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Understanding Multiple Independent Functions of the Tip60 Acetyltransferase in Embryonic Development

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UNDERSTANDING MULTIPLE INDEPENDENT FUNCTIONS OF THE TIP60

ACETYLTRANSFERASE IN EMBRYONIC DEVELOPMENT

A Dissertation Presented

By

DIWASH ACHARYA

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UNDERSTANDING MULTIPLE INDEPENDENT FUNCTIONS OF THE TIP60

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This work was undertaken in the Graduate School of Biomedical Sciences
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ABSTRACT

Chromatin is a dynamic structure, and chromatin remodeling enzymes regulate chromatin structure to control gene expression and proper lineage specification. Tip60-p400 is a multi-subunit chromatin remodeling complex containing two biochemical activities: the Tip60 subunit is a lysine acetyltransferase (KAT) that targets histones and non-histone proteins, and p400 catalyzes ATP-dependent incorporation of histone variant H2AZ into chromatin. Both of these chromatin modifications have been widely studied with respect to gene expression, DNA damage repair, and apoptosis. Ablation of these catalytic subunits causes defects in normal embryonic development, ESC self-renewal, and gene expression. My goal has been to understand the multiple independent functions of Tip60-p400 acetyltransferase in ESC maintenance and embryonic development.

I showed that Tip60 KAT function is dispensable for gene expression, chromatin accessibility, and ESC self-renewal, which is different from *Tip60* knockdown phenotype. Interestingly, KAT deficient mutants exhibited defect in differentiation towards mesoderm and endoderm lineages. Consistent with this defect, I also observed gastrulation defect in mice lacking *Tip60* KAT activity. Together, these data demonstrate that Tip60 KAT dependent function is only required during later stages of embryonic development, and it is dispensable for ESC self-renewal and pre-implantation development.

Tip60 KAT contains four isoforms generated from alternative splicing, whose individual functions are poorly characterized. In the second part of this thesis, I investigated the developmental role of one of the isoforms of *Tip60*, called *Tip55*. Unlike *Tip60* knockout mice, which lack all the isoforms and causes pre-implantation lethality, I found that ablation of *Tip55* results in post-implantation lethality. I further found that loss of *Tip55* causes defects in heart, and neural tube development, demonstrating the essential function of *Tip55* isoform for organogenesis during embryonic development.

Together, these studies have provided new insight into the functions of Tip60-p400 and the mechanisms by which this complex regulates gene expression, ESC pluripotency, and embryonic development. Furthermore, these studies set the stage for future work to identify how the catalytic and non-catalytic functions are directed to perform distinct regulatory functions, as well as how each Tip60 isoform individually contributes to formation of the mammalian body plan.

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LIST OF ABBREVIATIONS AND NOMENCLATURE

ESCs	Embryonic Stem Cells
LIF	Leukemia Inhibitory Factor
STAT3	Signal Transducer and Activator of Transcription 3
PRC2	Polycomb Repressive Complex 2
TE	Trophectoderm
EPI	Epiblast Cells
PrE	Primitive Endoderm
T	Brachyury
FGF	Fibroblast Growth Factor
MEK	Mitogen-Activated Protein Kinase
ERK	Extracellular Signal-Regulated Kinase
TGFβ	Transforming Growth Factor beta
CRISPR	Clustured Regularly Interspread Short Palindromic Repeats
HR	Homologous Recombination
NHEJ	Non-Homologous End Joining
MLL	Mixed Lineage Leukemia
HSA	Helicase-SANT-Associated
KAT	Lysine Acetyltransferase
HAT	Histone Acetyltransferase
TIP	TAT Interactive Protein

TAT	Trans-activating Regulatory Protein
SRCAP	Snf2 Related CREB Binding Activator Protein
DSB	Double Strand Break
AP	Alkaline Phosphatase
EB	Embryoid Body
GCN5	General Control Nonderepressible-5
CBP	CREB Binding Protein
PCAF	p300/CBP Associated Factor
SAS2	Something About Silencing 2
MOZ	Monocytic Leukemic Zinc Finger
ESA1	Essential SAS2 Related Acetyltransferase
AR	Androgen Receptor
ATM	Ataxia Telangiectasia Mutated Kinase
MRN	Mre11, Rad50, Nbs1 complex
ATAC-seq	Assay for Transposase-Accessible Chromatin using sequencing
SSEA-1	Stage Specific Embryonic Antigen 1
CHIP-seq	Chromain Immunoprecipitation followed by genome-wide sequencing
RNA-seq	Genome-wide RNA sequencing
SRF	Serum Response Factor
ANF	Atrial Natriuretic Factor
MEF	Mouse Embryonic Fibroblast

CHAPTER I

Introduction

In all eukaryotes, DNA is present inside the cell nucleus in the form of chromosomes. Chromosomal DNA is negatively charged and is wrapped around positively charged histone proteins (H2A, H2B, H3 and H4) in the form of nucleosomes, which are further condensed into higher-order chromatin structures (**Figure I.1**) (Finch et al., 1977; Luger et al., 1997). Nucleosomes influence gene regulation by affecting accessibility of the regulatory proteins in their DNA binding sites (Lodén and van Steensel, 2005). There are several chromatin-remodeling complexes (discussed later in detail) that influence both nucleosome architecture and higher order chromatin structure to access or restrict DNA for gene expression.

Embryonic stem cells (ESCs), which give rise to all the cell types present in adult mammals, possess euchromatin (more relaxed and less compact chromatin) and heterochromatin (densely packed chromatin) structures distinct from differentiated cells (**Figure I.1**) (Meshorer and Misteli, 2006; S.-H. Park et al., 2009). Undifferentiated ESCs have fewer heterochromatic domains in comparison to differentiated cells that contain numerous condensed heterochromatin foci (Meshorer and Misteli, 2006; S.-H. Park et al., 2009). Chromatin remodeling complexes maintain chromatin organization and regulate proper differentiation into specific cell lineages (Bultman et al., 2000; Fazzio et al., 2008; Kaji et al., 2006; Kurisaki et al., 2005; Meshorer and Misteli, 2006).
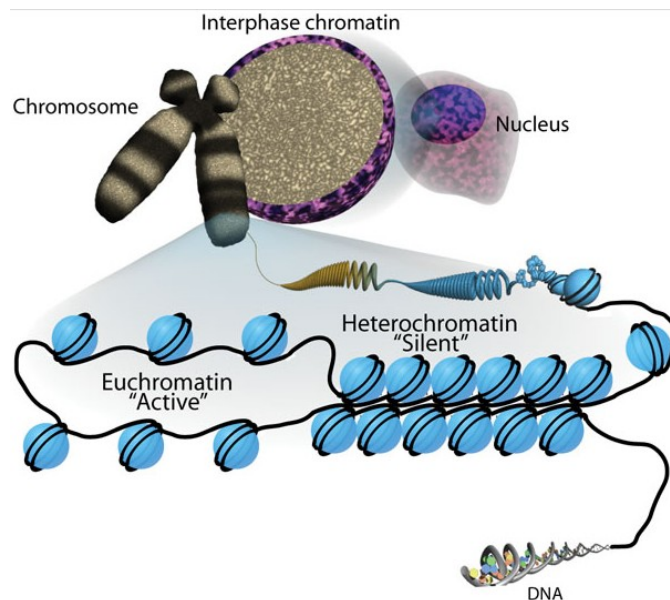
Figure I.1: Basics about chromatin organization. 147 bp of DNA is wrapped around a ball like structure of nucleosomes composed of core histone proteins, giving rise to euchromatin and heterochromatin structure to fit genetic information into nucleus. Euchromatin is associated with open chromatin structure to facilitate transcription whereas heterochromatin is tightly packed chromatin structure that restricts transcription. Adapted from (Boyer, 2009).

Characteristic features of mouse embryonic stem cells

Mouse embryonic stem cells (mESCs) are derived from cells of the inner cell mass (ICM) of blastocyst stage embryos at embryonic day 3.5 (E3.5) (M. J. Evans and Kaufman, 1981; Martin, 1981). It has two characteristic features: self-renewal and pluripotency. Self-renewal is the ability of ESCs to proliferate indefinitely in an undifferentiated state. Pluripotency is the ability of ESCs to give rise to all three embryonic germ layers ectoderm, mesoderm, and endoderm, which are the precursors for all cell types in the embryo. Because of these properties, ESCs hold great promise for studying early embryonic development and for regenerative medicine.

ESCs are cultured on feeder cells *in vitro* to maintain in the undifferentiated state. Feeder cells are derived from mouse embryonic fibroblasts

that provide essential nutrients for ESC self-renewal and proliferation (Suda et al., 1987).   Later, it was identified that ESCs can be maintained in gelatin coated plates, and doesn't required feeders if provided with differentiation inhibiting factor secreted by feeder cells, called leukemia inhibitory factor (LIF) (A. G. Smith et al., 1988; Williams et al., 1988). LIF is an interleukin 6 class cytokine that interacts with its heterodimer receptor, LIF receptor/glycoprotein 130 (LIFR/gp130), which then phosphorylates and activates Janus kinase (JAK) (Davis et al., 1993; Murakami et al., 1993; Narazaki et al., 1994). Activated JAK further phosphorylates and activates the signal transducer and activator of transcription 3 (STAT3) transcription factor, which then translocates into the nucleus to activate transcription of target genes that maintain ESC self-renewal (Hirai et al., 2011; Kristensen et al., 2005). Absence of LIF or disruption of the LIF mediated JAK-STAT pathway causes ESCs to differentiate (Hirai et al., 2011; Kristensen et al., 2005; Niwa et al., 1998). Therefore, LIF is important for ESC self-renewal, without which ESCs lose their self-renewing capability and cause undirected differentiation (A. G. Smith et al., 1988; Stewart et al., 1992).

Core pluripotency factors that control ESC self-renewal

The pluripotent state of the ESCs is controlled by a network of core transcription factors, octamer-binding transcription factor 4 (OCT4), sex determining region box-2 (SOX2), and NANOG (Chambers and A. Smith, 2004; Niwa, 2007; Silva and A. Smith, 2008). OCT4 (also called OCT3) belongs to the

POU family transcription factors encoded by *Pou5f1* gene, and is expressed during the oocyte stage of embryonic development. It is also called the first pluripotency factor identified that regulates ESC pluripotency (Nichols et al., 1998; Okamoto et al., 1990; Schöler et al., 1990).

SOX2 is a SRY-box transcription factor that is first expressed in cells at the morula stage (E2.5) during embryogenesis, and mostly restricted to the ICM of the blastocyst (E3.5) (Avilion et al., 2003). Mouse embryos lacking *Sox2* expression show a defect in ICM formation and produce only trophectoderm cells causing peri-implantation lethality. Interestingly, *Sox2* deficient ESCs also exhibit self-renewal defects and differentiate towards trophectoderm-like cells as observed *in vivo* (Avilion et al., 2003; Masui et al., 2007). Similar to the *Sox2* phenotype, knockout of *Oct4* in embryos and ESCs also causes peri-implantation embryonic lethality and differentiation towards the trophectoderm lineage, respectively (Nichols et al., 1998). These data suggested a functional relationship between SOX2 and OCT4. As expected, SOX2 was found to interact with OCT4 and the two factors co-occupy an overlapping set of target genes (Ambrosetti et al., 1997; Boyer et al., 2005; X. Chen et al., 2008; J. Kim et al., 2008; van den Berg et al., 2010). In addition, SOX2 positively regulates *Oct4* expression (Masui et al., 2007).

Another regulatory protein important for pluripotency is NANOG. Similar to *Sox2* and *Oct4* knockout phenotypes, *Nanog* deficient embryos exhibit peri-implantation lethality. In contrast to *Oct4 and Sox2* knockout phenotypes, ESCs

lacking *Nanog* expression can self-renew, but are defective in cell proliferation and prone to ESC differentiation towards endoderm-like cells (Mitsui et al., 2003). These results demonstrate that OCT4 and SOX2 are essential factors required for ESC maintenance at an earlier stage than NANOG; however, all three transcription factors are ultimately required for ESC pluripotency.

Gene regulatory network of core pluripotency factors

Oct4, Sox2, and *Nanog* expression control ESC pluripotency by forming a core regulatory network. All of these transcription regulators co-occupy regulatory regions of hundreds of protein coding genes, which are either expressed or silenced in ESCs (Loh et al., 2006). Interestingly, they also co-occupy their own promoter regions and positively regulate their expression forming interconnected auto-regulatory and feed forward loops for ESC self-renewal (Boyer et al., 2005; Loh et al., 2006; Young, 2011). ESCs lacking any of these transcription factors disrupt the interconnected regulatory loop and cause repression of ESC specific genes including *Oct4, Sox2,* and *Nanog*, and expression of normally silent lineage specific genes (Boyer et al., 2005; Loh et al., 2006). Therefore, these factors play crucial roles in ESC gene regulation to maintain pluripoteny.

Recent studies have shown that co-occupancy by these factors at enhancers promotes direct interactions with co-activators such as Mediator and p300, which are commonly found at enhancer regions and regulate the expression of lineage specific genes (X. Chen et al., 2008; Kagey et al., 2010).

The pluripotency factors also functionally interact with several chromatin

remodelers such as the INO80 and esBAF (ESC specific BAF) complexes to

regulate the expression levels of ESC specific genes (Ho et al., 2009; L. Wang et

al., 2014). In addition to gene activation, OCT4, SOX2, and NANOG are also

involved in repression of genes that are normally silent in ESCs such as lineage

specific transcription factors. Chromatin regulators such as histone-lysine N-

methyltransferase SetDB1 and Polycomb Repressive Complex 2 (PRC2) have

been shown to play important roles in repression of lineage specific transcription

factors. OCT4 interacts with sumoylated SetDB1 that catalyzes the incorporation

of the repressive histone mark H3K9me3. This repressive mark is recognized by

the PRC2 complex, which subsequently incorporates another repressive mark,

H3K27me3, therefore enhancing the repression of lineage specific

developmental regulators in ESCs (Bilodeau et al., 2009; Margueron et al.,

2009).

Because of these characteristic features, ESCs are useful models to

understand the mechanisms underlying regulation of chromatin structure, and the

importance of chromatin regulation in proper differentiation.

Mouse development

Mouse is a powerful model system to study human diseases due to its

strong genomic concordance with humans. In addition, mice are small, reproduce

quickly, cost-effective, and can be genetically manipulated to study the

importance of genes and its connection with several diseases. In developmental biology, mouse development has been studied in detail during pre-implantation and post-implantation development, both of which are discussed in detail below.

Pre-implantation embryonic development

The pre-implantation period of the embryo refers to the early embryonic developmental stages where cell division and the first cell differentiation process is observed, before the embryo attaches or implants in the uterus and starts forming specialized tissues and organs (**Figure I.2**). Early mouse development begins after the male and female gametes fuse with each other in an oviduct to form one cell stage embryo (Kojima et al., 2014). This one cell stage embryo undergoes three rounds of cell division to become eight cells at E2.5, the embryonic structure called a morula (Fleming, 2001; Kojima et al., 2014). A compaction event occurs within the morula, where loosely packed cells are transformed into tightly packed cells to generate the embryonic blastocyst by E3.5 (**Figure I.2**). Blastocysts contain two distinct cell types: trophectoderm (TE) cells that give rise to placenta during embryonic development, and ICM, which contributes to the embryo (Fleming, 2001; Kojima et al., 2014). As the blastocyst develops within the uterus and reaches E4.5, the ICM further differentiates into epiblast cells (EPI), which produces the embryo, and primitive endoderm (PrE), which contributes to the embryonic yolk sac and other extra-embryonic tissues (**Figure I.2**) (Fleming, 2001; Kojima et al., 2014). Although cells of the morula

can differentiate into all three cell-types (TE, ICM and PrE) and contribute to the embryonic and extra-embryonic regions, cells of the ICM of the blastocyst contribute to epiblast cells that form embryo, and PrE but not TE (Lu and Y. Zhang, 2015; Mitalipov and Wolf, 2009). Therefore, cells of the morula are considered totipotent while the ICM is pluripotent. ESCs are derived from the ICM of the blastocyst stage embryo, and are pluripotent (M. J. Evans and Kaufman, 1981; Martin, 1981).

OCT4, SOX2, and NANOG are highly expressed as early as the morula stage and play significant roles in early embryogenesis (Avilion et al., 2003; Dietrich and Hiiragi, 2007). Another transcription factor required for early embryonic differentiation is CDX2 (caudal like transcription factor) (Beck et al., 1995). Studies have demonstrated the opposing functions of OCT4 and CDX2 in TE and ICM differentiation to form the blastocyst. Both of these transcription factors are initially expressed in the morula, but eventually *Cdx2* expression becomes restricted to TE cells only, whereas *Oct4* is expressed exclusively in the ICM (Dietrich and Hiiragi, 2007; Niwa et al., 2005). It is still unclear how these factors specify the first lineage specific differentiation to form the blastocyst. Although *Nanog* and *Sox2* are expressed at similar stages of early embryonic development as *Cdx2* and *Oct4*, their roles, if any, in the formation of TE and ICM are yet to be identified.

