UTX and UTY Demonstrate Histone Demethylase-Independent Function in Mouse Embryonic Development

Karl B. Shpargel1, Toru Sengoku2, Shigeyuki Yokoyama2,3, Terry Magnuson1*

1 Department of Genetics, Carolina Center for Genome Sciences, and Lineberger Comprehensive Cancer Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States of America, 2 RIKEN Systems and Structural Biology Center, Tsurumi, Yokohama, Japan, 3 Laboratory of Structural Biology and Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Tokyo, Japan

Abstract

UTX (KDM6A) and UTY are homologous X and Y chromosome members of the Histone H3 Lysine 27 (H3K27) demethylase gene family. UTX can demethylate H3K27; however, in vitro assays suggest that human UTY has lost enzymatic activity due to sequence divergence. We produced mouse mutations in both Utx and Uty. Homozygous Utx mutant female embryos are mid-gestational lethal with defects in neural tube, yolk sac, and cardiac development. We demonstrate that mouse UTY is devoid of in vivo demethylase activity, so hemizygous X<sup>Utx<sup>−</sup>-</sub><sup>Y</sup> mutant male embryos should phenocopy homozygous X<sup>Utx<sup>−</sup>-</sub><sup>Y</sup> females. However, X<sup>Utx<sup>−</sup>-</sub><sup>Y</sup> mutant male embryos develop to term; although runted, approximately 25% survive postnataally reaching adulthood. Hemizygous X<sup>Utx<sup>−</sup>-</sub><sup>Y</sup> mutant males are viable. In contrast, compound hemizygous X<sup>Utx<sup>−</sup>-</sub><sup>Y</sup> males phenocopy homozygous X<sup>Utx<sup>−</sup>-</sub><sup>Y</sup> females. Therefore, despite divergence of UTX and UTY in catalyzing H3K27 demethylation, they maintain functional redundancy during embryonic development. Our data suggest that UTX and UTY are able to regulate gene activity through demethylase independent mechanisms. We conclude that UTX H3K27 demethylation is non-essential for embryonic viability.

Introduction

Post-translational modifications of histones establish and maintain active or repressive chromatin states throughout cell lineages. Thus, the enzymes that catalyze these modifications often have crucial roles in establishing genomic transcriptional states in developmental decision-making. Histone methylation can stimulate gene activation or repression depending on which residues are targeted. Methylation of histone H3 on Lysine 4 (H3K4me) is an active chromatin modification, while methylation on histone H3 Lysine 27 (H3K27me) is associated with repression of gene activity [1].

The polycomb repression complex 2 (PRC2) methylates H3K27 [2,3,4,5]. Within this complex, enhancer of zeste homolog 2 (EZH2) catalyzes di and tri-methylation of H3K27. Embryonic ectoderm development (EED) and suppressor of zeste homolog 12 (SUZ12) are additional PRC2 core components indispensable for PRC2 activity [6,7,8]. EZH1 is a secondary, less efficient H3K27 methyl-transferase that shares some overlapping redundancy with EZH2 in ES cells and epidermal stem cells [9,10,11,12]. The PRC1 complex is recruited through H3K27 trimethylation for additional histone modification and chromatin compaction [13]. In embryonic stem (ES) cells, PRC2 targets and represses genes essential for developmental events [14,15,16,17]. The promoters of these PRC2 targets typically contain “bivalent” chromatin marks with both active H3K4 and repressive H3K27 methylation [18,19,20]. Loss of PRC2 activity de-represses these genes but does not alter ES cell pluripotency [14]. However, mouse mutations in any of the three PRC2 core components are early embryonic lethal with gastrulation defects [7,21,22].

H3K27 trimethylation is reversible as a family of histone demethylases catalyzes the removal of this epigenetic mark [23,24,25,26]. JMJD3 (KDM6B) is an autosomal H3K27 demethylase upregulated during specific differentiation events [25,27]. UTX (KDM6A) is a broadly expressed X-linked H3K27 demethylase that escapes X-inactivation [23,24,26,28]. UTY is the Y chromosome homolog of UTX. Both UTX and JMJD3 demethylate H3K27 di and tri-methyl residues; however, UTY lacks this activity in vitro [26,29]. Based on cell culture models, UTX and JMJD3 mediated H3K27 demethylation is vital in a wide array of functions including cell cycle regulation, M2 macrophage differentiation, neuronal stem cell specification, skin differentiation, and muscle differentiation [27,30,31,32,33,34,35]. In contrast, the biological function of UTY remains unknown.

