *Tip60*$^{ci/ci}$ cells, we observed reduced or delayed induction

of numerous genes with key roles in differentiation, including developmental transcription factors and mediators of growth factor signaling, within each of the three clusters (Figure 4B).

To test whether impaired induction of key signaling proteins hindered activation of their downstream targets, we examined activation of the FGF/MEK/ERK and TGF-β pathways using antibodies recognizing the phosphorylated (and activated) forms of ERK1/2 and Smad2/3, respectively (Tsang and Dawid, 2004; Whitman and Mercola, 2001). These factors act downstream of FGF and BMP signaling in differentiating ESCs and embryos, and are critical for differentiation (Sui et al., 2013). Although Smad2/3 phosphorylation was unaltered in differentiating *Tip60*$^{ci/ci}$ ESCs, we observed impaired ERK phosphorylation in these mutants after six days of differentiation (Figure 4C). Together, these data suggest that the differentiation defect observed in *Tip60*$^{ci/ci}$ ESCs is due to at least two overlapping defects: delayed or reduced activation of ERK, and impaired induction of key developmental transcription factors.

Discussion

Here we showed that Tip60 functions in ESC gene regulation and self-renewal, as well as pre-implantation development, independently of its KAT activity. This finding was unexpected because Tip60 depletion or knockout leads to a self-renewal defect in ESCs and pre-implantation lethality in mice (Fazzio et al., 2008a; Hu et al., 2009). Furthermore, KAT–impaired mutants of *esa1,* the yeast homolog of *Tip60*, are severely growth impaired (Selleck et al., 2005), suggesting the critical cellular functions of this KAT are dependent on its acetylation activity.

The fact that Tip60 is largely a repressor of transcription in ESCs (Fazzio et al., 2008a), and this repressive function is independent of its KAT activity, suggests that Tip60 regulates ESC gene expression in a manner that is distinct from other well-studied KATs, at least in part. Consistent with its role as a broadly acting repressor of transcription in ESCs, we found Tip60 functions by a KAT–independent mechanism to limit chromatin accessibility directly over its promoter-proximal binding sites at many target genes. Additional studies will be necessary to determine whether Tip60 also performs this function in somatic cell types.

In contrast, the Tip60 KAT activity is essential during ESC differentiation and post-implantation development. Consequently, these findings demonstrate separable, essential functions of Tip60: its KAT-independent function is sufficient for Tip60's essential role in ESC self-renewal and pre-implantation development, and its KAT–dependent function is required for post-implantation development and ESC differentiation. Interestingly, we found that the ATP-dependent histone exchange activity of p400 was also dispensable for gene regulation and self-renewal in ESCs, revealing that Tip60-p400 complex represses differentiation genes in ESCs independently of its known chromatin remodeling activities (Figure 4D). These findings necessitate a re-evaluation of current models of gene regulation by this essential chromatin regulatory complex.

What is the role of the Tip60 KAT activity during development? Given the defect of KAT-deficient ESCs and embryos in lineage specification, one possibility is that histone acetylation at differentiation genes in ESCs (as observed previously (Fazzio et al., 2008a)) facilitates their up-regulation when differentiation is induced. This provides a potential explanation for the counterintuitive role of Tip60 in repression of differentiation genes in ESCs–occupancy of Tip60-p400 at differentiation gene promoters helps repress these genes by reducing chromatin accessibility, while acetylation at these loci may allow more rapid induction after binding of differentiation-specific transcription factors. Together, these data show that not all functions of Tip60 are reliant on its KAT activity, and raise the possibility that KAT–independent gene regulation by Tip60 plays important roles in additional cell types.

Experimental Procedures

Antibodies

Antibodies used in this study: p400 (A300-541A; Bethyl), StainAlive™ SSEA-1 (09-0067; Stemgent); Smad2/3 (8685; Cell Signaling Technologies); Phospho-Smad2/3 (8828; Cell Signaling Technologies); Erk1/2 (9102; Cell Signaling Technologies); Phospho-Erk1/2 (9101; Cell Signaling Technologies); H2AZ (ab4174, Abcam); Acetyl-H4 (06-598; Millipore); FLAG-M2 (F1804; Sigma); IgG (ab37415; Abcam); β-actin (A5316; Sigma).