Post-implantation embryonic development

Attachment of the blastocyst at E4.5 to the maternal uterine wall, followed by cell proliferation, and gastrulation marks the initiation of post-implantation embryonic development (**Figure I.2**) (L. J. Smith, 1980). When the embryo implants into the uterus, there are only about 100-120 cells within the embryo, which undergo rapid proliferation to produce about 660 cells by E6.5, when gastrulation begins (**Figure I.2**) (Snow, 1977). Gastrulation is the major event that forms all three primary germ layers, ectoderm, mesoderm, and endoderm from the epiblast for the formation of vertebrate body plan (Nowotschin and Hadjantonakis, 2010; Tam and Behringer, 1997). Prior to gastrulation, mouse embryos at E5.5 develop into a cup-shaped or cylinder-shaped embryo, which remains (with specific alterations discussed below) until E7.5. At the junction between epiblast cells and extra-embryonic ectoderm, a structure is formed at E6.5 called the primitive streak, which marks the beginning of gastrulation (**Figure I.2**) (Hashimoto and Nakatsuji, 1989; Tam and Behringer, 1997). Epiblast cells undergo an epithelial to mesenchymal transition (EMT) and ingress into the primitive streak-giving rise to mesendoderm cells, which amass on the outside of the cup. These cells are precursors of mesoderm cells, which gives rise to structures like heart, kidney, and somites; and endoderm cells, which gives rise to the gut and associated organs such as lung and pancreas (Lawson and Pedersen, 1987; Parameswaran and Tam, 1995). The ectoderm cells that also originate from the epiblast cells at the primitive streak are located on the inside of the cup shaped embryo, and
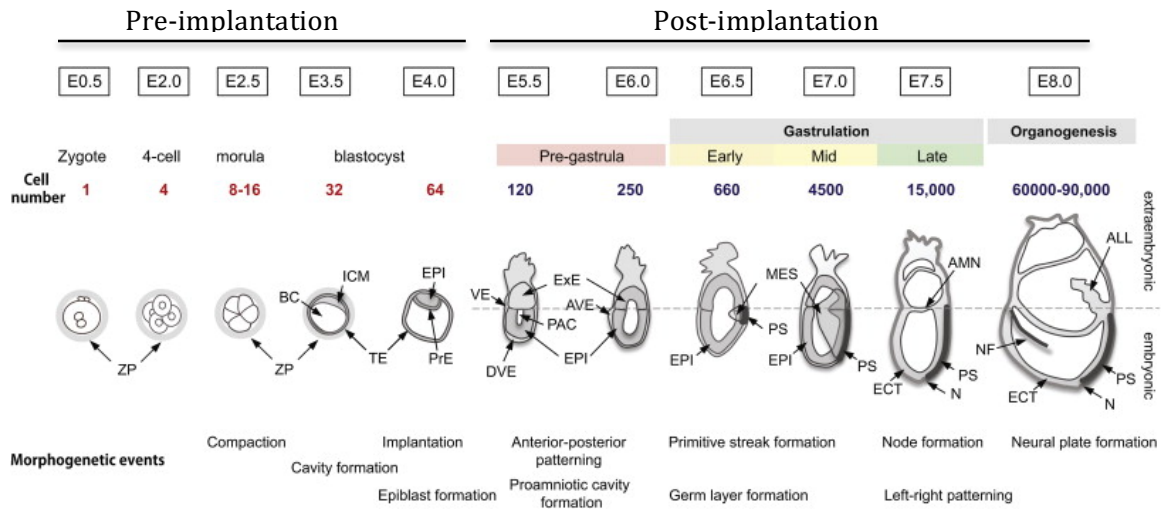
Figure I.2: Overview of pre-implantation and post-implantation embryonic development. Diagram representing embryo development from one cell stage (zygote) to thousands of cells (organ development). Zygote (E0.5) to blastocyst (E4.5) stage represent pre-implantation embryo development with the cell numbers highlighted in red. Events such as cell compaction, blastocoel cavity formation and first lineage specific cell differentiation occur during this stage. Stages from late blastocyst (E4.5) until organogenesis, before the pups are born, represent post-implantation embryo development with the cell number highlighted in blue. Events such as embryo implantation on the uterine wall, anterior posterior pattering, primitive streak formation, gastrulation and organogenesis happen during this stage of development. Abbreviations: ALL: allantois, AMN: amnion, AVE: anterior visceral endoderm, BC: blastocyst cavity, DVE: distal visceral endoderm, ECT: ectoderm, EPI: epiblast, ExE: extraembryonic ectoderm, ICM:inner cell mass, MES: mesoderm, N: node, NF: neuralfold, PAC: proamniotic cavity, PrE: primitive endoderm, PS: primitive streak, TE:trophectoderm,VE: visceral endoderm, ZP: zona pellucida. Adapted from (Kojima et al., 2014).

contribute to formation of the nervous system (Quinlan et al., 1995). Until E7.5, ectoderm is found inside the cup shaped embryo, while the endoderm germ layer comprises the outside layer. After E7.5, the embryonic germ layers undergo an inversion process called "turning" whereby the endoderm layer is moved to the inside while the ectoderm becomes the outside layer. (Fujinaga, 1997; Melloy et al., 1998).

During gastrulation, one of the earliest genes that is expressed is *Brachyury* (*T*). T is the first T-box transcription factor identified, and is required for proper development of primitive streak and induction of mesodermal precursors (Herrmann et al., 1990). It is specifically expressed in mesodermal cells and notochord (Wilkinson et al., 1990). Mice that are homozygous null for *T* die mid-gestation because of the inability of epiblast cells to migrate from the primitive streak and are also defective in formation of the notochord (Yanagisawa et al., 1981). How does *T* regulate proper gastrulation? Chromatin immunoprecipitation (ChIP) studies have shown that T binds the promoter regions of genes with important functions in development such as *Wnt3a* and *Fgf-8*, two essential signaling molecules (discussed later) and regulates their expression (A. L. Evans et al., 2012).

Signaling pathways involved in mouse embryonic development

Multiple signaling pathways such as fibroblast growth factor (FGF), wingless (WNT), and NODAL are required for pre- and post-implantation

embryonic development to control many cellular processes including cell proliferation, migration, and differentiation. The FGF family contains several secreted signaling proteins (FGFs) that bind to its specific tyrosine kinase receptors and mediate intracellular signaling through mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) pathway during various stages of embryonic development. Similarly, the WNT signaling pathway is activated by binding of WNT ligands to the Frizzled family of G-protein coupled receptors. In addition to FGF and WNT, NODAL signaling is very important during early embryonic development. NODAL belongs to the transforming growth factor beta (TGFβ) superfamily that binds to ACTIVIN receptors and regulates gene expression through SMAD proteins. Genetic experiments have shown that all of these signaling pathways exhibit essential functions during embryogenesis and proper ESC differentiation.

There are twenty-two FGF ligands and four FGF receptors, which are expressed during different stages of embryonic development (Ornitz and Itoh, 2015). One of the early FGF ligands is FGF-4, primarily expressed in the 8-cell stage morula and later restricted to the ICM. Unlike *Fgf4* ligand, its receptor *Fgfr2* expression is restricted to TE cells. Knockout of the ligand or the receptor exhibit similar embryonic lethality during early post-implantation, as well as defects in cellular differentiation and maintenance of the ICM (Arman et al., 1998; Feldman et al., 1995). This suggests that FGF-4 mediated signaling through FGFR2 is important for the first lineage specification of TE and ICM cells during early

embryogenesis (Arman et al., 1998; Goldin and Papaioannou, 2003). ESCs

lacking FGF-4 or its downstream target MEK/ERK also exhibit defect in

differentiation, suggesting that FGF-4 mediated activation of MEK/ERK pathway

is essential for proper ESC differentiation (Kunath et al., 2007; Rossant, 2008).

These *in vivo* and *in vitro* results demonstrate that FGF signaling is required

during early development. FGF-8 is another secreted ligand that is expressed

only during post-implantation embryonic development suggesting it may have a

role later during development. Mouse embryos lacking *Fgf-8* and *Fgfr1*

expression exhibit defect in mesodermal cells formation and cell migration during

gastrulation (Deng et al., 1994; X. Sun et al., 1999; Yamaguchi et al., 1994).

These studies suggest that FGF/FGFR/ERK signaling pathway is required

throughout embryonic development and ESC differentiation.

WNT3 and WNT3A are among the components of the WNT signaling

pathway that has been widely studied for its role in gastrulation. *Wnt3* is initially

expressed at E5.5 prior the formation of primitive streak in the posterior visceral

endoderm, and later restricted in the primitive streak at E6.5 where the early

gastrulation marker *T* is expressed (Rivera-Pérez and Magnuson, 2005). Ablation

of *Wnt3* specifically in epiblast cells affect the formation of primitive streak as

marked by the reduced or weaker expression of primitive streak markers *T, Fgf8,*

and *Sp5* (Tortelote et al., 2013). In comparison, *Wnt3a* is expressed later than

Wnt3 in mesodermal cells, and notochord of the primitive streak, and loss of

which also affects the expression of the *T* (Wilkinson et al., 1990; Yamaguchi et

al., 1999). Mice homozygous null for *Wnt3a* are embryonic lethal at E9.5 exhibiting defects in formation of mesodermal cells and notochord, phenocopying *T* null mice. Together these results suggest that WNT signaling mediated primitive streak formation, and *T* expression is critical for gastrulating mouse embryos (Takada et al., 1994; Yoshikawa et al., 1997).

Similarly, NODAL signaling is also required for early embryonic development. Unlike FGF and WNT, NODAL is not required for mesoderm specification because *T* is broadly expressed in the null mutants for *Nodal* compared to wild type controls. However, mice lacking *Nodal* affect the formation of appropriate primitive streak. Likewise, ESCs lacking *Nodal* expression also do not exhibit defects in mesodermal differentiation. These *in vivo* and *in vitro* results demonstrate that NODAL signaling is not required for mesoderm induction, but instead is required to induce or maintain proper primitive streak (Conlon et al., 1994).

Generation of knockout mice

Because of the ability to easily manipulate the mouse genome, mice are powerful experimental models for the study of development and human diseases. Homologous recombination has been used to delete or insert mutations in genes, insert reporter genes and many other manipulations (Bouabe and Okkenhaug, 2013; Sternberg, 1981). One of the most widely used techniques to generate conditional knockout mice is the Cre-loxP system (Gu et al., 1994; Sauer, 1998).

LoxP is a 34 basepair (bp) sequence that consists of an asymmetric 8bp sequence flanked by 13bp inverted repeats. CRE is a site-specific recombinase that acts as a molecular scissor and mediates recombination between loxP sites in the same orientation, efficiently removing the portion of gene flanked by the loxP (Babinet and Cohen-Tannoudji, 2001; Bouabe and Okkenhaug, 2013; Sternberg, 1981).

Recently scientists have adapted the bacterial host defense system, CRISPR/Cas9 (clustured regularly interspread short palindromic repeats/CRISPR associated nuclease) system, for gene targeting. CRISPR/Cas9 is fast, highly efficient, and multiplexable as compared to traditional approaches to generate knockout mice or mutant ESC lines for biomedical research (Cong et al., 2013; Mali et al., 2013; H. Wang et al., 2013; H. Yang et al., 2013). The CRISPR/Cas9 system that is adapted for genetic engineering contains a guide RNA (gRNA) and endonuclease Cas9. The gRNA is designed to be complementary to the target DNA sequence and directs Cas9 to the site of DNA cleavage (Cong et al., 2013; Mali et al., 2013). Expression of CRISPR/Cas9 components in mammalian systems (as well as other eukaryotes) has been demonstrated to edit the genome through homologous recombination or non-homologous end joining (Cong et al., 2013; Mali et al., 2013). In addition, the CRISPR/Cas9 system can be used to generate knockout mice by directly injecting Cas9 mRNA, gRNA and an oligonucleotide template (for HR) into the zygote, and transferring the manipulated zygotes into pseudo-pregnant females

(L.-F. Jin and J.-S. Li, 2016; H. Wang et al., 2013; H. Yang et al., 2013; Zuo et al., 2017).

Chromatin structure plays important roles to influence gene expression during embryonic development or during cell differentiation into specific lineages. There are multiple chromatin remodeling factors and chromatin modifying enzymes that regulate chromatin structure by various mechanisms to control the expression of genetic information encoded in DNA during development.

Packaging of eukaryotic DNA into chromatin

The fundamental unit of chromatin is the nucleosome, which consists of a complex of positively charged histone proteins around which negatively charged DNA is wrapped. Each canonical nucleosome consists of two copies of histones H2A, H2B, H3, and H4 (Luger et al., 1997). These histones contain a histone fold and histone tail regions. The histone fold region forms the structure of each histone and is important for histone-histone interactions, such as the formation of H2A-H2B dimers and H3-H4 tetramers. The two H2A-H2B dimers bind the H3-H4 tetramer through the interactions between H2B and H4 (Luger et al., 1997). Approximately 147bp of negatively charged DNA is wrapped around the positively charged histone octamer and binds tightly to form nucleosomes (**Figure I.1**). Nucleosomes are connected by linker DNA of varying length between 10 – 70bp resulting in "beads on a string" chromatin structure (Luger et al., 1997; Thoma, 1979; Woodcock and Ghosh, 2010). Linker histone H1, which

is not a core histone protein, interacts with nucleosomes and the linker DNA entering and exiting the nucleosome. Histone H1 influences chromatin compaction (Bednar et al., 1998; Harshman et al., 2013; Thoma, 1979), but it is not entirely clear how it promotes the higher order chromatin folding. On the other hand, histone tails are flexible regions of histone proteins that protrude from the nucleosome core, forming docking sites for chromatin regulatory complexes and influencing higher order folding. They interact with each other among different nucleosomes or with the DNA within nucleosomes to provide dense or compact chromatin structure (Hansen, 2002).

Chromatin structure affects several aspects of transcription, such as binding of transcription factors, formation of pre-initiation complex and transcription elongation (Workman and Kingston, 1998). Therefore, chromatin structure is regulated dynamically to facilitate proper gene regulation, with factors that promote heterochromatin formation repressing transcription and factors that increase accessibility working as activators. There are several multi-subunit protein complexes that regulate the chromatin structure through covalent modifications of histones, and/or the use of energy from ATP hydrolysis to alter the position or structure of nucleosomes. Primary nucleosome positioning and occupancy is regulated through several mechanisms: histone exchange, histone dissociation, and sliding or relocation of nucleosomes (Kassabov et al., 2003; Lusser and Kadonaga, 2003).

Histone modifications and histone variants

The N-terminal histone tails are subject to various post-translational modifications such as acetylation, phosphorylation, methylation and ubiquitination (Strahl and Allis, 2000; Turner, 1998). These post-translational modifications provide binding sites for chromatin regulators that move nucleosomes around and either allows or restricts access of transcription machinery causing either an open (transcriptionally active) or closed (transcriptionally inactive) chromatin state. Covalent modifications of histone tails such as H3K4me3, H3K9ac and H3K14ac are associated with actively transcribed genes, whereas H3K9me3 and H3K27me3 are associated with repressed or silent genes (Kraushaar and Zhao, 2013; Young, 2011). In ESCs, active H3K4me3 and repressive H3K27me3 marks are identified at the promoter region of developmentally silent genes and primed for activation during normal differentiation (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Mikkelsen et al., 2007). Two independent chromatin-modifying enzymes catalyze these functionally opposing bivalent marks: PRC2 and MLL complexes. PRC2 catalyzes the incorporation of H3K27me3 for gene silencing, and MLL complex catalyzes the incorporation of H3K4me3 for gene activation (Azuara et al., 2006; Boyer et al., 2006; Denissov et al., 2014; Dou et al., 2006). During ESC differentiation into specific lineages, the bivalent marks are resolved and harbor either active H3K4me3 mark for lineage specific gene expression or inactive H3K27me3 mark for repression of genes (Bernstein et al., 2006; Kraushaar and

Zhao, 2013). Covalent modifications also act as a prerequisite signal that regulate the binding of transcription factors as well as chromatin remodeling enzymes such as SWI/SNF or ISWI to regulate the chromatin structure during several cellular processes like DNA repair, transcription, recombination and replication (Guccione et al., 2006).

In addition to histone modifications, histone variants also play important role to regulate nucleosome structure and gene regulation. All histones have variants except for H4. Some of the variants are tissue specific and only expressed in germ line cells such as testis or oocyte (TH2A and TH2B variants of histones H2A and H2B respectively), and some of the variants are expressed during different stages of embryonic development (H3.3 and H2AZ variants of histones H3 and H2A respectively) (Buschbeck and Hake, 2017). For instance, H2AZ that is expressed during pre-implantation development is a highly conserved histone variant and shares 60% homology with H2A (Eberharter and Becker, 2004; Fischle et al., 2003). Nucleosomes containing the H2AZ variant are similar in structure to those with H2A, although there are multiple amino-acids substitutions in H2AZ compared to H2A, including the docking domain that interact between H2A-H2B dimer and H3-H4 tetramer (Suto et al., 2000). With 40% of the sequence divergent from H2A, this suggests H2AZ has functions that are at least partially distinct from H2A (Jackson, 2000). In ESCs, H2AZ largely occupies regulatory regions. Two studies from Kaestner and Zhao labs showed that H2AZ incorporation enhances chromatin accessibility, and decreases

nucleosome occupancy (G. Hu et al., 2013; Z. Li et al., 2012). During

differentiation, overall nucleosome density increases and chromatin accessibility

decreases at regulatory regions occupied by H2AZ in ESCs. Interestingly, upon

ESC differentiation towards the endoderm lineage, H2AZ enrichment decreases

at promoter regions, facilitating the binding of the lineage specific transcription

factor FOXA2, which activates expression of many endoderm-specific genes (Z.

Li et al., 2012). Upon differentiation into the neuronal lineage, regulatory regions

bound by the transcription factor RARα were enriched at regions occupied by

H2AZ in ESCs (G. Hu et al., 2013). It still remains to be determined if the

enrichment of RARα correlates with depletion of H2AZ enriched nucleosome

during neuronal differentiation. Together, these data suggest that H2AZ mediated

chromatin structure is important for proper gene regulation and lineage

specification. The biological functions of H2AZ, and how H2AZ is incorporated

into nucleosomes will be detailed below.

Mechanistic roles of chromatin remodeling enzymes

Nucleosome positions and occupancy are dynamically regulated in order

to permit or restrict access to DNA for controlled gene expression (B. Li et al.,

2007). There are four families of chromatin remodeling enzymes: SWI/SNF,

ISWI, CHD, and INO80 that utilize energy from ATP hydrolysis to disrupt DNA-

histone contacts, with several outcomes such as nucleosome sliding, histone

eviction or histone variant exchange (Clapier and Cairns, 2009; Narlikar et al.,

2013). Along with a conserved ATPase domain these enzymes also contain different accessory domains such as a bromodomain, helicase-SANT-associated (HSA) domain, and chromodomain. These domains are used to further divide chromatin remodeling enzymes into different families (**Figure I.3**) (Clapier and Cairns, 2009). The ATPase domain includes Walker A and Walker B motifs, which are required for ATP binding and hydrolysis (Clapier and Cairns, 2009; Walker et al., 1982). The Walker A motif consists of conserved GXXXXGKT/S (where X is any amino acid) amino acid sequence. The lysine (K) residue in this motif is crucial for ATP binding. Mutation of the invariant K in the Walker A motif eliminates ATP binding and hydrolysis (Hanson and Whiteheart, 2005). The Walker B motif contains conserved acidic residues following a stretch of hydrophobic residues with a consensus of hhhhDE (where h is any hydrophobic amino acid) (Hanson and Whiteheart, 2005). Aspartate (D) coordinates with a magnesium ion (acts as a cofactor that bridges between D and gamma phosphate of ATP) and glutamate (E) activates a water molecule (acts as a nucleophile) for ATP hydrolysis (Hanson and Whiteheart, 2005). These enzymes bind chromatin through accessory domains such as the bromodomain (which recognizes acetylated histones), HSA domain (which recognizes nucleosomal DNA) or chromodomain (which recognizes methylated histones). After binding the nucleosome substrate, the Walker A motif binds to ATP, hence causing a conformational change from an open to closed enzyme structure (Hanson and Whiteheart, 2005). This conformational change positions the gamma phosphate
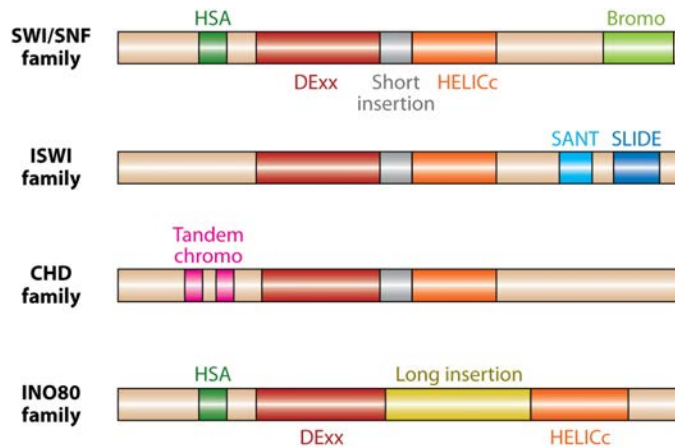
Figure I.3: Classification of chromatin remodeling families. ATP dependent chromatin remodeling families are classified based on the presence of ATPase domain and associated unique accessory domains. The ATPase domain is splitted into two parts by DExx and HELICs, with short insertion in between SWI/SNF, ISWI and CHD family whereas long insertion in the INO80 family. Each of these families has unique domains: HSA, Bromo, SANT, SLIDE, Chromo as shown. Adapted from (Clapier and Cairns, 2009).

for hydrolysis by the Walker B motif, resulting in physical work such as

nucleosome movement, histone eviction or histone variant exchange.