UTX and UTY are genetically amenable to delineate H3K27me3 demethylation dependent versus demethylase independent function in mouse development. Comparative amino acid sequence analysis of UTX and UTY reveals 88% sequence similarity in humans (83% identity) and 82% sequence similarity in mouse. Across the annotated JmjC histone demethylase domain, the similarity is at 98% and 97% for human and mouse
results. In the TPR (tetrapeptide repeat) domain, the similarity is at 94%. So while UTY is reported to have lost H3K27 demethylase activity, it is remarkably well conserved with respect to UTX. Recent discoveries have revealed that UTX remains as a histone demethylase domain that renders the protein incapable of H3K27 demethylation. Therefore, the overlapping function of UTX and UTY in embryonic development is due to H3K27 demethylase independent mechanism. Moreover, the presence of UTY allows UTX-deficient mouse embryos to survive until birth. Thus, H3K27 demethylase is not essential for embryonic viability. These intriguing results raise new questions on how H3K27me3 repression is removed in the early embryo.

Author Summary

Trimethylation at Lysine 27 of histone H3 (H3K27me3) establishes a repressive chromatin state in silencing an array of crucial developmental genes. Polycomb repressive complex 2 (PRC2) catalyzes this precise posttranslational modification and is required in several critical aspects of development including Hox gene repression, gastrulation, X-chromosome inactivation, mono-allelic gene expression and imprinting, stem cell maintenance, and oncogenesis. Removal of H3K27 trimethylation has been proposed to be a mechanistic switch to activate large sets of genes in differentiating cells. Mouse Utx is an X-linked H3K27 demethylase that is essential for embryonic development. We now demonstrate that Uty, the Y-chromosome homolog of Utx, has overlapping redundancy with Utx in embryonic development. Mouse UTY has a polymorphism in the JmjC demethylase domain that renders the protein incapable of H3K27 demethylation. Therefore, the overlapping function of UTX and UTY in embryonic development is due to H3K27 demethylase independent mechanism. Moreover, the presence of UTY allows UTX-deficient mouse embryos to survive until birth. Thus, H3K27 demethylase is not essential for embryonic viability. These intriguing results raise new questions on how H3K27me3 repression is removed in the early embryo.

Results

Hemizygous Utx mutant male mice have reduced perinatal viability

We developed mutant mouse lines to assess the contribution of UTX H3K27 demethylase function in mouse development. Two alleles for Utx were obtained from public resources. The BayGenomics gene trap line Kdm6aGt(RRA094)Byg is designated as X\(^{Utx\text{-}GT1}\) (Figure 1A). RT-PCR and PCR genotyping verified the identity of this allele in both ES cells and mutant embryos (Figures S1 and S2A–S2C). Additionally, we obtained the EUCOMM Kdm6a knockout line (project 26585, Kdm6a\(^{+/−}\)EUCOMM(Wtsi)) designated as X\(^{Utx\text{-}GT2}\), which inserts a gene trap in intron 2 along with a floxed 3\(^{\text{rd}}\) exon (Figure 1A). Southern blotting and PCR genotyping verified the identity of this allele (Figures S1 and S2D–S2F). Notably, quantitative RT-PCR comparison of tail RNA from X\(^{Utx\text{-}GT1}\)Y\(^{+\text{-}}\) versus X\(^{Utx\text{-}GT2}\)Y\(^{+\text{-}}\) mice demonstrated that Utx gene trap 1 is more effective than gene trap 2 (a 96% reduction compared to a 61% reduction in Figures S2C and S2F). Because X\(^{Utx\text{-}GT1}\) demonstrated incomplete trapping, the 3\(^{\text{rd}}\) exon was deleted with Cre recombinase to establish X\(^{Utx\text{-}GT2D}\) (containing both the gene trap and deleted 3\(^{\text{rd}}\) exon, Figure 1A). Deletion of the third Utx exon produces a frameshift and introduction of a translational stop codon when Utx is spliced from exon 2 to exon 4. X\(^{Utx\text{-}GT2D}\) and X\(^{Utx\text{-}GT2\text{del}}\) are null alleles as UTX protein was eliminated in western blotting of these embryonic lysates (Figure 1B, 1C). Consistent with RT-PCR data, X\(^{Utx\text{-}GT2}\) exhibits a reduction but not absence of UTX protein (Figure 1D).