Cell Lines

Mouse ESC lines were derived from E14 (129/Ola) (Hooper et al., 1987) and grown as described (P. B. Chen et al., 2013). *Tip60*$^{ci/ci}$ ESCs were derived from floxed Tip60-H3F cells (P. B. Chen et al., 2013), by introduction of Cre recombinase (Addgene, 20781) to loop out wild type *Tip60* regions upstream of exon 11 that harbors two substitution mutations (Q377E and G380E) that eliminate acetyl CoA binding (Ikura et al., 2000) (figure S1A).

Catalytically inactive mutants of p400 (*Ep400*$^{ci/ci}$) were generated using homologous recombination stimulated by CRISPR/Cas9-mediated cleavage (Cong et al., 2013; Mali et al., 2013). A repair template (Table S3) was synthesized (Integrated DNA Technologies), cloned into pCR2.1, and introduced together with the CRISPR/Cas9 vector (a variant of plasmid pX330 that expresses puromycin resistance). The *Ep400*^{hypo} mutant line, described previously (P. B. Chen et al., 2015), was generated using the same CRISPR/Cas9 construct, but without the repair template, resulting in a homozygous 135bp in-frame deletion that disrupts the ATPase domain and results in lower expression of p400 protein (Figure S1E).

ESC differentiation

Embryoid bodies (EBs) for growth/morphology assays were formed using hanging drops containing 100 cells in 10 ul of differentiation medium. Morphology was examined after 48 hours. For gene expression assays, 10^6 ESCs were plated on non-adherent plates for 48 hours to form EBs, and then transferred into gelatinized 6-well plates at a low density. Cells were harvested using TRIzol reagent (Invitrogen) at indicated time points. RNA was prepared and RT-qPCR was performed as described (P. B. Chen et al., 2013), using primers listed in Table S1.

Cell Staining

10^5 ESCs were grown on gelatin-coated 6-well plates for 48 hours. Alkaline phosphatase (AP) staining was performed using a kit (EMD Millipore, SCR004), following the manufacturers' guidelines. SSEA-1 staining of live ESCs was also performed per the manufacturers' instructions (Stemgent, 09-0067).

Tip60-p400 Purification

Tip60-p400 complex was purified from nuclear extracts of WT, $Tip60^{ci/ci}$, $p400^{ci/ci}$, and $p400^{hypo}$ cells with endogenous 6Xhis/3XFLAG tags at the *Tip60* locus, as described previously (P. B. Chen et al., 2013). Purified proteins were separated on SDS-PAGE gels, and Silver Staining was performed using a Silver Staining Kit (ThermoFisher, LC6100).

Western Blotting

30ug of nuclear extract per lane (prepared using the NE-PER kit; ThermoFisher, 78833) were used for Western blotting.

Generation of *Tip60$^{ci/ci}$* mice

Tip60$^{ci/+}$ heterozygous mice were generated by crossing *Tip60* floxed mice (P. B. Chen et al., 2013) with the allele described above with Tg(EIIa-cre) mice, which broadly express Cre recombinase (Dooley et al., 1989; Lakso et al., 1996). Mice were genotyped by PCR with primers listed in Table S2. *Tip60$^{ci/+}$* mice were maintained as heterozygotes on an inbred FVB/N background and intercrossed to generate *Tip60$^{+/+}$*, *Tip60$^{ci/+}$*, and *Tip60$^{ci/ci}$* embryos. Animal studies were performed in accordance with guidelines of the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School (A-2165) and NIH.