All of these chromatin-remodeling enzymes are important to regulate

higher order chromatin structure and gene regulation in eukaryotes. The major

focus of my work has been to understand the importance of Tip60-p400 complex

in ESC maintenance, gene regulation, and development. Tip60-p400 (~1.5 MDa)

is a 17-subunit chromatin remodeling complex, which has histone modifying and

chromatin remodeling functions widely studied in the context of DNA repair and

apoptosis (J. H. Park et al., 2010). Tip60 is a lysine acetyltransferase (KAT) that acetylates histones and non-histone proteins, whereas the ATPase activity of p400 catalyzes replacement of H2A with H2AZ within a nucleosome (Ikura et al., 2000; J. H. Park et al., 2010; Y. Xu et al., 2012). As the p400 and the Tip60 subunits of Tip60-p400 belongs to the INO80 family and MYST family respectively, I will discuss the mechanisms of action of INO80 family remodeling factors, and MYST family acetyltransferase in detail below. In addition, I will also focus on the biology and function of Tip60, p400 and H2AZ in various cellular processes such as gene regulation, DNA repair, apoptosis, and development.

INO80 family is highly conserved in all eukaryotes from yeast to mammals. This family of remodeling enzymes contains INO80 and SWR1 complexes in yeast.   All INO80 family members contain a long insertion in the middle of the ATPase domain, which acts a platform for binding other subunits in the complex (Clapier and Cairns, 2009). INO80 complex contains 15 subunits in yeast, and functions in mobilization of nucleosomes for processes such as DNA repair, DNA replication and gene regulation (Ebbert et al., 1999; Jónsson et al., 2004; Papamichos-Chronakis and Peterson, 2008; Shen et al., 2000). Mammalian INO80 complex is similar in composition to yeast INO80 complex and was shown to exhibit DNA and/or nucleosome mediated ATPase activity which promotes nucleosome sliding in vitro (J. Jin et al., 2005; Willhoft et al., 2016). Recently, mammalian INO80 complex has been shown to regulate ESC

pluripotency. Knockdown of *Ino80* causes a defect in ESC self-renewal and

reduced expression of pluripotnecy genes (L. Wang et al., 2014). These data

underscore the importance of INO80 complex in both yeast and higher

eukaryotes. SWR1 complex from the same family consists of 14 subunits in

yeast, including the catalytic SWR1 protein, which has an ATPase domain

(Mizuguchi et al., 2004). Most chromatin remodeling enzymes use ATP

hydrolysis to slide or evict nucleosomes within chromatin structure, whereas

SWR1 complex functions to catalyze the incorporation of the H2AZ histone

variant in place of the canonical H2A histone through a dimer exchange

mechanism (Mizuguchi et al., 2004).

Recently, extensive biochemical studies have elucidated the mechanism

by which SWR1 complex binds the nucleosome substrate and uses energy from

ATP hydrolysis for H2AZ exchange. The SWR1 and SWC2 (another subunit in

the complex) proteins, as well as a linker DNA length of more than 50bp between

nucleosomes, were shown to be required for the recruitment of SWR1 complex

to the promoter region of target genes (Ranjan et al., 2013). These and other

studies suggested a mechanism by which SWR1 complex catalyze the exchange

of H2AZ from *in vitro* biochemical experiments. SWR1 complex binds the A/A

nucleosome (nucleosome with two H2A histones), which causes a

conformational change of the SWR1 complex and activates ATP hydrolysis (Luk

et al., 2010). An H2AZ-H2B dimer then binds to the SWR1 complex as shown in

Figure I.4. Further ATP hydrolysis causes the incorporation of H2AZ and
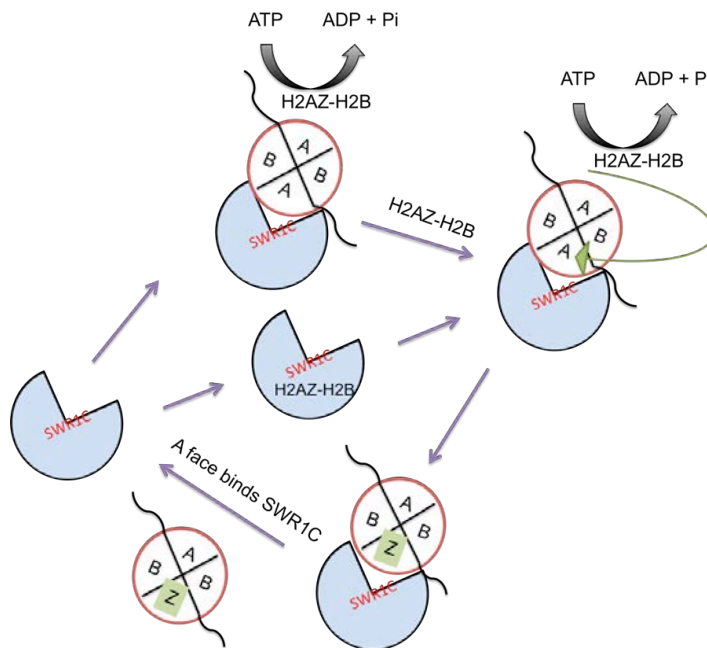
Figure I.4: Mechanism for SWR1 mediated H2AZ exchange. SWR1 complex binds the A/A nucleosome and catalyze H2AZ exchange through ATP hydrolysis forming A/Z nucleosome. A face of the hetero-nucleosome then binds the catalytic domain of SWR1C for another round of histone replacement to form Z/Z nucleosome. Adapted from (Luk et al., 2010).

removal of one H2A from the nucleosome through a dimer exchange mechanism, resulting in A/Z nucleosome (nucleosome with one H2A and one H2AZ histone) (Luk et al., 2010). After the H2AZ exchange, catalytic activity of SWR1 complex is inactivated and A/Z nucleosome is released from the catalytic core of the enzyme. It has been proposed that the A/Z nucleosome may be subjected to another round of exchange mechanism that will result in Z/Z nucleosome (nucleosome with two H2AZ histones) (Luk et al., 2010). Interestingly, the other major constituent of the INO80 family of chromatin remodeling complexes, INO80 complex, was shown to catalyze the reverse reaction to regulate H2AZ distribution (Papamichos-Chronakis et al., 2011). In

the presence of ATP and H2A-H2B dimer, purified INO80 complex catalyzes the exchange of H2AZ for H2A within nucleosomes (Papamichos-Chronakis et al., 2011; Watanabe and Peterson, 2011). From these studies, it is clear that the two ATP dependent chromatin-remodeling complexes of the INO80 family, SWR1 and INO80, regulate H2AZ localization.

The mammalian homologs of SWR1 complex are SRCAP and Tip60-p400, multi-subunit complexes containing distinct SWR1 homologs, with Tip60-p400 also containing the Tip60 histone acetyltransferase (HAT) activity (Cai et al., 2005; Clapier and Cairns, 2009). The SRCAP and p400 subunits that contain conserved bipartite ATPase domain are homologous to the yeast SWR1 protein, which catalyze H2AZ exchange in mammalian cells like its yeast counterpart (Eissenberg et al., 2005; March-Díaz and Reyes, 2009; Ruhl et al., 2006; Wong et al., 2007; Y. Xu et al., 2012). These catalytic subunits also contain similar HSA domain at the N-terminus and distinct domain at the C-terminus: SRCAP contains multiple A/T hook, whereas p400 contains SANT domain (Eissenberg et al., 2005; March-Díaz and Reyes, 2009). Despite these structural differences, it is likely that SRCAP and Tip60-p400 complexes use a similar mechanism for H2AZ exchange in higher eukaryotes; however, this has not been tested. Like Tip60-p400 (described below), SRCAP mediated H2AZ exchange also play important roles in gene expression, and DNA damage repair (Bowman et al., 2011; Dong et al., 2014). But, it still need to be identified if there is redundancy between SRCAP and Tip60-p400 complexes for H2AZ exchange and various

cellular functions. Recently, p400 was also found to catalyze incorporation of histone variant H3.3 in place of canonical histone H3.1 into chromatin. H2AZ and H3.3 occupancy highly correlate with p400 occupancy at regulatory regions such as promoters and enhancers, and both are important for regulation of gene expression (Pradhan et al., 2016). The mechanism of H3.3 exchange is yet to be identified. Below, I have discussed in detail about the biological functions of Tip60 and p400 in various processes including gene transcription, DNA repair, apoptosis, and development.

Biological functions of p400

The p400 subunit of Tip60-p400 complex was identified as part of an adenovirus E1A oncoprotein binding complex (Fuchs et al., 2001). This protein is highly conserved from yeast to human and has been shown to be important in various cellular processes like DNA repair, senescence, and transcription (Gevry et al., 2007; Kusch, 2004). p400 has also been identified as an essential protein for ESC self-renewal (Fazzio et al., 2008).

In normal cells (human fibroblasts), p400 occupies the promoter region of the *p21*, a gene involved in cell cycle inhibition and senescence, and acts as a negative regulator for expression of *p21* (Chan et al., 2005; Gevry et al., 2007). Knockdown (KD) of *p400* causes up-regulation of *p21* and cell cycle arrest in human fibroblasts, suggesting that p400 is important for p21 mediated cellular senescence (Chan et al., 2005). p400 and H2AZ were shown to co-localize at the

promoter region of *p21*, and KD of *p400* reduces H2AZ occupancy, consistent

with this function (Gevry et al., 2007). KD of *H2AZ* also affects *p21* expression,

causing its up-regulation and resulting in premature senescence of human

fibroblasts, similar to what is observed upon *p400* KD (Chan et al., 2005; Gevry

et al., 2007). Upon DNA damage, both p400 and H2AZ occupancy are reduced

at the promoter of *p21*, allowing the upregulation of *p21* expression for cellular

senescence, and efficient DNA repair (Gevry et al., 2007).

p400 also plays an important role in double strand break (DSB) repair

along with the Tip60 acetyltransferase. Upon DSB production in human cells,

p400 catalyzes incorporation of H2AZ into nucleosomes at the break site. The

Tip60 acetyltransferase, which is a part of Tip60-p400 chromatin remodeling

complex then acetylates the N-terminal tail of histone H4 (to be discussed later)

to enhance H2AZ exchange, which is necessary for efficient double strand DNA

break repair through homologous recombination (HR) or non-homologous end

joining (NHEJ) pathways (Price and D'Andrea, 2013; Y. Xu et al., 2012). In

Drosophila, Tip60 acetylates phospho-H2Av (the Droshophila homolog of both

H2AZ and H2A.X) following DSB, resulting in the removal of phospho-H2Av and

incorporation of unmodified H2Av by p400/Domino (Kusch, 2004). These data

from different model systems suggest that p400 and Tip60 work together to

facilitate chromatin remodeling for DNA repair through H2AZ exchange and

histone acetylation activities respectively (Kusch, 2004; Y. Xu et al., 2012).

In addition to its role in transcription, DNA repair, and senescence, p400 is also essential for mouse embryonic development and ESC self-renewal. From an RNAi screen for chromatin remodeling enzymes, p400 has been identified as an essential protein for ESC self-renewal. *p400* KD exhibit flattened and elongated ESC morphology, and decreased cell proliferation compared to wild type ESCs (Fazzio et al., 2008). *p400* KD also show reduced alkaline phosphatase (AP) staining (an established marker of pluripotent stem cells), smaller and fewer embryoid body (EB) formation (*in vitro* readout for ESC differentiation), and a defect in teratoma development (*in vivo* readout for ESC differentiation). In addition, p400 occupies the promoter region of both active and silent genes in ESCs, and *p400* KD largely causes derepression of genes normally silent in ESCs and minimally affects the expression of active genes (Fazzio et al., 2008). These data suggest that p400 largely acts as a repressor of silent genes (e.g. developmental genes) for ESC maintenance. To understand the role of p400 in embryonic development, Exon2 of the *p400* gene was deleted to generate *p400* KO mice. Interestingly, deletion of exon2 resulted in aberrant splicing of the *p400* transcript that resulted in the expression of N-terminally truncated p400 protein. Mice homozygous for this mutation exhibited embryonic lethality at E9.5 (Ueda et al., 2007). Interestingly, the phenotype of a true null mutation has yet to be determined. Whether it causes a pre-implantation defect (similar to *Tip60* KO mice and consistent with the phenotype of *p400* KD ESCs) or a post-implantation

defect (similar to the *p400* exon delete mouse phenotype) remains an open question.

Biological functions of H2AZ

H2AZ is highly conserved among eukaryotes and it plays an essential role in mammalian development. *H2AZ* KO mice are embryonic lethal and die during the developmental stage around E7.5 (Faast et al., 2001). H2AZ incorporation plays an important role in various cellular processes such as gene activation, heterochromatin silencing, and DNA damage repair. Genome-wide localization analyses in yeast have shown that SWR1 and H2AZ co-localize at the promoter regions of many repressed genes and some active genes, and regulate their expression (Guillemette et al., 2005; B. Li et al., 2005; Meneghini et al., 2003; Mizuguchi et al., 2004; H. Zhang et al., 2005). H2AZ is also enriched near telomeres and its occupancy prevents the spread of heterochromatin towards euchromatic regions (Guillemette et al., 2005; Meneghini et al., 2003). In ESCs, H2AZ largely occupies the promoter region of silent genes, such as developmental genes (Creyghton et al., 2008; G. Hu et al., 2013). In contrast, H2AZ mostly occupies the promoter regions of active genes but not silent genes in somatic cells (Hardy et al., 2009). Upon *H2AZ* KD in ESCs, expression of H2AZ enriched silent genes are up-regulated similar to what is observed in *p400* KD (Creyghton et al., 2008; Fazzio et al., 2008; G. Hu et al., 2013). These data suggest that the catalytic function of p400 might be required for ESC

maintenance and repression of developmental genes by incorporating H2AZ, a prediction that we address in this work.

The MYST family of acetyltransferases

Histone acetylation was first reported in 1964 by Alfred Mirsky's laboratory (Allfrey et al., 1964). Histone acetyltransferases (HAT) are enzymes that catalyze the covalent addition of acetyl group from acetyl-coenzyme A to amine groups of lysine residues on histone tails. Acetylation changes the overall positive charge on histone tail to neutral and creates binding sites for proteins with bromodomains (Dhalluin et al., 1999; Marmorstein and Zhou, 2014). There are different HAT families such as GCN5 related N-acetyl transferases (GNATs), MYST, and p300/CBP found in higher eukaryotes that catalyze the HAT activity.

The GNAT family includes HAT1, GCN5, and PCAF members. GCN5 is the most widely studied HAT that mostly acetylates lysine residues on H3 (K9, K14, K18, K23 and K27) and H2B (K11 and K16) tails (Suka et al., 2001). The p300/CBP family is only present in higher eukaryotes and acetylates all core histones: H2A (K5), H2B (K12 and K15), H3 (K4 and K18), and H4 (K5 and K8) (Kimura, 2005). The MYST family of acetyltransferases includes founding members MOZ, YBF2/SAS3, SAS2, and Tip60 all of which consists of conserved MYST domain containing acetyl-CoA binding site (Sapountzi and Côté, 2011; Voss and Thomas, 2009). SAS2 was the first member of MYST family discovered in yeast that specifically acetylates H4K16 to prevent spreading of

heterochromatin and maintenance of euchromatin (Kimura et al., 2002; Lafon et al., 2007; Suka et al., 2002). The acetyl-CoA binding domain of SAS2 was found to be closely related to SAS3 in yeast and two other mammalian proteins MOZ and Tip60. SAS3, which acetylates H3K16, is one of the less studied members of the MYST family (Lafon et al., 2007; Voss and Thomas, 2009). MOZ targets H3 and plays important roles in gene regulation and hematopoietic stem cell (HSCs) maintenance (Crump et al., 2006; Katsumoto, 2006; Thomas et al., 2006; Voss and Thomas, 2009). The most widely studied MYST family protein is Tip60, which acetylates lysine 5 (K5) on the amino-terminal tails of histone H2A and H2A.Z, as well as K5, K8, K12, and K16 residues on histone H4 (Ikura et al., 2000; Kimura and Horikoshi, 1998). In addition to mammals, Tip60 is also found in other organisms such as Drosophila (dTip60), and Caenorhabditis elegans (ceTip60/MYS1), and is important for their development (Ceol and Horvitz, 2004; Voss and Thomas, 2009; Zhu et al., 2007). Tip60 is also closely related to another MYST family member in yeast called Esa1 based on similar protein domains, histone targets, interacting proteins, and various cellular functions including cell cycle progression and DNA damage repair (Bird et al., 2002; Ikura et al., 2000; Sapountzi et al., 2006; Voss and Thomas, 2009).

Most of these HAT enzymes are multi-subunit proteins that not only acetylate histone tails, which correlates with transcription activation, but also acetylate lysine residues of non-histone proteins such as HIV-1 TAT, p53, and androgen receptor (AR) and act as transcriptional co-activators (Verdone et al.,

2005; X.-J. Yang, 2004). Lysine acetylation generates docking sites for regulatory proteins and protein complexes containing bromodomains such as Gcn5, PCAF, and SWI/SNF complex to control gene expression (Verdone et al., 2005; X.-J. Yang, 2004). Here, I will discuss biological functions and general mechanism of action of HAT enzymes, particularly focusing on Tip60.

Mechanism of lysine acetylation by Tip60/Esa1

The catalytic mechanism of the GCN5 HAT family was the first to be characterized. It was shown that GCN5 forms a ternary complex between acetyl-CoA, histone substrate, and enzyme. The glutamate (Glu) residue at the catalytic domain acts as a general base, which abstracts a proton from the lysine residue on the histone tail substrate. Deprotonated lysine becomes unstable and acts as a nucleophile to attack the carboxyl group on the acetyl-CoA within the ternary complex. This leads to the formation of an unstable tetrahedral intermediate structure and its collapse finally gives acetylated histone tail as the final product (Tanner et al., 2000; 1999). It is important to note that ternary complex formation is necessary before the catalysis occurs. During this process, acetyl-CoA binds first with the enzyme, and then with the histone substrate and CoA is released along with an acetylated substrate. In contrast, p300/CBP uses different amino acids as catalytic residues (Tyr1467 and Trp1436) for catalysis. From structural analysis, Tyr1467 in the catalytic domain is proposed to act as a general acid for catalysis and Trp1346 is proposed to orient the lysine residue

from the histone substrate into the active site of p300/CBP (Yuan and

Marmorstein, 2012).