Heterozygous Utx female mice were crossed to wild type male mice to produce hemizygous Utx mutant males. At weaning, the hemizygous X\(^{Utx\text{-}GT1}\)Y\(^{+\text{-}}\), X\(^{Utx\text{-}GT2}\)Y\(^{+\text{-}}\), and X\(^{Utx\text{-}GT2D}\)Y\(^{+\text{-}}\) mice all exhibited reductions of 68%, 83%, and 55% respectively from the expected genotype frequencies based on these crosses, yet expected genotype frequencies were observed at embryonic day E18.5 (Table 1). At E18.5, most of the hemizygous Utx mice appeared phenotypically normal; however a small percentage of the fetuses exhibited exencephaly. At birth, the hemizygous Utx males were small and exhibited a failure to thrive phenotype. Those males that survived through this phenocritical phase reached adulthood and were fertile. Hemizygous Utx mutant males were runted compared to wild type littermates and remained smaller than controls throughout their lifespan (Figure 2A, 2B). Backcross of the Utx allele onto a C57BL/6j or 129/SvJ background affected postnatal viability, but hemizygous Utx male embryos were still readily obtained at E18.5 (Table S1).
**Figure 1. Utx mutant alleles.** (A) Schematics of mouse mutations in Utx. Included are annotations and locations of where the protein would be mutated. Two Utx mutant alleles included a gene trap in intron 3 (X_UtxGT1) and a gene trap/floxed exon 3 (X_UtxGT2fl). A UTX protein annotation is illustrated at the top to indicate to positions of Utx alleles. A germline Cre recombinase deleted exon 3 in the X_UtxGT2fl background to create X_UtxGT2. Additionally, the gene trap of X_UtxGT2fl was excised with Flp recombinase to create a standard floxed exon 3 (X_Utxfl) and Cre recombination created X_UtxD. (B) Western blotting of E18.5 liver demonstrates a complete loss of UTX in X_UtxGT1 Yuty+ lysates. RbBP5 was used as a loading control. (C) Western blotting of E10.5 whole embryo demonstrates a complete loss of UTX in X_UtxD Yuty+ and X_UtxD X_UtxD lysates. RbBP5 was used as a loading control. (D) Western blotting of E12.5 primary MEFs demonstrates a reduction of UTX in X_UtxGT2fl Yuty+ and X_UtxGT2fl X_UtxGT2fl lysates. RbBP5 was used as a loading control. doi:10.1371/journal.pgen.1002964.g001
Homozygous Utx females are mid-gestational embryonic lethal

Human UTY lacks demethylase activity based on in vitro assays, so we hypothesized that X<sup>Utx<sup>2</sup>Y<sup>Uty</sup></sup> homozygous females will phenocopy X<sup>Utx<sup>2</sup>Y<sup>Uty</sup></sup> hemizygous males in demethylase dependent function (UTX specific), but may demonstrate a more severe phenotype in demethylase independent roles. Homozygous X<sup>UtxGT1</sup>Y<sup>Uty</sup> and X<sup>UtxGT2</sup>Y<sup>Uty</sup> females were never observed at weaning or embryonic day E18.5 (Table 1), but were observed at expected genotype frequencies at E10.5. However, these embryos were dead and resorbed by E12.5 (Table 1). Notably, at E10.5 all homozygous X<sup>UtxGT1</sup>Y<sup>Uty</sup> and X<sup>UtxGT2</sup>Y<sup>Uty</sup> females were smaller in size and had open neural tubes in the midbrain region (Figure 3A-ii, iii, vi, vii). Variation in severity of the Utx homozygous phenotypes was observed in mutant embryos, ranging from medium sized with typical E10.5 features (Figure 3A-ii, vi) to much smaller embryos resembling the E9.5 timepoint (Figure 3A-iii, vii). The X<sup>UtxGT1</sup> and X<sup>UtxGT2</sup> alleles failed to complement, as trans-heterozygous X<sup>UtxGT1</sup>X<sup>UtxGT2</sup>Y<sup>Uty</sup> female embryos resembled individual homozygous alleles (Figure 3A-viii). Homozygous X<sup>UtxGT1</sup>Y<sup>Uty</sup> male embryos appeared phenotypically normal at E10.5 (Figure 3A-iv). Homozygous X<sup>UtxGT2fl</sup>Y<sup>Uty</sup> females exhibited a slight reduction in phenotypic severity; about half of the mutant embryos had open neural tubes and some survival to E12.5 (Table 1).