RNA in situ hybridization

Whole mount in situ hybridization was performed as previously described (Rivera-Pérez and Magnuson, 2005), using a full-length cDNA probe of *T* (Herrmann, 1991). Embryos were genotyped after staining by PCR, using primers listed in Table S2.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation and deep sequencing were performed as described previously (P. B. Chen et al., 2013; Hainer et al., 2015). ChIP-qPCR was performed using SYBR FAST (KAPA Biosystems) with primers described previously (Fazzio et al., 2008a).

RNA-seq

Strand specific library construction and RNA-seq were performed by Applied Biological Materials, Inc. and the UCLA Clinical Microarray Core for ESCs and differentiating ESCs, respectively. Data analysis is described in Supplemental Experimental Procedures.

ATAC-seq

ATAC-seq was performed essentially as described (Buenrostro et al., 2013; 2015). Two independent ATAC reactions per biological replicate were performed, using 35,000 and

70,000 ESCs each. After library preparation, the two reactions were found to have indistinguishable distributions of fragment sizes, and were therefore combined for sequencing. (Therefore, each biological replicate consisted of two ATAC reactions.) Data analysis is described in Supplemental Experimental Procedures.

Statistical Methods

For non-genomic in vitro experiments, two tailed t-tests were used to calculate statistical significance. A chi-square test was used to evaluate genotypes of offspring from Tip60ci/+ intercrosses. Adjusted p-values were calculated for RNA-seq data using DEseq2. Significance of differences in ATAC-seq read enrichment were calculated by a hypergeometric test using the *dhyper* package in R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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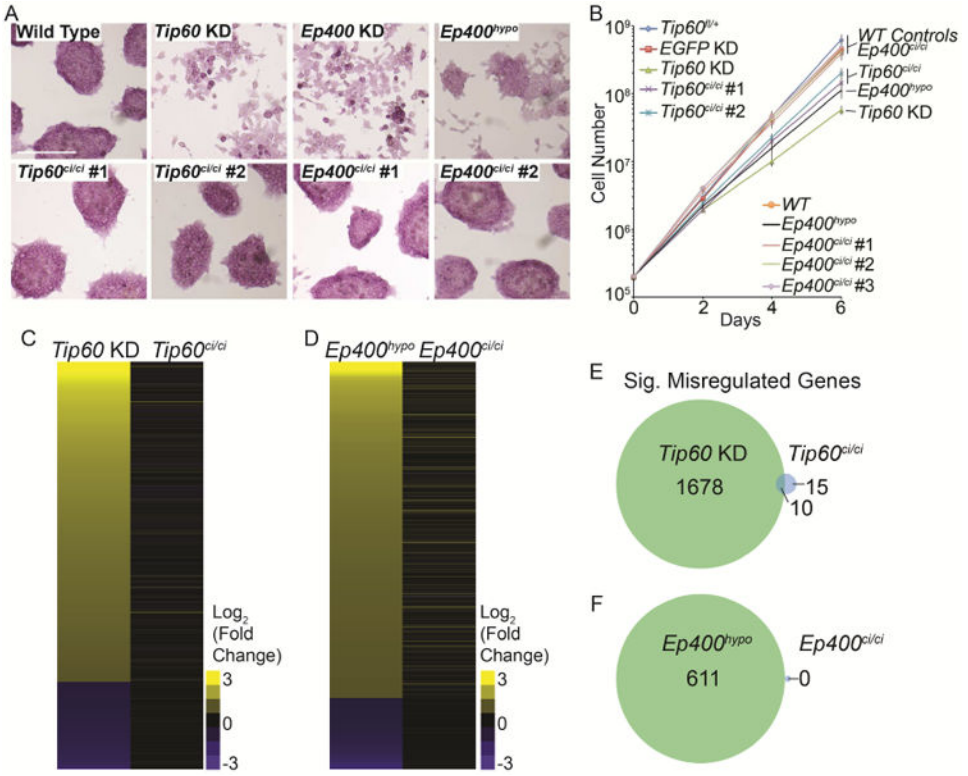

Figure 1. Tip60 KAT and p400 ATPase activities are dispensable for ESC self-renewal and gene regulation