Members of the MYST family of HATs, which include Tip60, contain a

conserved MYST catalytic domain, and other accessory domains such as zinc

fingers (within MYST domain) and chromo domains for substrate specificity

(**Figure I.5)** (Brown et al., 2016; Kuo and Allis, 1998). The catalytic mechanism of

MYST with regard to lysine acetylation has been well studied with regard to

Esa1, the Tip60 homolog in yeast, suggesting similar mechanism might exist in

Tip60. Motif A present within MYST domain is a highly conserved motif among

HATs that contains the amino acid sequence R/Q-X-X-G-X-G/A (where X is any

amino acid). This conserved sequence binds the cofactor acetyl-CoA, thereby

catalyzing the transfer of an acetyl group onto the histone tails (Kuo and Allis,

1998). Like Gcn5, ESA1/ Tip60 also forms a ternary complex between acetyl-

CoA and histone substrates for catalysis (**Figure I.6)** (Berndsen et al., 2007).

Biological functions of Tip60 acetyltransferase

Tip60 not only acetylates histone tails, but also acetylates and activates

non-histone substrates, such as ATM or p53, during DNA repair and apoptosis

(Sapountzi et al., 2006). In addition, Tip60 also acetylates the nuclear receptor

AR, and the transcription factor MYC to enhance activation of their target genes

(Patel et al., 2004). Upon DNA damage, Tip60 has been shown to acetylate and

activate p53 and trigger apoptotic cell death if the damaged DNA is not repaired
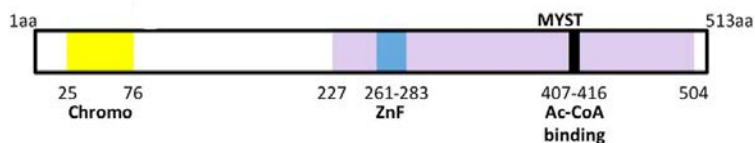
Figure I.5: Tip60 protein structure. Diagram representation of Tip60 domains that belong to MYST family of histone acetyltransferase. Tip60 contains chromo-domain and MYST domain. Within MYST domain contains zinc finger (ZnF) domain and acetyl-CoA binding domain. Adapted from (Brown et al., 2016).
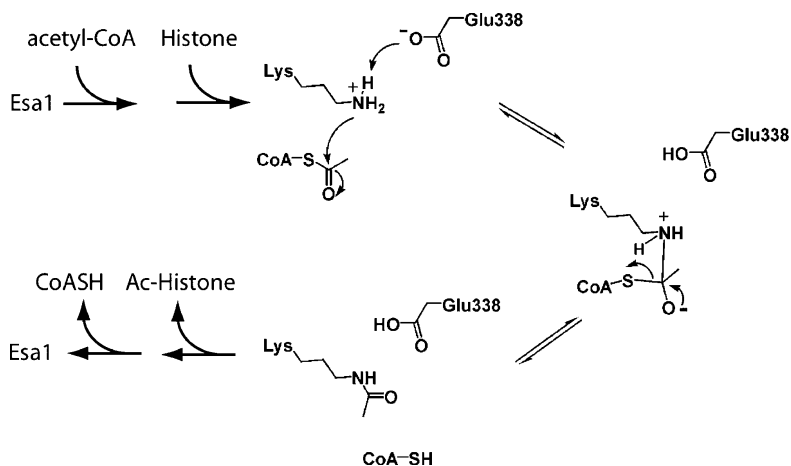
Figure I.6: Schematic for the ESA1/Tip60 HAT activity. Direct attack mechanism of ESA1/Tip60 protein to acetylate the N-terminal histone tail forming ternary complex with acetyl-CoA and histone substrate. Adapted from (Berndsen et al., 2007).

(Ikura et al., 2000). Expression of mutated *Tip60* lacking catalytic activity in HeLa cells fails to promote apoptosis after gamma irradiation indicating the catalytic activity of Tip60 is required to promote apoptosis (Ikura et al., 2000).

During dsDNA break repair, Tip60 is directly recruited to the break site along with ATM. Tip60 acetylates ATM, which leads to auto-phosphorylation and activation of ATM kinase activity, resulting in phosphorylation of histone variant H2A.X (called γH2A.X) (Y. Sun et al., 2005). Acetylation of histone tails by Tip60 is also thought to facilitate chromatin decompaction to allow spreading of γH2A.X and efficient repair of damaged DNA (Price and D'Andrea, 2013). The γH2A.X variant acts as a signal for recruitment of DNA damage repair proteins such as MRN complex or Ku70/Ku80 complex. The recruitment of these repair proteins mediate dsDNA break repair using error free homologous recombination or error prone non-homologous end joining pathways (Price and D'Andrea, 2013).

In addition to its role in DNA damage and apoptosis, Tip60 is required for ESC maintenance and mammalian development (Fazzio et al., 2008; Y. Hu et al., 2009). Tip60 was first isolated as a HIV-1 Tat interactive protein (Tip) that acts as a co-activator and enhances the expression of the HIV-1 promoter (Kamine et al., 1996). It consists of four isoforms, which are alternative splice variants of the *Tip60* gene (**Figure I.7**) (M.-S. Kim et al., 2006; Legube and Trouche, 2003; Ran and Pereira-Smith, 2000). The functional roles of each of these specific isoforms are currently unknown. In this study, we have characterized the importance of one isoform called Tip55 during embryonic
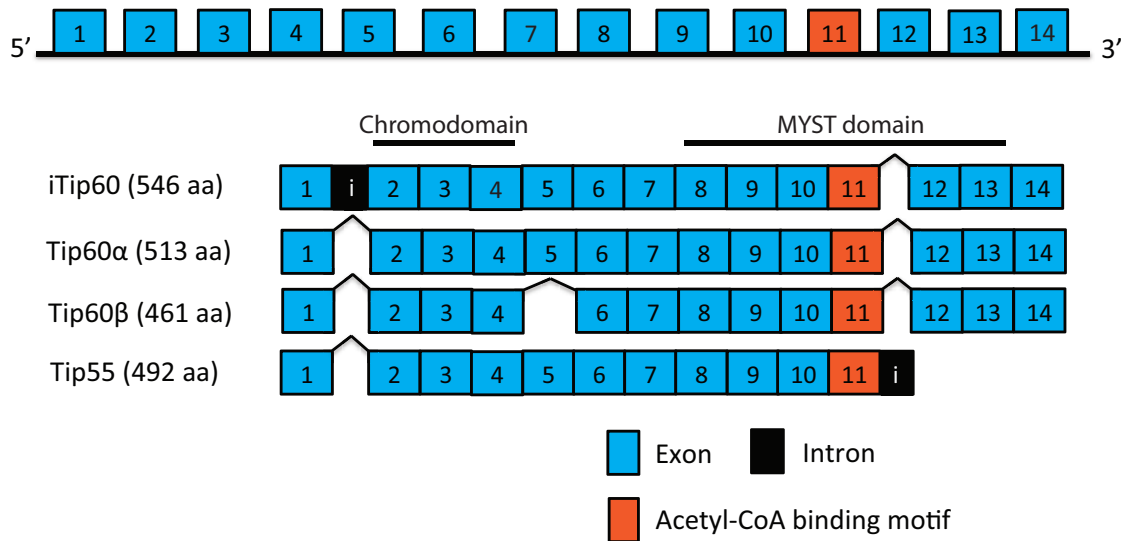
Figure I.7: Tip60 isoforms. *Tip60* gene contains four alternatively spliced isoforms. *iTip60* retains intron 1, *Tip60α* retains all exons (no introns), *Tip60β* lacks exon 5, and Tip55 retains short intron between exon11 and exon12 (doesn't retain exons 12, 13 and 14).

development, which will be discussed in chapter III. Previous work found that

Tip60 KO mice that lack all four isoforms are embryonic lethal and die due to

proliferation defects in the inner cell mass that lead to apoptosis (Y. Hu et al.,

2009). In addition, an RNAi screen looking for proteins involved in ESC self-

renewal identified Tip60 as an essential protein for ESC maintenance (Fazzio et

al., 2008), consistent with the phenotype of *Tip60* KO mice. *Tip60* KD exhibits a

similar phenotype to *p400* KD with flattened and elongated ESC morphology,

reduced cell proliferation, decreased alkaline phosphatase (AP) staining, and

defects in embryoid body (EB) formation and teratoma development (Fazzio et

al., 2008). Tip60 also occupies and acetylates the promoter region of both active and silent genes in ESCs. Upon *Tip60* KD, there is decrease in acetylation level and de-repression of developmental genes normally silent in ESCs, indicating that Tip60 acts as a repressor of differentiation or developmental genes for ESC self-renewal (Fazzio et al., 2008). This is a surprising finding because histone acetylation is associated with gene expression, but these data suggest that Tip60 mediated histone acetylation might be responsible for repressing silent genes for ESC maintenance and proper embryonic development. We have tested this possibility and report the findings below, in chapter II.

CHAPTER II

PREFACE

Data presented in this chapter are published in Cell Reports:

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

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Author Contributions

Diwash Acharya, Jaime Rivera-Pérez, and Thomas Fazzio designed experiments. Diwash Acharya performed most experiments with help from Yeonsoo Yoon and Jaime Rivera-Pérez (early embryo dissection and staining), Feng Wang and Ingolf Bach (late embryo dissection), and Thomas Fazzio (ATAC-seq). Sarah Hainer and Diwash Acharya analyzed the deep sequencing data.

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.

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, 2013). 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., 2008). 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 (Fazzio et al., 2008). 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) (Y. 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). 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; Jiang et al., 2011; Kusch, 2004; Sykes et al., 2006; Tang et al., 2006; Van Den Broeck et al., 2011). 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 (Gevry 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 (Gevry et al., 2007; Papamichos-Chronakis et al., 2011; Y. 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 (Fazzio et al., 2008). 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 (Fazzio et al., 2008). 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 (Y. 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 II.S1A-B), both of which were previously shown to block enzymatic activity (Ikura et al., 2000). We confirmed that these mutations broadly reduced H4 acetylation and H2A.Z deposition, respectively, in ESCs (Figure II.S1C-D). Since *Tip60* or *Ep400* depletion in ESCs causes loss of self-renewal (Fazzio et al., 2008), 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 II.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 II.1A), and SSEA-1 (Figure II.S1F), whereas *Tip60* KD or *Ep400^{hypo}* cells exhibited reduced AP and SSEA-1 staining and flattened colony morphologies, as observed previously (Fazzio et al., 2008). *Tip60^{ci/ci}* and *Ep400^{ci/ci}* cells proliferated more rapidly than *Tip60* KD and *Ep400^{hypo}* cells

(Figure II.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 II.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 (Fazzio et al., 2008), *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 II.1C-F, Figure II.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 II.1C-

F, Figure II.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.

A. Wild Type, Tip60 KD, Ep400 KD, Ep400^hypo, Tip60^ci/ci #1, Tip60^ci/ci #2, Ep400^ci/ci #1, Ep400^ci/ci #2

B. Cell Number vs Days. Legend: Tip60^fl/+, EGFP KD, Tip60 KD, Tip60^ci/ci #1, Tip60^ci/ci #2, WT Controls, Ep400^ci/ci, Tip60^ci/ci, Ep400^hypo, Tip60 KD, WT, Ep400^hypo, Ep400^ci/ci #1, Ep400^ci/ci #2, Ep400^ci/ci #3

C. Tip60 KD, Tip60^ci/ci. Log$_2$ (Fold Change) 3, 0, -3

D. Ep400^hypo, Ep400^ci/ci. Log$_2$ (Fold Change) 3, 0, -3

E. Sig. Misregulated Genes. Tip60 KD 1678, Tip60^ci/ci 15, 10

F. Ep400^hypo 611, Ep400^ci/ci 0

Figure II.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 ǂ m.
(**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 |$\log_2$ (fold change)| > 1 and their multiple testing-adjusted p value < 0.05.

A Initial Targeting Construct

B *Ep400* Targeting Construct

C H4ac ChIP-seq

D H2A.Z ChIP-seq

E

F SSEA-1 / Brightfield

G Wild Type / *Tip60^ci/ci Ep400^ci/ci* #1 / *Tip60^ci/ci Ep400^ci/ci* #2
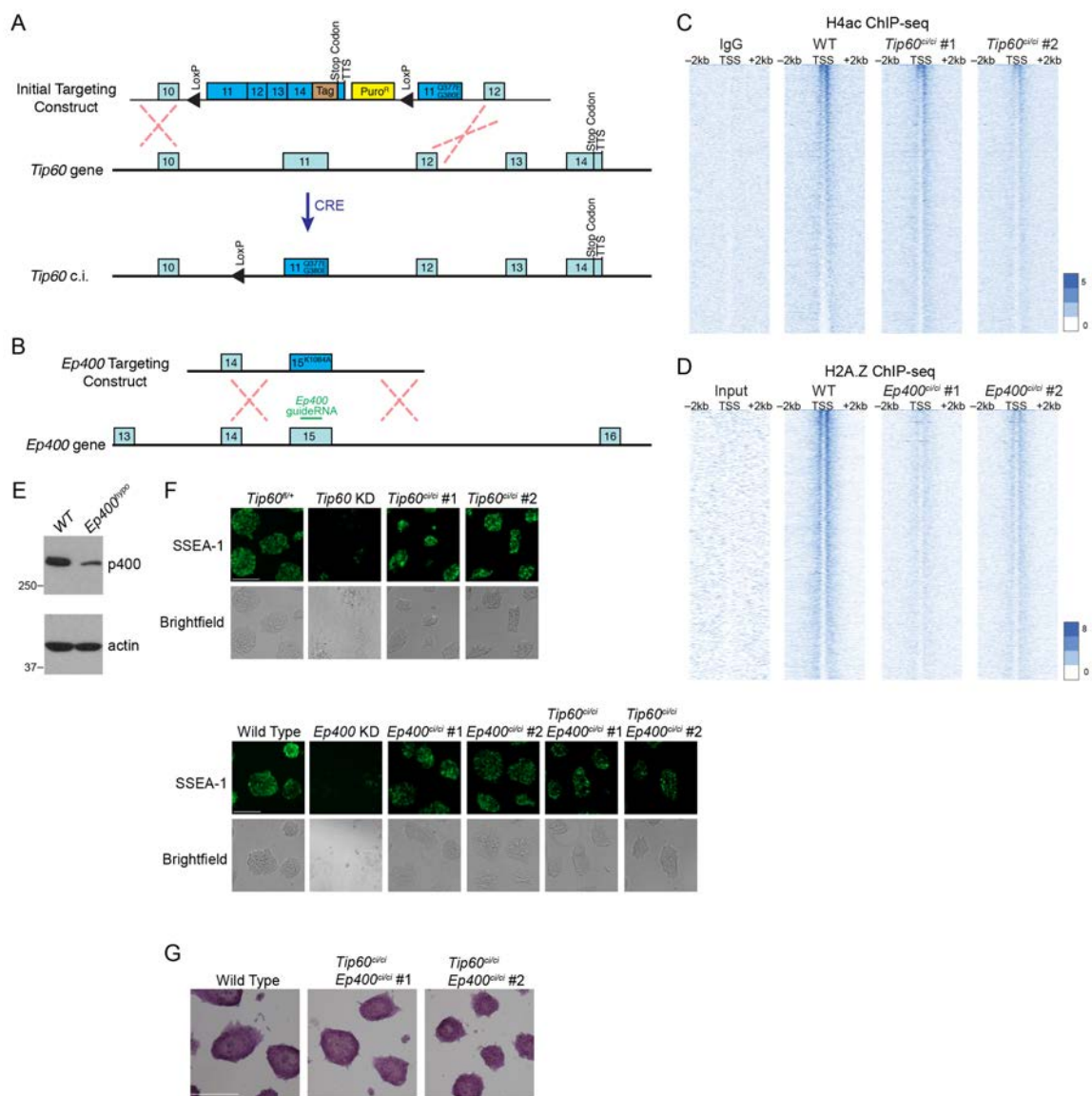
Figure II.S1: Generation and phenotypes of *Tip60$^{ci/ci}$* and *Ep400$^{ci/ci}$* mutants, Related to Figure II.1.

(**A**) Schematic representation of *Tip60$^{ci/ci}$* lines generated using homologous recombination of the construct, followed by Cre-LoxP–mediated excision of the wild type *Tip60* sequence.

(**B**) Schematic for generation of *Ep400$^{ci/ci}$* mutants using CRISPR/Cas9 mediated homologous recombination.

(**C**) ChIP-seq of tetra-acetylated H4 (K5/8/12/16) in wild type and two *Tip60$^{ci/ci}$* lines. Heatmaps are over TSS-proximal regions (+/- 2kb), sorted from highest H4ac to lowest. IgG is a specificity control.

(**D**) H2A.Z ChIP-seq in wild type or two *Ep400$^{ci/ci}$* mutant ESC lines, as in (**C**).

(**E**) Western blot confirmation of *Ep400^{hypo}* lines, generated using CRISPR/Cas9 without the repair template.

(**F**) SSEA-1 live cell staining of *Tip60$^{ci/ci}$*, *Ep400$^{ci/ci}$*, and *Tip60$^{ci/ci}$Ep400$^{ci/ci}$* mutants, compared to their respective controls *Tip60$^{fl/+}$*, *Tip60* KD, Wild Type and *Ep400* KD.

(**G**) AP staining of *Tip60$^{ci/ci}$Ep400$^{ci/ci}$* mutants as in Figure II.1A. Scale bars equal 200 μm in both (**F**) and (**G**).
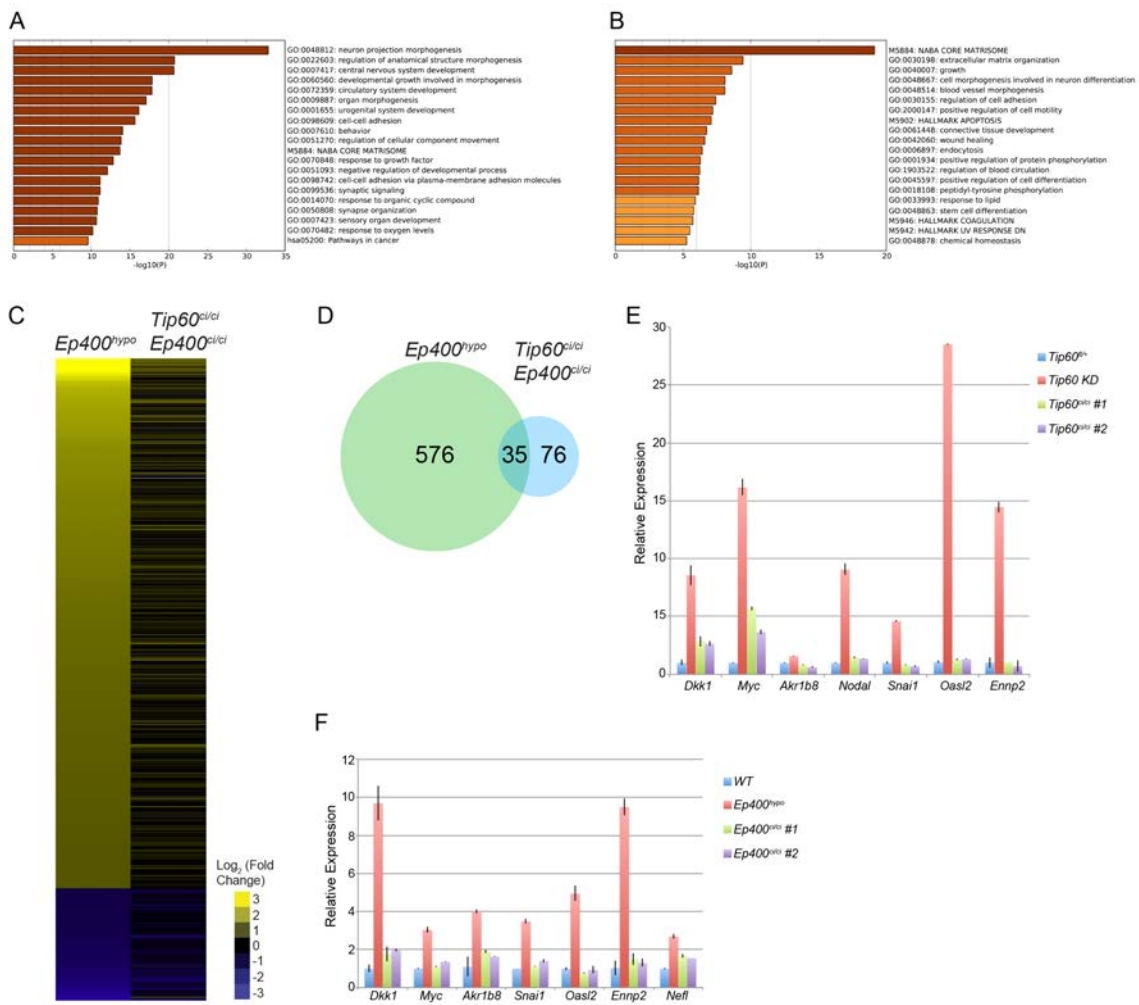
Figure II.S2: Catalytic activity-independent gene regulation by Tip60-p400, Related to Figure II.1.

(**A, B**) GO (Gene ontology) terms enriched within genes upregulated in *Tip60* KD (**A**), and *Ep400^{hypo}* ESCs (**B**), as measured by RNAseq. Shown are histograms depicting the significance (-log$_{10}$ p value) of GO categories enriched in each gene set (generated by Metascape; http://metascape.org).
(**C**) Heatmaps of differentially expressed genes in *Tip60$^{ci/ci}$ Ep400$^{ci/ci}$* ESCs relative to *Ep400^{hypo}* control cells, as in Figure II.1.
(**D**) Venn diagram showing number of significantly misregulated genes commonly misregulated in *Tip60$^{ci/ci}$Ep400$^{ci/ci}$* and *Ep400^{hypo}* ESCs, as in Figure II.1.
(**E, F**) RT-qPCR measuring mRNA levels of Tip60-p400 target genes in mutants or control ESCs as indicated. mRNA levels were normalized to *GAPDH*.

Table II.S3. *Ep400* repair template, related to Figures S1-S3 and Figure 1.
 (**K1084A mutation**; <u>silent PAM mutation</u>; *guideRNA sequence*)
TAGGCTCATAAAACTCACAGCAGTCTGAGTTGTGTCTATTTCATTGTTGTTGT
AGATGTAGAAGACTGTCCTAGTGACAGGGAGAGCCGGAGGGACTCCGTTC
TCATTGACTCACTCTTCATCATGGATCAGTTTAAAGCTGCAGAGAGAATGAG
CATTGGAAAATCCAACACCAAGGACATCACAGAAGTTACTGCTGTGGCTGA
AGCCATCCTCCCTAAGGGCAGTGCCCGAGTCACAACTGCGGTGAGGAAAG
CCTTTCCTGCCTCCCAAACACGCTCCATAGGAATGCCTAGAAAAGGCAGTT
CTTGTGTCCTTATGTTCTGTAAATCATTTGGGATAGTCTCTCGATTTAGGCTC
TGAGAAGGTGTGTGCCAATTACTCACTCTTTGGCTGGTCTGTCTGTCTCTAT
AGGTGAAGTTTAGTGCTCCATCTTTGTTGTATGGTGCTCTCCGAGACTATCA
GAAGATAGGCCTGGACTGGTTGGCCAAGCTATACCGGAAGAATCTCAATGG
CATAT*TGGCTGATGAAGCAGGGCTT*<u>GGC</u>**GCC**ACTGTGCAGATCATTGCTTT
TTTTGCTCACCTTGCCTGTAATGAAGGTAAGATCCTCTCAGTCTCCACTAAG
AGCGTGTGTTAGATCTGAGAGAAAGAAATTGTCAGCCTCTTTTGCTCATCT
CTCTTTCTTGAGCCAAGAAATGACTCTCCTTTTTAAAATTTTTATTTATTTTTTT
ATTCTTTGCATACATTATATCTCGACCACACCATTCTTCCCAGAGTCTTCCTC
CATCATCTTCTCCCCAGGAGCTTCCATTACCATAAAAAAATAAAACCAACAAA
TAACAGCAACAAAAACAAAAGCAGGCATCCCAGGGATATCCACCATACAT
GGCATAACAAGTTACAGTGAGACTAGGCACAAACCCTCATCTCAAGGCTGG
ATGAGGCAGCCCAGTAGA

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 II.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 II.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 II.2A-B), consistent with the observed enrichment of Tip60 near gene regulatory elements such as promoters and enhancers (Fazzio et al., 2008). Interestingly, we observed significantly increased chromatin accessibility upon *Tip60* KD, but minimal changes in accessibility in KAT–deficient ESCs (Figure II.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 II.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 II.2D) (P. B.

Chen et al., 2015; 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 II.2E). Consistent with these findings, KAT–deficient Tip60

bound to Tip60-p400–target genes at levels similar to wild type (Figure II.S3C).

These data demonstrate Tip60 functions independently of its KAT activity to

regulate promoter-proximal chromatin accessibility in ESCs.

A

B

C

D

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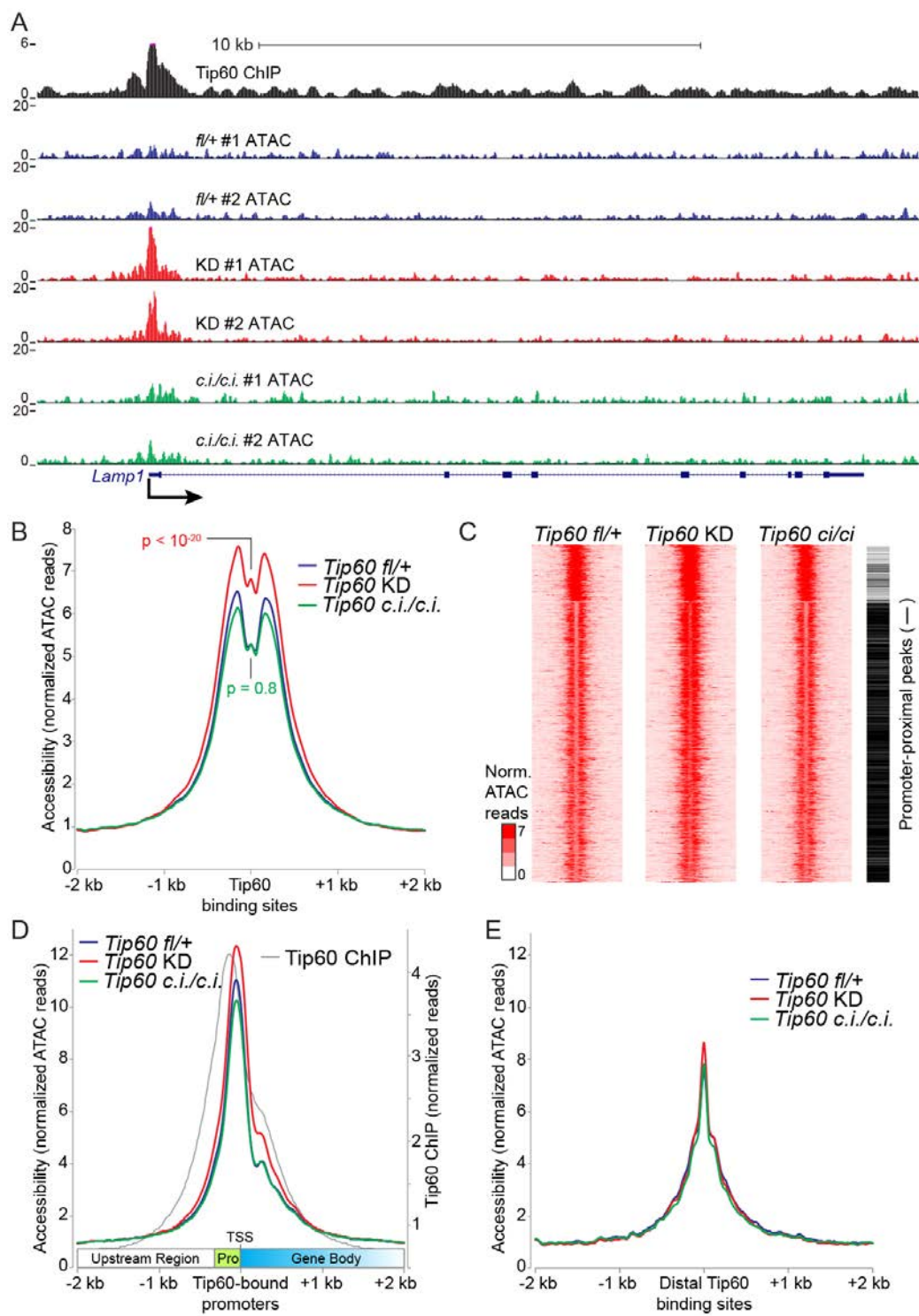
Figure II.2: KAT-independent regulation of chromatin accessibility at Tip60 target loci.

(**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.

A

Tip60-FLAG

Tip60^{ci/ci}
p400^{ci/ci}

WT #1 #2

WB:
p400

250—

75—

FLAG

50—

50—

Actin

37—

B

Tip60-FLAG

WT *p400^{hypo}* *Tip60^{ci/ci}* *p400^{ci/ci}*

220—

120—

80—

50—

15—

C

Tip60-FLAG ChIP-qPCR

■ WT
■ *p400^{ci/ci}*
□ *Tip60^{ci/ci}*

Log2 Fold Change (Rel. to WT)

2

1.5

1

0.5

0

Rps9 *Gata6* *Gata4* *Zfp37* *Lars* *Nodal*
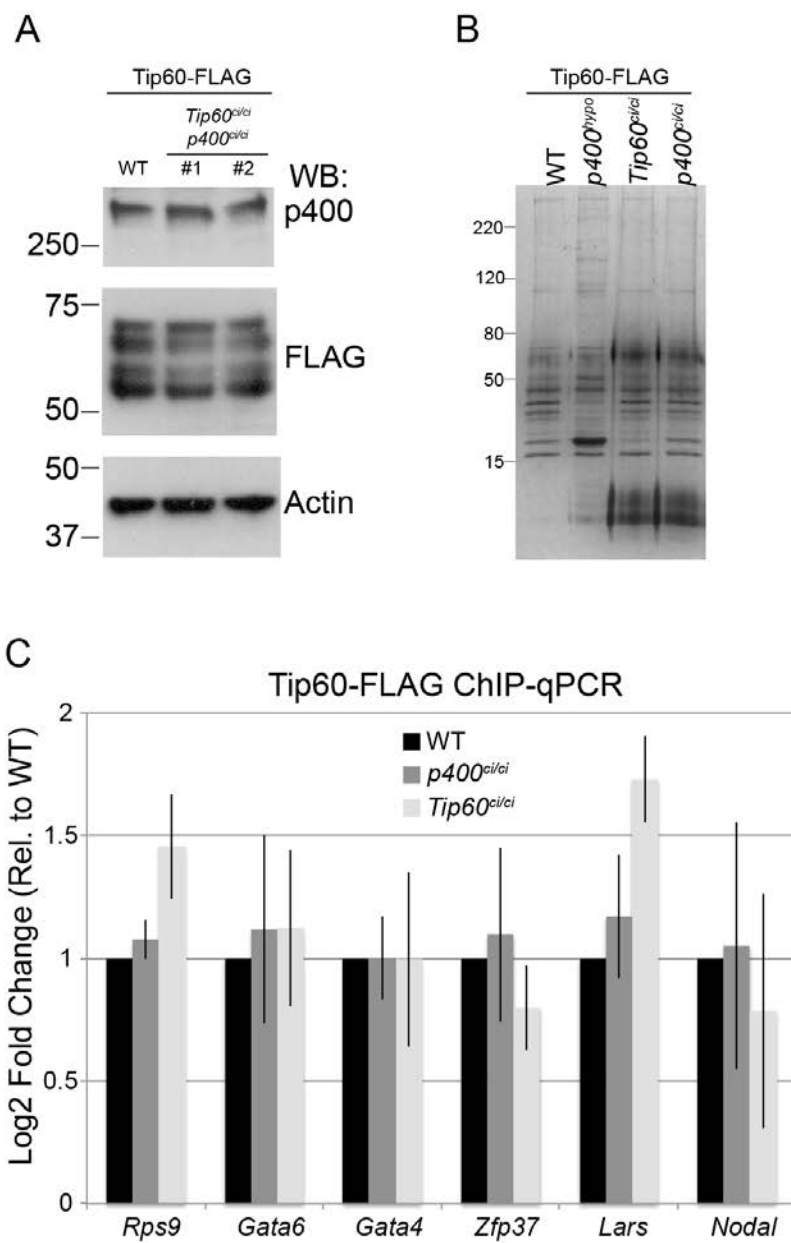
Figure II.S3: Catalytically inactive Tip60-p400 mutations do not compromise complex integrity, Related to Figure II.2.

(**A**) Western blots indicating equal expression of catalytic subunits of Tip60-p400 in wild type or double catalytically inactive mutant ESCs. Actin is a loading control.
(**B**) Silver stain of Tip60-p400 complex purified from lines with genotypes indicated at top. In each case, Tip60 is FLAG-tagged at both copies of its endogenous locus.
(**C**) Tip60-FLAG ChIP-qPCR from WT, $Tip60^{ci/ci}$, and $Ep400^{ci/ci}$ ESCs show similar Tip60 occupancy in each. Shown are biological triplicate ChIP-qPCRs from each line, normalized to untagged control ESCs, and expressed relative to WT.

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

Consistent with the self-renewal defect of *Tip60* KD ESCs (Fazzio et al., 2008), *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 (Y. 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 II.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 II.3A), but were much smaller than *Tip60$^{+/+}$* or *Tip60$^{ci/+}$* littermates (Figure II.3B), and exhibited morphological abnormalities as early as E6.5 (Figure II.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 (Fazzio et al., 2008), 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 II.3C-D). However, induction of mesodermal and endodermal markers was delayed and/or reduced in *Tip60$^{ci/ci}$* EBs (Figure II.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 II.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*.
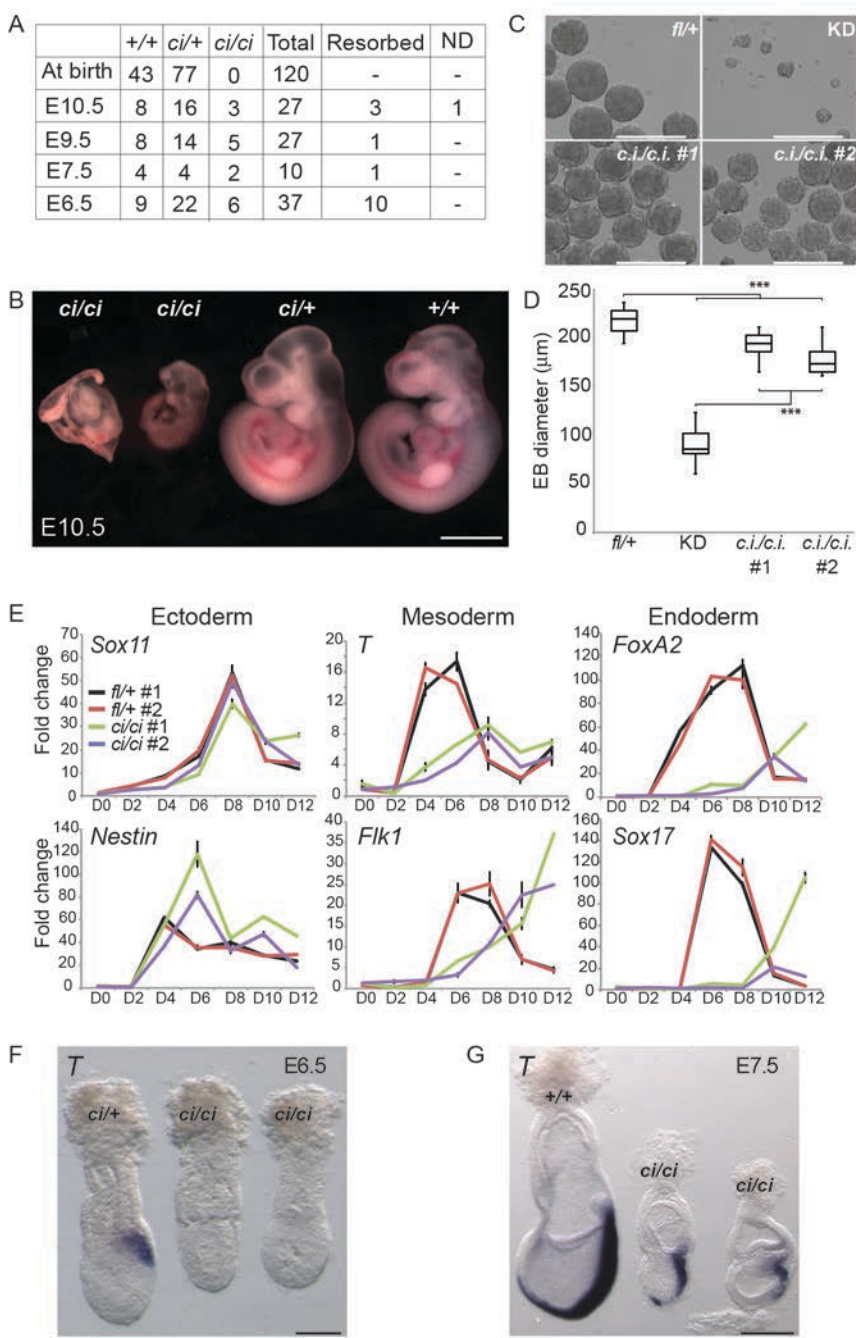
Figure II.3: The *Tip60* catalytic activity is required for differentiation and post-implantation development.