(**A**) Alkaline phosphatase staining (AP) of *Tip60*$^{ci/ci}$ and *Ep400*$^{ci/ci}$ mutants and controls (*Tip60*$^{fl/+}$, *Tip60* KD, *Ep400* KD, and *Ep400*^{hypo}). Scale bars equal 200 μm. (**B**) Growth curve, measuring the proliferation rates of the indicated mutant and control ESCs. (**C, D**) Heatmaps of differentially expressed genes in *Tip60*$^{ci/ci}$ and *Tip60* KD ESCs relative to *Tip60*$^{fl/+}$control cells (**C**), or *Ep400*$^{ci/ci}$ and *Ep400*^{hypo} ESCs relative to wild type (E14) control ESCs (**D**). Genes in the heatmaps are sorted from the most upregulated to the most down regulated genes in the *Tip60* KD and *Ep400*^{hypo} controls, respectively. (**E, F**) Venn diagrams showing number of genes commonly misregulated in *Tip60*$^{ci/ci}$ and *Tip60* KD ESCs (**E**), or *Ep400*$^{ci/ci}$ and *Ep400*^{hypo} ESCs (**F**). Genes were considered significantly misregulated in each KD or mutant if their $|\log_2 (\text{fold change})| > 1$ and their multiple testing-adjusted p value < 0.05.

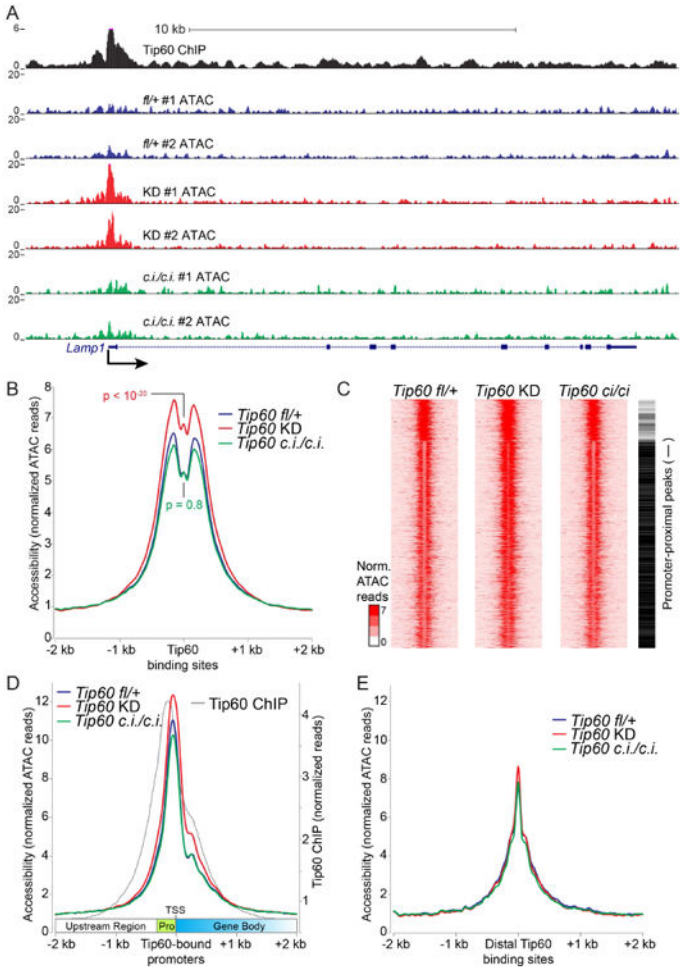

Figure 2. KAT-independent regulation of chromatin accessibility at Tip60 target loci