(**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**).
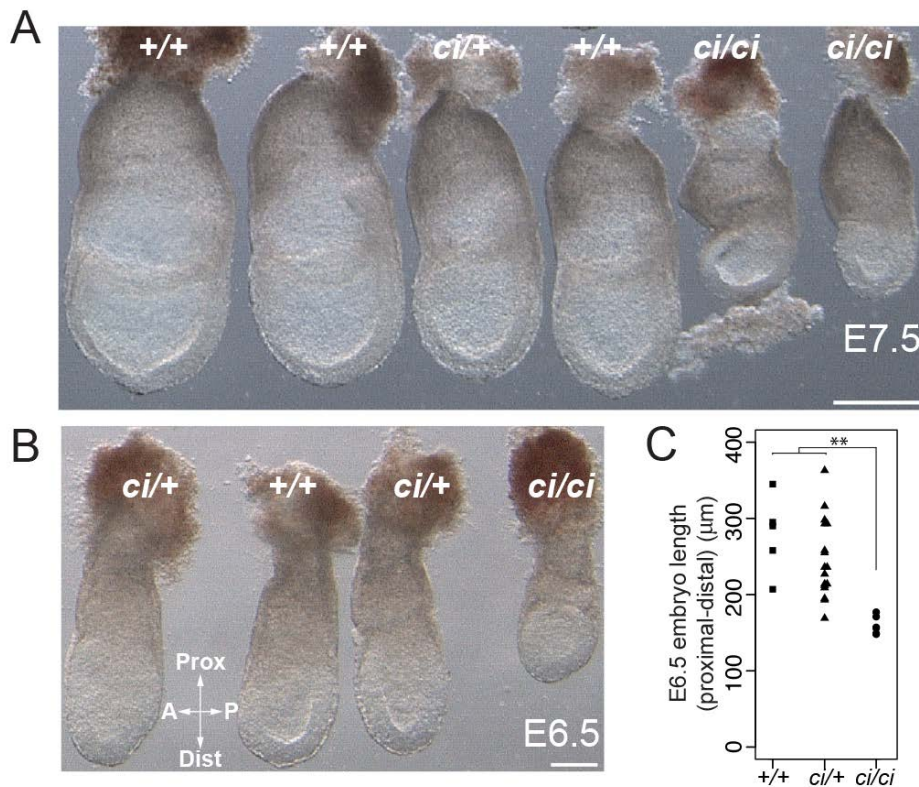
Figure II.S4. Phenotypes of *Tip60^{ci/ci}* embryos, Related to Figure II.3.

(**A, B**) Brightfield images of E7.5 and E6.5 embryos from *Tip60^{ci/ci}* intercrosses, with their genotypes (determined after imaging) indicated. Scale bars equal 100 μm.
(**C**) Measurement of the length of the proximal distal axis of embryos (epiblast + extraembryonic endoderm) of the indicated genotypes (**p < 0.01, calculated using a two sided t-test).

Table II.S1. RTqPCR primers, Related to Figure 3.

Nestin	Forward: TGGCACACCTCAAGATGTCCCTTA
	Reverse: AAGGAAATGCAGCTTCAGCTTGGG
Sox11	Forward: ACGACCTCATGTTCGACCTGAGCT
	Reverse: CACCAGCGACAGGGACAGGTTC
T	Forward: CCAAGGACAGAGAGACGGCT
	Reverse: AGTAGGCATGTTCCAAGGGC
Flk1	Forward: GCTTGCTCCTTCCTCATCTC
	Reverse: CCATCAGGAAGCCACAAAGC
Sox17	Forward: CTCGGGGATGTAAAGGTGAA
	Reverse: GCTTCTCTGCCAAGGTCAAC
FoxA2	Forward: CCCTACGCCAACATGAACTCG
	Reverse: GTTCTGCCGGTAGAAAGGGA
Dkk1	Forward: ACTCAAATGGCTTTGGTAATATGG
	Reverse: ATAATCTCTTCTGAATTCTGCCCA
Myc	Forward: AGCTGTTTGAAGGCTGGATTTC
	Reverse: GCAACATAGGATGGAGAGCAGA
Akr1b8	Forward: TACTGTCACTCGAAGGGCATCT
	Reverse: ATCTCCTCGTCACTCAACTGGA
Nodal	Forward: TCCTTCTTCTTCAAGCCTGTTG
	Reverse: CCAGATCCTCTTCTTGGCTCA
Snai1	Forward: CTTGTGTCTGCACGACCTGTG
	Reverse: AGACTCTTGGTGCTTGTGGAG
Oasl2	Forward: TTGTGCGGAGGATCAGGTACT
	Reverse: TGATGGTGTCGCAGTCTTTGA
Ennp2	Forward: ATGGCAAGACAAGGCTGTTTC
	Reverse: TTGACGCCGATGGCAAAAGT
Scamp1	Forward: CCTTGAGGTCTGTGGTATTGGA
	Reverse: TACACCCTTAGTGACCTCAGTGTC
Nefl	Forward: AGCTAGAGGACAAGCAGAATGC
	Reverse: GCAAGCCACTGTAAGCAGAAC

Table II.S2. Genotyping primers, related to Figure 3.

Tip60^{ci/+} or *Tip60*^{ci/ci} (mice and ESCs)	Forward: GTGGGCTACTTCTCCAAGGTC
	Reverse: TGTGAAGCACAGATGAGGGT

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 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 II.4A; e.g. *FoxA2, Gata4, Sox17, T, Hand1, Flk1*), expanding on our preliminary analyses (Figure II.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 II.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 II.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 II.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.
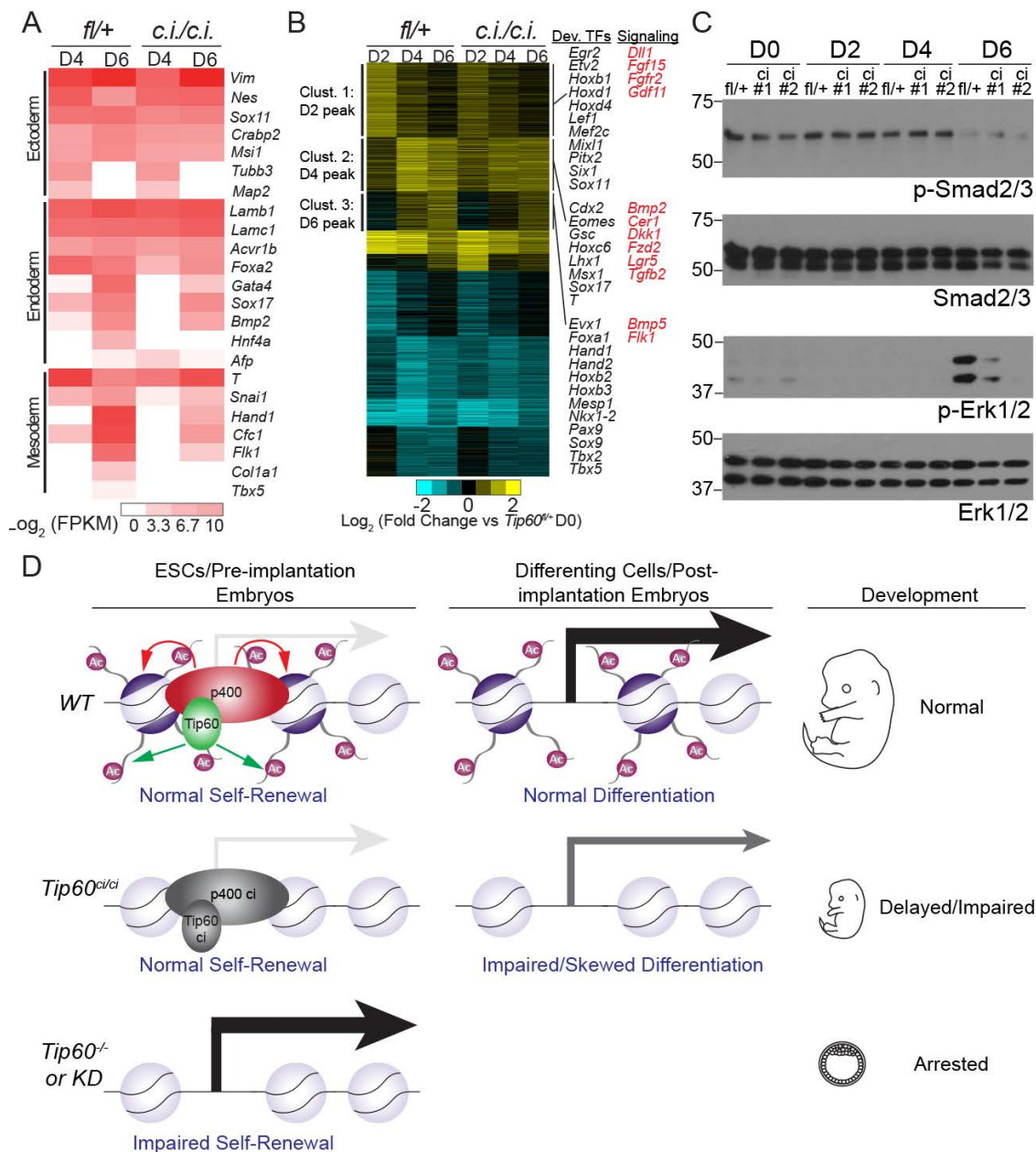
Figure II.4: Delayed/impaired expression of developmental regulators in

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.

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., 2008; Y. 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., 2008), 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 II.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., 2008)) 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[TM] 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 II.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 II.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 II.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 II.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 II.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 II.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., 2008).

ChIP-seq analysis

Single-end raw FastQ reads were collapsed, adaptor sequence were removed, and reads were mapped to the mouse mm10 genome using bowtie, allowing one mismatch. Aligned reads were used for downstream analysis using the "annotatePeaks" command in HOMER (Heinz et al., 2010) to make 20 bp bins over promoter proximal regions and summing the reads within each bin. Experiments were aligned over high quality (peak score > 6) promoter-proximal Tip60 peaks called from (Ravens et al., 2015). After anchoring mapped reads over the reference site, heatmaps for biological replicates were generated using Java Treeview.

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.

RNA-seq analysis

TopHat2 (D. Kim et al., 2013) was used to map the RNAseq reads to the mouse genome (mm10) using parameters (--library-type fr-firststrand --segment-length 38). The bam files from the Tophat output were used for downstream analysis using HOMER (Heinz et al., 2010). DESeq2 (Love et al., 2014) was used to identify the differentially expressed genes. Heatmaps were generated using Java TreeView (Saldanha, 2004). K-means clustering was performed using Cluster 3.0 (de Hoon et al., 2004), and GO term enrichment was calculated using Metascape software (http://metascape.org) (Tripathi et al., 2015).

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.

ATAC-seq analysis

Paired-end 75 bp reads were trimmed to 24 bases and reads were then aligned to mm10 using Bowtie2 with the parameter -X 2000 to ensure that fragments up to 2 kb were allowed to align. Duplicates were then removed using Picard (http://broadinstitute.github.io/picard/). Reads with low quality score (MAPQ < 10) and reads mapping to the mitochondrial genome (chrM) were removed. Reads were separated into size classes as described (Buenrostro et al., 2013) and only nucleosome free reads (less than 100 bp) were used for subsequent analyses. These reads were processed in HOMER (Heinz et al., 2010). Genome browser tracks were generated from mapped reads using the "makeUCSCfile" command. Mapped reads were aligned over specific regions using the "annotatePeaks" command to make 20 bp bins over regions of interest and sum the reads within each bin. Experiments were aligned over high quality (peak score > 6) Tip60 peaks called from (Ravens et al., 2015), that were subsequently separated into promoter-proximal and –distal groups. After anchoring mapped reads over reference sites, aggregation plots were generated by averaging data obtained from biological replicates. Heatmaps were ordered based on clustering of reads

summed over -100 bp to +100 bp from the Tip60 peak center through K-means clustering using Cluster 3.0.

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.

Accession numbers

Deep sequencing data are available at Gene Expression Omnibus (accession: GSE85505).

CHAPTER III

PREFACE

Data presented in this chapter are not published. Currently, we are working to prepare manuscript for submission:

The Tip55 HAT supports cellular proliferation and multilineage development

Diwash Acharya, Bernadette Nera, Zachary J. Milstone, Lauren Bourke, Chinmay M. Trivedi, Thomas G. Fazzio

Author Contributions

Diwash Acharya, Chinmay M. Trivedi, and Thomas Fazzio designed experiments. Diwash Acharya performed most experiments with help from Bernadette Nera, Zachary J. Milstone, and Lauren Bourke (embryo dissection and staining).

Summary

Tat-interactive protein 60 (Tip60) belongs to the MYST family of histone acetyltransferases (HATs), and is important for various cellular functions including gene regulation, DNA repair, apoptosis, and early embryonic development. Four splice variants of Tip60 are expressed, *Tip60α, Tip60β, iTip60,* and *Tip55.* The extent to which these isoforms perform overlapping or distinct functions has not been addressed, with most studies focusing solely on the most abundant isoform, *Tip60α.* In this study, we ablate the *Tip55* isoform in mice and find that homozygous mutation of *Tip55* results in embryonic lethality around embryonic day E11.5. We further show that loss of *Tip55* causes a defect in cellular proliferation in heart, and increased cell death in the neural tube. Our findings demonstrate that the *Tip55* isoform is essential for organogenesis during early embryonic development.

Introduction

Tip60 was originally identified from a yeast two hybrid screen as a 60

kDa, HIV-1 Tat interactive protein (Kamine et al., 1996). Tip60, which is the

mammalian ortholog of the essential yeast HAT Esa1, is highly conserved among

eukaryotes (Doyon et al., 2004). In mammalian cells, Tip60 exists mainly within

the Tip60-p400 multi-subunit complex, which regulates gene expression and has

key roles in DNA repair (Fazzio et al., 2008; Ikura et al., 2000). In addition, Tip60-

p400 contains a second catalytic subunit, the p400 ATPase, which functions in

exchange of canonical H2A for H2A.Z within nucleosomes, and has been

reported to function in incorporation of H3 variant H3.3 (Pradhan et al., 2016; Y.

Xu et al., 2012). The components and functions of Tip60-p400 are largely

conserved, although the HAT and ATPase functions comprise separate

complexes in yeast, NuA4 and SWR1, respectively (Auger et al., 2008; Fuchs et

al., 2001; Ikura et al., 2000).

Tip60 is one of the founding members of the MOZ, YBF2, SAS2, TIP60

(MYST) family of histone acetyltransferase (HAT) proteins that contains a

chromodomain and MYST domain (Sapountzi et al., 2006). The MYST domain of

Tip60 contains a HAT domain that acetylates lysine residues on histone tails

(Kimura and Horikoshi, 1998; Yamamoto and Horikoshi, 1997). In addition, Tip60

binds and acetylates non histone proteins such as p53, ATM, and the androgen

receptor and acts as a coactivator with numerous transcription factors (Verdone

et al., 2005; X.-J. Yang, 2004). In addition, Tip60 has also been observed to

interact with repressor proteins such as CREB and ZEB and acts as a corepressor at some loci (Gavaravarapu and Kamine, 2000; Hlubek et al., 2001). Therefore, the Tip60-p400 complex has a complex array of context-dependent functions in gene regulation. In addition to regulation of gene expression, Tip60 mediated acetylation is important for additional functions within the nucleus, such as DNA repair (Ikura et al., 2000; Kusch, 2004). Not surprisingly, given its diverse array of cellular functions, *Tip60* is essential for mouse embryonic development (Acharya et al., 2017; Y. Hu et al., 2009), and is mutated in a variety of cancer types (Gorrini et al., 2007; Sakuraba et al., 2009; 2011).

The *Tip60* gene expresses four splice variants: (i) isoform1 (*iTip60*), which retains an intron between exon 1 and 2; (ii) isoform2 (*Tip60α*), the best characterized isoform, which retains all 14 exons and no intronic regions; (iii) isoform3 (*Tip60β*), which lacks exon5; and (iv) isoform4 (*Tip55*), which retains a short intron between exon11 and exon12 (M.-S. Kim et al., 2006; Legube and Trouche, 2003; Ran and Pereira-Smith, 2000). Studies have demonstrated that knockdown (KD) of all four isoforms causes self-renewal and gene regulation defects in ESCs (Acharya et al., 2017; P. B. Chen et al., 2013; Fazzio et al., 2008). Consistent with this phenotype, homozygous knockout of all four isoforms of the *Tip60* gene results in arrest of blastocyst stage (E3.5) embryos, owing to increased apoptosis and poor proliferation within the inner cell mass (ICM) (Y. Hu et al., 2009). All of these studies suggest the importance of Tip60 in various

cellular functions including cell proliferation and mouse embryonic development, but the functional roles of *Tip60* individual isoforms have not been deconvolved.

Given its early embryonic lethality, there are relatively few studies that have examined the roles of Tip60 at later stages of development. *Tip60* is highly expressed during multiple stages of heart development, as assessed by an antibody that recognizes all four isoforms (M.-S. Kim et al., 2006; Lough, 2002). These isoforms have been shown to interact with serum response factor (SRF) to induce transactivation of the promoter of ANF (atrial natriuretic factor), a heart specific gene (M.-S. Kim et al., 2006). However, it is unclear which specific isoform(s) of *Tip60* are important for early heart development.

Here we characterize the developmental roles of the *Tip55* isoform of *Tip60*. Using an allele that removes intron 11, which is uniquely retained in the Tip55 isoform, we show that *Tip55* is necessary for embryonic development, with *Tip55*$^{\Delta/\Delta}$ animals dying at or around E11.5. Development prior to this stage was grossly normal; however, fibroblasts isolated from E9.5 embryos proliferated slowly and exhibited premature senescence. Closer examination of E8.5 embryos revealed defects in morphology of both heart and neural tube. Interestingly, *Tip55* loss results in reduced proliferation in heart and increased apoptosis in neural tube, suggesting distinct functions in these two tissue types. These data reveal a critical function of *Tip55* in development that is distinct from those observed upon loss of all *Tip60* isoforms.

Results

Tip55 is essential for embryonic development

The Tip55 isoform of Tip60 protein encodes a unique 103 amino acids at the C-terminus relative to the most abundant and well studied Tip60α isoform (figure III.1.A) (M.-S. Kim et al., 2006). In the course of generating catalytically inactive _Tip60_ mutant mice described previously (Acharya et al., 2017), we generated an allele of _Tip60_ with a deletion of intron 11 (which encodes the unique C-terminal 103 amino acids in Tip55 only). In this allele, exons 11-14, which are retained in all other _Tip60_ isoforms, are fused together, such that all _Tip60_ isoforms except _Tip55_ are intact (figure III.1.B; see experimental procedure for details). We intercrossed mice heterozygous for this allele (hereafter, _Tip55_$^{\Delta/+}$) to generate _Tip55_$^{\Delta/\Delta}$ homozygotes as shown in figure III.1.B. However, we recovered no _Tip55_$^{\Delta/\Delta}$ pups at birth (χ^2= 38.76; _P_ < 0.001), suggesting that the _Tip55_ isoform is essential for embryonic development (figure III.1.C). Mice lacking all four isoforms of _Tip60_ do not survive past pre-implantation stages and embryonic stem cells cannot be recovered from blastocysts isolated from these mice (Y. Hu et al., 2009). To determine when during embryonic development _Tip55_$^{\Delta/\Delta}$ mice were blocked, we dissected and genotyped embryos from E8.5 to E11.5 (figure III.1.C and III.1.D). In addition, we also performed RT-PCR to confirm _Tip55_$^{\Delta/\Delta}$ homozygotes are not expressing _Tip55_ specific isoform (figure III.1.E). We observed no morphological differences between _Tip55_$^{\Delta/\Delta}$ and _Tip55_$^{+/+}$ at E8.5 (figure III.2.A). Interestingly, _Tip55_$^{\Delta/\Delta}$ homozygous null embryos

at E9.5 and beyond were smaller as compared to *Tip55*^{+/+}, although they appeared morphologically normal (figure III.2.A). Although we found that *Tip55*^{Δ/Δ} homozygous null embryos could readily be recovered as late as E10.5 and occasionally as late as E11.5 (figure III.1.C), these embryos are morphologically abnormal and lack beating hearts (figure III.2.A). These data reveal that *Tip55*^{Δ/Δ} mice progress normally through early developmental stages, in contrast to *Tip60* null mice, but die during mid-gestation, potentially due to deficiency in heart development.
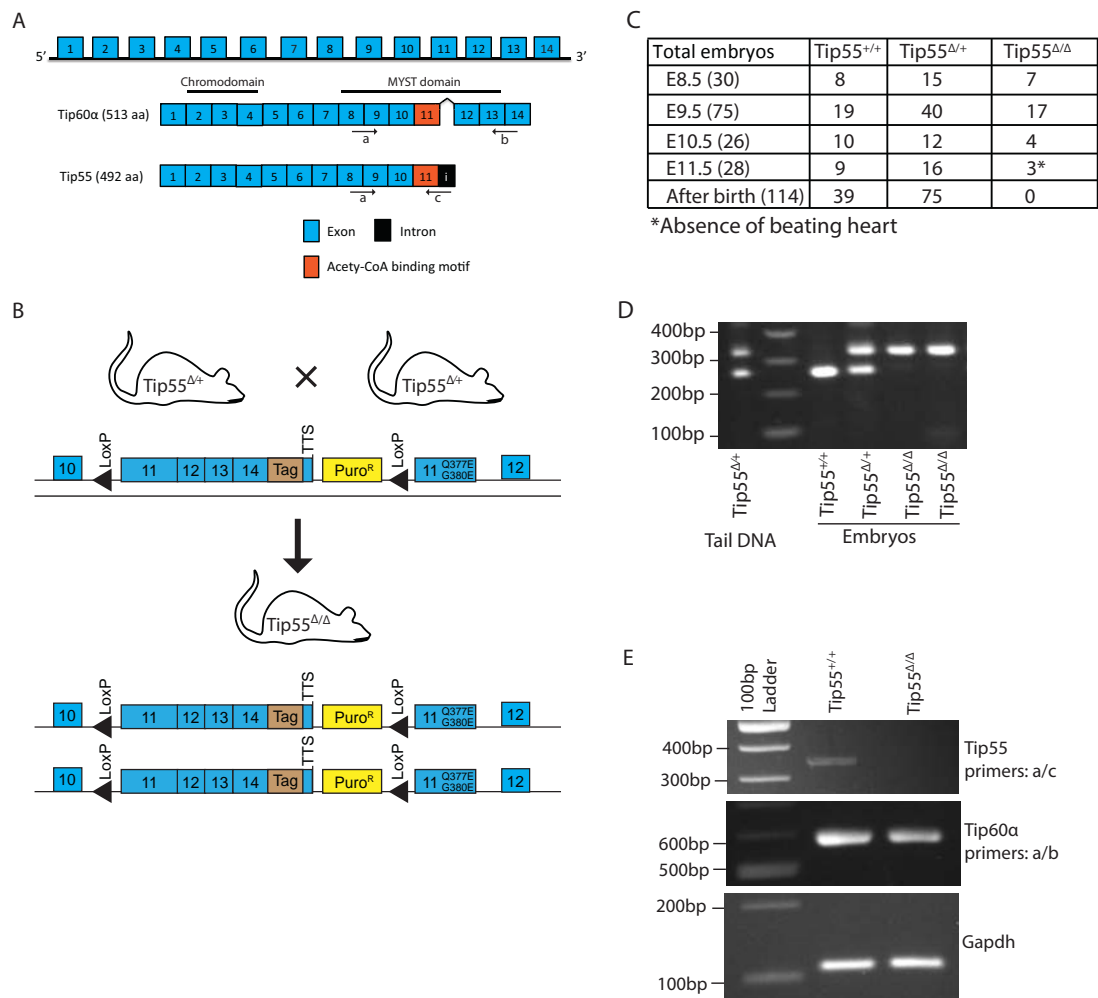
Figure III.1. *Tip55* knockout mice are embryonic lethal.

(A) Schemetic representation of the *Tip60* gene along with its two isoforms. *Tip60α* retains all the exons, whereas *Tip55* isoform retains exons 1 through 11 and small intronic region (103 amino acids) between exons 11 and 12.
(B) Schematic for generation of *Tip55^{Δ/Δ}* embryos.
(C) Genotypes of embryos from *Tip55^{Δ/+}* intercrosses at different developmental stages.
(D) PCR genotyping of *Tip55^{+/+}*, *Tip55^{Δ/+}*, and *Tip55^{Δ/Δ}* embryos.
(E) RT-PCR showing *Tip55^{Δ/Δ}* mutants express all isoforms but *Tip55*.
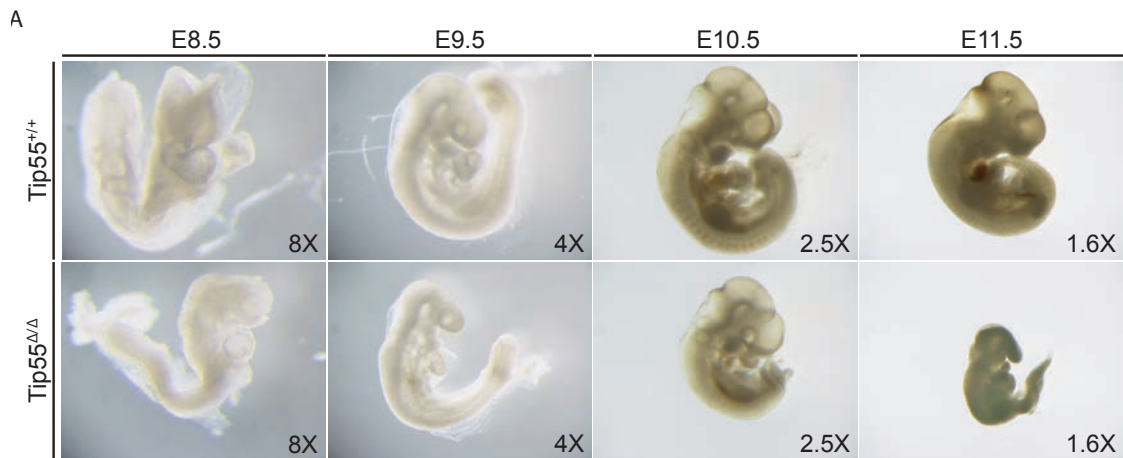
Figure III.2. *Tip55* isoform is necessary for normal embryonic development.

(A) Images of embryos of the indicated genotypes during development from E8.5 to E11.5. Images are taken in different magnifications as indicated.

Table III.1. Genotyping primers, Related to Figure 1.

Tip60 isoforms (all)	Forward (a):	ACGCCACTTGACCAAATGTGA
	Reverse (b):	TACTGGCCCTTGTAGTAATTG
Tip55 specific isoform	Forward (a):	ACGCCACTTGACCAAATGTGA
	Reverse (c):	TACTCACTGAACTCAATAAGC
Tip55 tail/embryo PCR	Forward :	CAAGTGTTTCCTGGACCACA
	Reverse :	TTGCCATAGCCCCGGCGCT

Loss of *Tip55* causes premature senescence in MEFs

The data in figure III.2.A suggested that the cause of developmental

defects and embryonic lethality in *Tip55*$^{\Delta/\Delta}$ mice might be due to impaired cellular

proliferation. To test this possibility, we intercrossed *Tip55*$^{\Delta/+}$ heterozygotes,

isolated *Tip55*$^{+/+}$ and *Tip55*$^{\Delta/\Delta}$ mouse embryonic fibroblasts (MEFs) from E9.5

embryos, and measured their proliferation rates. As shown in figure III.3.A,

Tip55$^{\Delta/\Delta}$ MEFs stop increasing in number after two days in culture, and have

reduced cell numbers by four days, suggesting these cells may be undergoing

premature senescence. To test this possibility, we stained for senescence-

associated β-galactosidase activity. We observed that *Tip55*$^{\Delta/\Delta}$ MEFs exhibit

increased β-galactosidase staining relative to *Tip55*$^{+/+}$ controls (figure III.3.B).

These observations indicated that the *Tip55*$^{\Delta/\Delta}$ MEFs undergo premature

senescence in culture, resulting in arrested proliferation.

Figure III.3. Loss of *Tip55* causes premature senescence in MEFs.

(A) Growth curve, measuring the proliferation rates of the indicated mutant and control MEFs.
(B) β-galactosidase activity looking into cellular senescence in mutant and control MEFs. Images are taken in 10X magnification.

Tip55 null embryos exhibit heart and neural tube defects
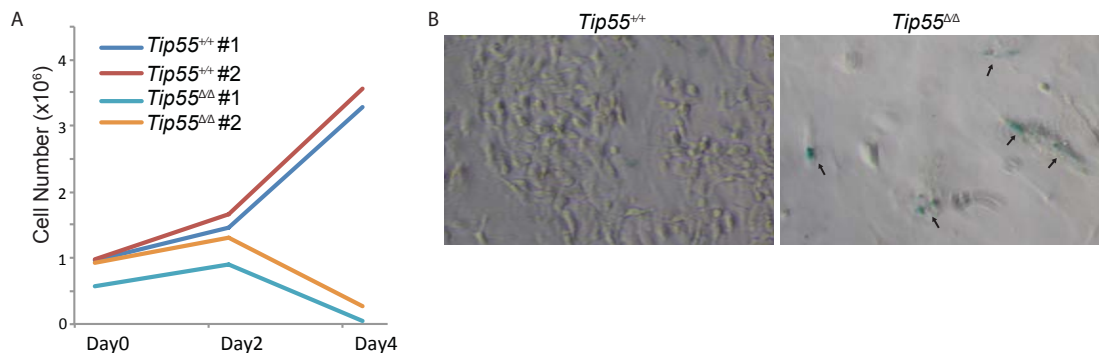
Our results that $Tip55^{\Delta/\Delta}$ embryos are smaller during embryonic

development, and that $Tip55^{\Delta/\Delta}$ MEFs undergo premature senescence suggest

the possibility that impaired cell proliferation underlies the observed defects in

organ development. Alternatively, loss of Tip55 may affect different tissues

differently, leaving open the possibility that increased cell death or premature

differentiation might cause these phenotypes. To investigate these possibilities,

sagittal sections of $Tip55^{+/+}$ and $Tip55^{\Delta/\Delta}$ embryos were obtained from E8.5

embryos. Haemotoxylin and Eosin (H&E) staining of the embryo revealed no

obvious morphological differences between $Tip55^{+/+}$ and $Tip55^{\Delta/\Delta}$ at this stage

(figure III.4.A), as we observed for whole E8.5 embryos (figure III.2.A). Next, we

performed immunohistochemistry (IHC) using antibodies against phospho-

Histone H3 (p.Histone H3), and cleaved caspase 3 (Casp3) to test for

proliferation defects or elevated apoptosis, respectively, in embryo sections. We

observed no obvious defects in embryo sections with the exception of two

tissues, heart and neural tube, which develop at these early stages of embryonic

development (figure III.4.B). We observed significant reduction in proportion of

p.Histone H3 positive cells ($p<0.022$), in $Tip55^{\Delta/\Delta}$ embryos relative to wildtype,

but no obvious changes in Casp3 staining in the heart (figures III.4.B and III.4.C).

These data suggest that cellular proliferation in developing heart tissue is

impaired in $Tip55^{\Delta/\Delta}$ mice. In contrast, we observed significant increase in Casp3

staining ($p<0.041$), but no significant changes in p.Histone H3 positive cells, in

the neural tube regions of $Tip55^{\Delta/\Delta}$ embryos relative to $Tip55^{+/+}$ controls, suggesting increased apoptosis in this tissue (figures III.4.B and III.4.C). These data suggest that embryonic lethality caused by *Tip55* loss is due to combined defects in the development of the heart and the neural tube, which manifest as impaired proliferation and increased apoptosis, respectively.
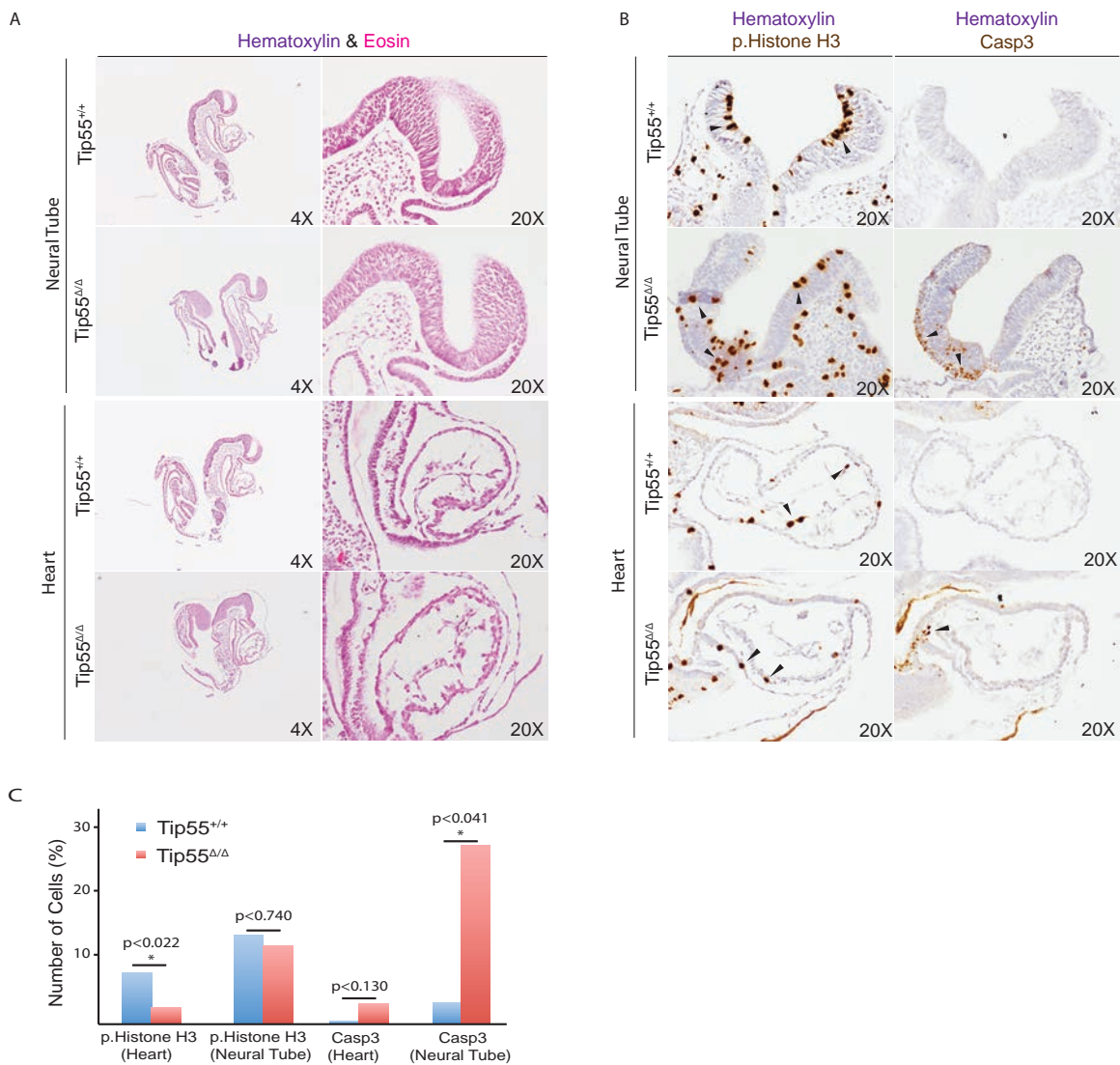
Figure III.4. *Tip55* null embryos exhibit heart and neural tube defects.

(A) H&E stained E8.5 embryo sections of *Tip55$^{+/+}$* and *Tip55$^{\Delta/\Delta}$* looking into the morphology of whole embryo, and specific organs heart and neural tube.
(B) Cleaved caspase 3 (Casp3) and phospho-Histone H3 (p.Histone H3) immunostaining of E8.5 embryo sections of *Tip55$^{+/+}$* and *Tip55$^{\Delta/\Delta}$* looking into cellular proliferation and apoptosis within heart and neural tube. Arrowhead showing some of the positively stained cells.
(C) Quantification of percentage of phospho-Histone H3 and cleaved caspase 3 positive cells in neural tube and heart. N=3 embryo sections were used for the two-sided t test (*p<0.05).

Discussion

In this study, we generated mice lacking sequences specific for the *Tip55* isoform of *Tip60*. We found that loss of *Tip55* causes a cell proliferation defect in heart and fibroblasts from mutant embryos, as well as increased apoptosis in neural tube. *Tip55*$^{\Delta/\Delta}$ mutant embryos fail to develop beating hearts, which may account for their mid-gestation lethality.

As reported previously, *Tip60* is required for mouse ESC maintenance and embryonic development (Acharya et al., 2017; Fazzio et al., 2008; Y. Hu et al., 2009). In addition to *Tip60,* ablation of *Esa1* (the yeast homolog of *Tip60*) or drosophila *Tip60* (*dTip60*) causes defects in proliferation and embryogenesis, respectively, consistent with the essential function of Tip60 in mammals (E. R. Smith et al., 1998; Zhu et al., 2007). Although alternative *Esa1* and *dTip60* isoforms have not been reported, mammalian *Tip60* gene contains four isoforms, several of which remain uncharacterized (M.-S. Kim et al., 2006; Legube and Trouche, 2003; Ran and Pereira-Smith, 2000). Here we demonstrated that the *Tip55* isoform of *Tip60* is required for normal embryogenesis, leaving open the possibility that other *Tip60* isoforms have important functions during embryonic development that remain to be identified.

Prior to E9.5, *Tip55* mutant embryos exhibit no apparent morphological phenotypes. Starting around E9.5, homozygous *Tip55* mutants appear smaller but grossly normal, suggesting that the observed proliferation defect might contribute to the defects in heart and neural tube development in *Tip55* null

embryos. Our *in vitro* results revealed *Tip55* null MEFs undergo premature senescence, consistent with the growth defect in mutant embryos. Unlike previous findings that homozygous *Tip60* null mutants were peri-implantation lethal and catalytic inactive mutants exhibited defects at approximately the gastrulation stage, loss of *Tip55* causes much later developmental defects that manifest during organogenesis. These data suggest that one or more of the remaining *Tip60* isoforms play critical roles in early development that are unaffected by *Tip55* loss.

What are the molecular defects underlying embryo lethality in *Tip55* mutants? The poor growth of embryos, premature senescence of MEFs, and reduced proliferation of cells within the developing heart suggest that cell cycle inhibitors may be upregulated in the absence of Tip55, proteins that stimulate cell cycle progression are downregulated or both. Ongoing work in the lab will address these possibilities. In addition, the increase in apoptotic markers in *Tip55* mutant neural tube raises the possibility that DNA damage or other pro-apoptotic stimuli may accumulate in mutant cells of this lineage. The reasons why some cell types may grow slowly and others may undergo apoptosis due to *Tip55* loss is not known at this time.

Together, these studies identify the essential role of *Tip55* isoform during post-implantation embryonic development. These findings are the first to demonstrate the importance of *Tip55* during *in vivo* development, which are different from previously reported null phenotype for *Tip60* that lacks all its

isoforms and resulted lethality during blastocyst stage. Our observations that

Tip55 null embryos die mid-gestation while exhibiting reduced growth, premature

senescence, and increased apoptosis raise the question of what developmental

roles are played by the other remaining isoforms.