(**A**) Example Tip60 target gene showing increased promoter-proximal chromatin accessibility in *Tip60* KD but not $Tip60^{ci/ci}$ relative to $Tip60^{fl/+}$ control cells. Shown are normalized ATAC-seq reads ≤ 100bp for each biological replicate, and Tip60 ChIP-seq data from (Ravens et al., 2015). (**B**) Aggregation plot showing average ATAC-seq signal for two biological replicates of each mutant or KD aggregated over high-quality Tip60 binding sites. A Kolmogorov–Smirnov test of differences in ATAC profiles was used to calculate p values. (**C**) K-means clustering (K=3) for ATAC-seq data over Tip60 binding sites. Promoter-proximal peaks are marked with a black bar to the right, promoter-distal peaks with a white bar. (**D**) Aggregation plot of ATAC-seq data (as in B) over Tip60–bound promoter regions aligned such that all gene bodies are to the right. Promoter-proximal regions (pro) and transcription start sites (TSS) are indicated. Tip60 ChIP-seq data (Ravens et al., 2015) are shown for reference. (**E**) Aggregation plot over Tip60–bound gene-distal regions.

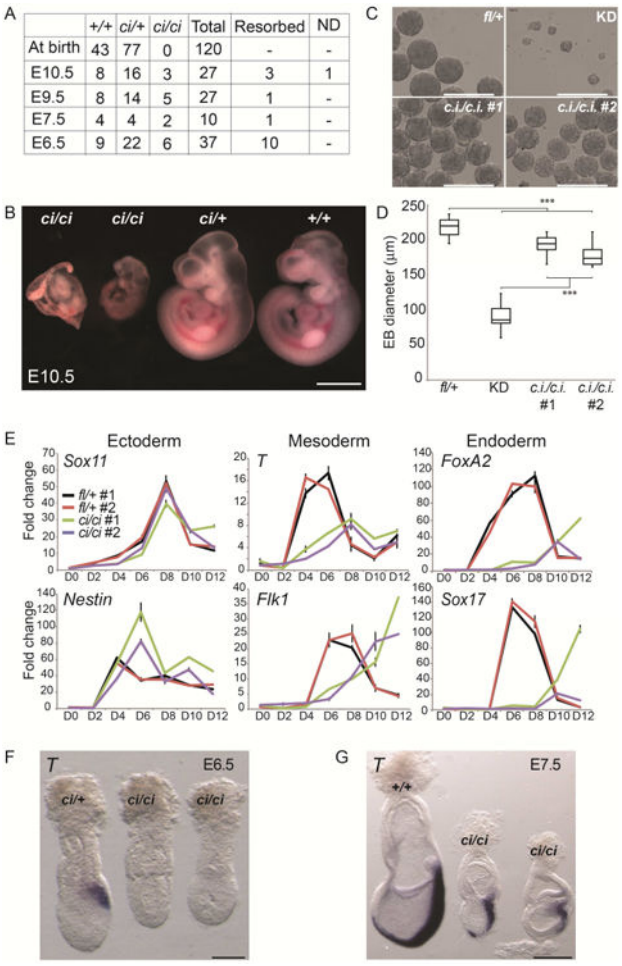

Figure 3. The *Tip60* catalytic activity is required for differentiation and post-implantation development

(**A**) Genotypes of embryos from *Tip60*$^{ci/+}$ intercrosses at different developmental stages. (**B**) Images of E10.5 embryos of the indicated genotypes. Scale bar equals 1 mm. (**C**) Embryoid body (EB) formation assay comparing EB morphology in *Tip60*$^{ci/ci}$ mutant ESCs to *Tip60*$^{fl/+}$ and *Tip60* KD controls. Scale bars equal 400 µm. (**D**) Quantification of EB size in indicated mutants and controls (n = 49 per genotype). Boxes range from the 25th to the 75th percentile, the dark lines indicate the median, and the whiskers indicate the lesser of either the extreme (max or min) value or 1.5 times the interquartile range (***$p < 0.001$, calculated using a two-sided t-test). (**E**) RT-qPCR analysis of indicated germ layer markers during a time course of EB differentiation. (**F, G**) Whole mount *in situ* hybridization in E6.5 and E7.5 mouse embryos staining for *T* transcript. Scale bars equal 100 µm (**F**) or 250 µm (**G**).