Experimental procedures

Antibodies

Antibodies uses in this study were as follows: phospho-Histone H3 (9701, Cell

Signaling Technologies), and Cleaved caspase 3 (9661, Cell Signaling

Technologies).

Generation of *Tip55* knockout mice

$Tip60^{fl/+}$ mice used to generate $Tip60^{ci/ci}$ mice lack *Tip55* isoform from floxed

allele (called $Tip55^{\Delta/+}$ mice) (Acharya et al., 2017; P. B. Chen et al., 2013). Mice

were genotyped by PCR with primers listed in Table III.1. $Tip55^{\Delta/+}$ mice were

maintained as heterozygotes on an inbred FVB/N background and intercrossed

to generate $Tip55^{+/+,}$ $Tip55^{\Delta/+}$ and $Tip55^{\Delta/\Delta}$ embryos. Animal studies were

performed in accordance with the recommendations of the Institutional Animal

Care and Use Committee at the University of Massachusetts Medical School (A-

2165) and NIH guidelines.

Derivation of MEFs

MEFs were generated from E9.5 day embryos as previously described (Todaro and Green, 1963). Briefly, E9.5 day embryos were dissected, and trypsin (0.05%) digested for 12 minutes at 37°C incubator. Embryos were pipetted up/down to obtain single cells, and cultured in a 12 well plate in the presence of MEF medium.

Senescence and cell growth assays

Two independent MEFs of $Tip55^{+/+,}$ $Tip55^{\Delta/+}$ and $Tip55^{\Delta/\Delta}$ genotypes were cultured in 12-well plate of varying numbers. Total number of cells were counted and re-plated into new 12-well plates every 48 hours for the total of four days to obtain growth curve. Same MEFs of equal number (1.5×10^{4}) were plated into a gelatin coated 24-well plate for another 48 hours, and β-galactosidase staining was performed to test for cellular senescence using a kit (EMD Millipore, KA002), following the manufacturers' protocol.

Hematoxylin and Eosin (H&E) Staining

E8.5 embryos were collected and sectioned at 8um thickness for morphological analysis as previously described (Milstone et al., 2017). Hematoxylin- and eosin-staining was performed by deparaffinizing sections in xylene, rehydrated through an ethanol gradient, 30s with 30% Harris modified hematoxylin, and a 30s

counterstain with eosin Y. Slides were rinsed and dehydrated with ethanol, cleared with xylene, and mounted using Vectashield mounting media.

Immunohistochemistry

Sections from E8.5 embryos were examined for proliferation and apoptosis defects following the protocols described previously (Milstone et al., 2017). Briefly, sections were rehydrated through an ethanol gradient, followed by heat antigen retrieval (Buffer A, Electron Microscopy Sciences). Immunostaining was conducted using the Vectastain Elite ABC and DAB Peroxidase Substrate kit according to manufacturer guidelines. Sections were incubated with phospho-Histone H3 (1:100) and cleaved caspase 3 (1:100) primary antibodies overnight at 4°C(Milstone et al., 2017). For counterstaining, slides were rinsed and then incubated with 30% hematoxylin for 30s after 3,3' DAB developing. All slides were ethanol-dehydrated, cleared with xylene, and mounted with Vectashield mounting medium.

Proliferation and Apoptosis Quantification

Immunostaining for phospho-Histone H3 or cleaved caspase-3 was utilized for defining cardiomyocytes undergoing mitosis or apoptosis, respectively. Digital images of E8.5 sections were taken using a Nikon Eclipse 80i microscope and the NIS-Elements 4.00.03 software. Postive stained cells were counted manually using 20X images and ImageJ (v 1.6.0_65). The percentage of phospho-Histone H3 positive

or cleaved caspase-3 positive cells relative to the total number of nuclei in the neural tube and heart, was calculated for a minimum of three embryos per genotype. Data collected was plotted and statistically analyzed using Microsoft Excel.

Chapter IV

Discussion and future directions

Chromatin structure plays important roles in gene expression and lineage specification of specific cell types. Tip60-p400 is a multi-subunit chromatin remodeling complex that has biochemical activities to modify histone tails and remodel nucleosomes, catalyzed by the Tip60 KAT and p400 ATPase subunits, which have been widely studied with respect to gene expression, DNA damage repair, and apoptosis (Gevry et al., 2007; Ikura et al., 2000; Mizuguchi et al., 2004). Our lab has previously shown that the Tip60-p400 complex largely acts as a repressor of developmental genes to control gene expression and self-renewal in ESCs (Fazzio et al., 2008). However, the molecular mechanism regarding the requirement of chromatin remodeling activities present within Tip60-p400 complex was largely unknown. In addition, while the Tip60 KAT contains multiple isoforms, their individual roles during development have not been studied. Therefore, in the first part of this thesis, I focused on understanding the catalytic and non-catalytic functions of Tip60-p400 with respect to ESC maintenance, gene expression, and embryonic development. In the second part, I investigated the developmental role of one of the isoforms of *Tip60*, called *Tip55*. Below, I discuss in detail my major findings, as well as critical analyses of the findings, limitations of the studies, and future directions.

KAT-independent function of Tip60 in ESC

I found that Tip60-p400 catalytic activities are dispensable for ESC maintenance and repression of developmental genes, and instead are required for ESC differentiation and embryonic development. Previous studies demonstrated that knockdown of *Tip60* KAT and *p400* ATPase subunits in ESCs causes self-renewal and gene regulation defects causing ESCs to differentiate and de-repression of normally silent genes such as developmental genes in ESCs (P. B. Chen et al., 2013; Fazzio et al., 2008). From these studies, we learned that Tip60-p400 is required for the ESC maintenance and repression of developmental genes. But, it was unknown if the Tip60-p400 catalytic functions are required, which was the major focus of my studies. The *p400* subunit of Tip60-p400 catalyzes H2AZ incorporation into chromatin using energy from ATP hydrolysis (Gevry et al., 2007; Mizuguchi et al., 2004). The Boyer lab demonstrated that *H2A.Z* KD ESCs self-renew normally, but fail to differentiate properly in EBs and neuronal cells, which is different from the *p400* KD that exhibit both self-renewal and differentiation defects (Creyghton et al., 2008; Fazzio et al., 2008). In addition, *H2AZ* KO and N-terminally deleted *p400* also causes post-implantation embryo lethality (Faast et al., 2001; Fujii et al., 2010; Ueda et al., 2007). It still remains to be determined whether complete ablation of *p400* causes pre-implantation lethality, as expected from the *p400* KD phenotype in ESCs, or causes post-implantation lethality. In this study, we generated independent ESC lines lacking *p400* ATPase activity to investigate its function in

ESC self-renewal. In correlation with *H2AZ* KD*,* we identified that *p400* catalytic

function is only required for normal ESC differentiation, not for ESC self-renewal.

Unlike *p400* KD, a hypomorphic *p400* mutant, and H2AZ KD, *p400* catalytic

mutants did not exhibit gene regulation defects, suggesting the possibility of

redundancy between p400 and SRCAP, another ATPase in higher eukaryotes

that also exchanges H2AZ for H2A through a dimer exchange mechanism (Ruhl

et al., 2006; Wong et al., 2007).

Next, we tested the possibility that *Tip60* KAT activity might be required

for ESC self-renewal and Tip60-p400 mediated repression of developmental

genes. *Tip60* acetylates histone proteins and histone acetylation is generally

known to activate gene expression (Allard et al., 1999; Kimura and Horikoshi,

1998; Utley et al., 1998; Verdone et al., 2005). Therefore, it would be surprising

to find the *Tip60* KAT activity was critical for repression of developmental genes.

When we generated independent lines lacking *Tip60* KAT activity, we found that

Tip60 catalytic function is also dispensable for repression of developmental

genes and ESC self-renewal. In addition, our results further showed that there is

no functional redundancy between *Tip60* KAT and *p400* ATPase activities in

ESCs. These results raised an interesting question: what non-catalytic function is

required for ESC maintenance? One possibility is that Tip60-p400 occupies the

promoter region of target genes and reduces the chromatin accessibility to

maintain repressive chromatin structure. To test this possibility, we performed

ATAC-seq and showed that *Tip60* KD causes increased chromatin accessibility

at the promoter region compared to the controls. In contrast, *Tip60* KAT deficient

ESCs exhibit minimal changes. Together, our studies demonstrate that Tip60-

p400 mediated repression of chromatin accessibility is important for repression of

normally silent developmental genes in ESCs, regardless of Tip60 KAT activity.

These are surprising findings that *Tip60* KAT and *p400* ATPase

nucleosome remodeling activities of Tip60-p400 complex are dispensable for

ESC maintenance and repression of developmental genes. Although these

catalytic activities are not required, these subunits possess other non-catalytic

domains such as chromodomain (*Tip60*), and SANT domain (*p400*) that

generally recognize and binds histone tails (Boyer et al., 2002; 2004; Jacobs and

Khorasanizadeh, 2002). Very little is known about these domains with respect to

Tip60 and *p400* function. It is possible that these chromo or SANT domains might

be important to recruit Tip60-p400 to their target genes for ESC self-renewal and

their repression, which need to be studied.

Apart from *Tip60* and *p400* nucleosome-remodeling subunits, Tip60-p400

also contains two other catalytic subunits Ruvbl1 and Ruvbl2. These proteins

belong to the highly conserved ATPase family that contain DNA helicase activity

in association with Tip60-p400 complex (Ikura et al., 2000; Jha and Dutta, 2009).

Ruvbl1 and Ruvbl2 is also part of nucleoprotein complexes such as INO80 and

SWR1, but very little is known about their cellular function in Transcription, DNA

damage response, and apoptosis (Huen et al., 2010; Jha and Dutta, 2009). Our

lab has also previously demonstrated that KD of *Ruvbl1* and *Ruvbl2* subunits

exhibit self-renewal defect in ESCs, similar to the phenotype observed in *Tip60* KD and *p400* KD (Fazzio et al., 2008). Therefore, future studies regarding the ATPase activities of these proteins are necessary to understand their role in ESC self-renewal and gene regulation.

Although we did not observe any phenotypic differences between controls and *Tip60* KAT mutants in ESCs, we were interested to pursue the importance of *Tip60* KAT activity during mouse embryonic development and ESC differentiation. Previously, it has been reported that ablation of *Tip60* causes embryonic lethality during the pre-implantation stage, which correlates with the failure of *Tip60* KD cells to maintain ESC pluripotency (Fazzio et al., 2008; Y. Hu et al., 2009). In addition, ablation of *Esa1* (*Tip60* homolog in yeast) or *dTip60* (*Tip60* homolog in drosophila) is lethal (Clarke et al., 1999; E. R. Smith et al., 1998; Zhu et al., 2007). Consistent with these phenotypes, we also observed embryonic lethality in our *Tip60* KAT deficient mutants. In contrast to the essential function of Tip60 during pre-implantation, the *Tip60* KAT deficient embryos fail to gastrulate properly and die during post-implantation development, suggesting the importance of Tip60 KAT activity for proper lineage specification. When we performed RNA-seq on wild type and *Tip60* KAT deficient ESCs during undirected differentiation, we observed delayed and reduced expression of multiple transcription factors associated during gastrulation (e.g. *FoxA2, Gata4, Sox17*, *T*, *Hand1, Flk1)* and signaling proteins associated with early development (e.g. *Fgfr2, Tgfb2, Bmp2, Fzd2)* in KAT deficient mutants. These *in vivo* and *in*

vitro results imply the importance of *Tip60* KAT activity in regulation of multiple lineage specific transcription factors and signaling pathways for embryonic development. Future studies should look more closely into global changes in Tip60-p400 occupancy and Tip60 mediated histone acetylation in wild type and *Tip60* KAT deficient mutants to understand the functional role of Tip60-p400 in gene regulation during ESC differentiation. In addition, we can also generate tissue specific *Tip60* KAT deficient mice, and study the function of Tip60 catalytic activity in various organs such as heart and brain where it is highly expressed.

Catalytic-independent functions of multiple chromatin regulators

Multiple chromatin regulators, including Gcn5 and RING1B, exhibit catalytic-independent functions in addition to their better understood catalytic roles in chromatin regulation. Gcn5, one of the most widely studied HATs in multiple eukaryotes, is essential for embryonic development in mammals and is a key regulator of gene expression from yeast to humans (Roth et al., 2001). Ablation of *Gcn5* in yeast displayed a decrease in expression of many target genes, whereas *Gcn5* HAT deficient mutants had much weaker effects on gene expression, suggesting a catalytic independent function of Gcn5 in gene regulation (Huisinga and Pugh, 2004). *Gcn5* deletion in mice results in embryonic lethality at around E10.5, due to a defect in specification of some mesodermal cell types (W. Xu et al., 2000). In contrast, mouse embryos homozygous for a mutation in the *Gcn5* catalytic domain survive until E16.5, where they exhibit

defects in neural tube closure (Bu et al., 2007). These data suggest that one or more HAT-independent functions of Gcn5 are required earlier in embryonic development than the HAT activity is required. Gcn5 is also required for gene expression in ESCs. Ablation of *Gcn5* in ESCs caused downregulation of target genes enriched for cell cycle regulation, which is similar to the phenotype observed in yeast (Hirsch et al., 2015; Huisinga and Pugh, 2004). It still remains to be addressed if the acetyltransferase activity of Gcn5 is required for this function.

In addition to Gcn5, RING1B, a critical subunit of the PRC1 complex, also possesses a catalytic independent function in gene regulation and embryonic development. RING1B is the catalytic subunit of the PRC1 complex that ubiquitylates histone H2A on K119, which is important to recruit PRC2 complex for gene silencing (Simon and Kingston, 2013). Ablation of *Ring1B* results in a gastrulation defect, causing embryo lethality around E10.5 (Voncken et al., 2003). In contrast, mouse embryos homozygous for a mutation in the *Ring1B* catalytic domain complete gastrulation and survive longer than *Ring1B* null embryos until E15.5 (Illingworth et al., 2015). RING1B also plays important role in gene regulation. Ablation of *Ring1B* caused de-repression of genes normally silent in ESCs, such as *Homeobox* genes, whereas *Ring1B* catalytic deficient mutants had much weaker effects on gene expression (Eskeland et al., 2010; Illingworth et al., 2015). These data together suggest the catalytic independent functions of RING1B during embryo development and gene regulation.

These findings, together with our findings that Tip60-p400 catalytic functions are dispensable for ESC gene regulation and pre-implantation development, suggest that the assumption that most chromatin remodeling enzymes function through their enzymatic activities is not always correct. Although additional studies are necessary, it is likely that additional chromatin regulatory enzymes also have important functions independent of their catalytic activities, which will be uncovered only when catalytic mutants are generated and compared with knockouts.

The *Tip55* isoform of *Tip60* is required during embryonic development

We found that *Tip55,* one of the four isoforms of *Tip60,* is essential for mouse embryonic development. Previous studies have clearly demonstrated that ablation of *Tip60* causes pre-implantation lethality, where the embryo fails to hatch from the zona pellucida (Y. Hu et al., 2009). This result is consistent with the ESC phenotype, where knockdown of *Tip60* causes a self-renewal defect (Fazzio et al., 2008). In these studies, the above-mentioned phenotypes were observed upon targeting all four isoforms. Here, we study the role of *Tip55* isoform during mouse embryonic development. Unlike the *Tip60* knockout phenotype, ablation of the *Tip55* isoform causes post-implantation lethality at E11.5, suggesting *Tip55* plays an important role during later stages of development. Our findings also raise the possibility that other isoforms might

have important functions during earlier stages of embryonic development, which will need to be tested.

We have identified that *Tip55* ablation impairs growth of mouse embryos starting from E9.5. Previous studies have shown that ablation of *Tip60* causes proliferation defects and increased apoptosis in mouse embryos during pre-implantation stages (Y. Hu et al., 2009). We also observed a cell proliferation defect marked by a decrease in phospho-histone H3, and an increase in apoptosis marked by an increase in cleaved caspase 3 in the heart and neural tube (that gives rise to brain) of *Tip55* mutant embryos respectively. But, these defects occurred much later than defects observed in *Tip60* knockout mutants. Therefore, our data clearly imply the role of the *Tip55* isoform in heart and brain development, where *Tip60* isoforms are highly expressed (M.-S. Kim et al., 2006; Lough, 2002; McAllister et al., 2002). Tip55 has been previously reported to regulate the activation of ANF, a heart specific gene suggesting its function in heart development (M.-S. Kim et al., 2006), which is also supported by our *in vivo* findings. In contrast, the role of Tip55 in brain development was not reported previously. Whether the Tip55 KAT activity is necessary for heart and brain development needs to be tested. In addition, whether or not the expression of heart and neural tube specific genes is altered in *Tip55* mutant embryos will need to be addressed.

This is the first report to show that a specific *Tip60* isoform is required for embryonic development. Our findings raise other interesting questions in addition

to the functions of other *Tip60* isoforms. What happen to the Tip60-p400 complex formation, their occupancy and gene regulation in the absence of *Tip55* isoform? We can use CRISPR-Cas9 system to delete novel 103 amino acids from *Tip55* isoform in ESCs, including other cell types to understand these biological questions.

Increased complexity of regulatory networks resulting from differential splice isoforms of regulatory proteins

Whole genome sequencing of various organisms has revealed protein complexity in higher eukaryotes as compared to Drosophila and yeast, due to alternative splicing of protein coding genes (Blencowe, 2006; Maniatis and Tasic, 2002). Generation of knockout mice using gene-targeting strategies have been used to study the *in vivo* functions of specific isoforms. For instance, FGFR2, one of the four receptor tyrosine kinases important for intracellular signaling contains two isoforms *Fgfr2 IIIb*, and *Fgfr2 IIIc*, generated by splicing of exon 8 and exon 9 respectively (Eswarakumar et al., 2005; Orr-Urtreger et al., 1993). Unlike *Fgfr2* knockout, which exhibits lethality due to a placental defect around E10.5, knockout of *Fgfr2 IIIb* causes lethality immediately after birth due to impairment in lungs, limbs and other tissues (Arman et al., 1998; De Moerlooze et al., 2000). In contrast, *Fgfr2 IIIc* knockout mice were born with defects in the skull and bone development (Hajihosseini et al., 2001).

Differential isoforms of chromatin remodeling proteins are also important during development. The p400 homolog in drosophila (called DOMINO) contains two alternatively spliced isoforms, *DomA* and *DomB* (Ruhf et al., 2001). Depletion of either of these isoforms results in non-redundant phenotypes during different stages of oogenesis (Börner and Becker, 2016). However, the individual functions of multiple isoforms of *p400* in mammals are yet to be identified.

Our findings that the *Tip55* isoform is essential during post-implantation development and the few examples provided above suggest that alternative isoforms of other regulatory proteins play key (yet unexplored) roles during various stages of embryonic development. Interestingly, the average number of distinct protein isoforms encoded by each gene in mammals is four, suggesting these studies are at the tip of the iceberg (Melamud and Moult, 2009). Therefore, in order to understand the entire repertoire of functions of each developmental regulator, one needs to examine mutants individually defective in each isoform.

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