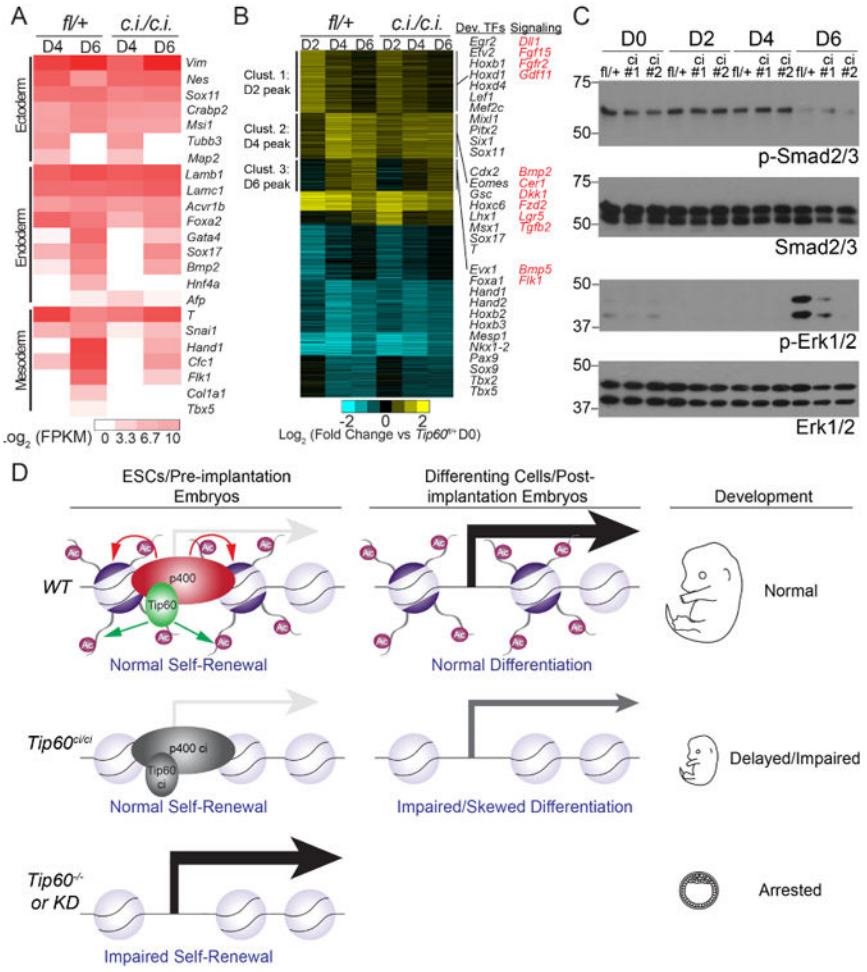

Figure 4. Delayed/impaired expression of developmental regulators in differentiating *Tip60*$^{ci/ci}$ ESCs

(**A**) Heatmap indicating induction kinetics of each germ layer markers during differentiation of *Tip60*$^{fl/+}$ controls or *Tip60*$^{ci/ci}$ mutant ESCs. (**B**) K-means clustering (K = 9) of differentially expressed genes [|log2 (fold change)| > 0.7; multiple testing-adjusted p value < 0.05] in *Tip60*$^{fl/+}$ controls or *Tip60*$^{ci/ci}$ mutant ESCs during the differentiation time course. Large up-regulated clusters are noted. Key regulatory proteins with impaired induction in *Tip60*$^{ci/ci}$ mutant ESCs are highlighted. (**C**) Western blots (one of two independent experiments with similar results) of phosphorylated and total Smad2/3 and Erk1/2 during differentiation in *Tip60*$^{fl/+}$ or *Tip60*$^{ci/ci}$ ESCs. (**D**) Model indicating the KAT-independent role of Tip60 in ESC self-renewal and gene regulation, as well as pre-implantation development, and the KAT-dependent role of Tip60 in differentiation and post-implantation development. See text for additional details.