To distinguish between embryonic...
and extraembryonic contribution of UTX towards the homozygous phenotype, we crossed the Sox2Cre transgene into the Utxfl background. In this cross, paternally inherited Sox2Cre expression will drive Utx deletion specifically in embryonic tissue [40]. No X\(^{Utxfl}\)X\(^{Utxfl}\), Sox2Cre female embryos were recovered at E18.5, whereas X\(^{Utxfl}\)Y\(^{Utxfl}\), Sox2Cre male embryos were recovered at expected frequencies (Table S2). At E10.5, X\(^{Utxfl}\)X\(^{Utxfl}\), Sox2Cre embryos produced phenotypes largely identical to Utx homozygotes. In summary, Utx homozygous females demonstrate a significantly more severe embryonic phenotype in comparison to Utx hemizygous males.

Mid-gestational lethality is typically associated with defective cardiovascular development. Accordingly, we observed both heart and yolk sac vasculature/hematopoietic phenotypes in Utx homozygotes. Utx homozygous mutant hearts were small and underdeveloped, and more severe embryos exhibited peri-cardial edema (Figure 3A-iii, vii). The yolk sac vasculature of Utx homozygotes was pale with a reduction in the amount of vascular blood (Figure 3B-ii). In more severe examples, homozygous yolk sacs were completely pale with an unremodeled vascular plexus (Figure 3B-iii). Thus, abnormal cardiovascular function may be a source of lethality and developmental delay in Utx homozygous mutant embryos.

UTX and UTY have redundant function in embryonic development

The most likely explanation for the disparity between Utx hemizygotes and homozygotes is that UTY can compensate for the loss of UTX in embryonic development. We tested Utx and Uty expression in embryonic development to assess any overlap in expression patterns. Utx expression was initially gauged utilizing the B-galactosidase reporter in X\(^{Utx\text{-GT1}}\)X\(^{Utx\text{-GT1}}\) and X\(^{Utx\text{-GT2}}\)D\(^{Utx\text{-GT2}}\) whole mount E10.5 embryos. Utx was expressed at lower levels throughout the E10.5 embryo with a particular enrichment in the neural tube and otic placode (Figure 3A-ii, iii, iv). In situ hybridization for both Utx and Uty demonstrated similar expression patterns characterized by widespread low-level expression with particular enrichment in the neural tube (Figure 3B-ii, iii, v, vi). Our analysis of publicly available RNA-seq data sets [41,42] revealed similar low-levels of expression for Utx and Uty.

Figure 3. Homozygous female Utx mutant embryos have mid-gestational developmental delay. (A) Compared to controls (A-i and A-v), homozygous female E10.5 X\(^{Utx\text{-GT1}}\)X\(^{Utx\text{-GT1}}\) (A-ii) and X\(^{Utx\text{-GT2}}\)D\(^{Utx\text{-GT2}}\) (A-vi) embryos have some developmental delay including smaller size, underdeveloped hearts (white arrows), and open neural tube in the head (arrowheads). More severe embryos resemble the size and features of E9.5 embryos with cardiac abnormalities and peri-cardial edema (A-iii, vii, red arrows). Hemizygous male X\(^{Utx\text{-GT1}}\)Y\(^{Utx\text{-GT1}}\) embryos appear phenotypically normal at this stage (A-iv). The X\(^{Utx\text{-GT1}}\) and X\(^{Utx\text{-GT2}}\) alleles fail to complement as female X\(^{Utx\text{-GT1}}\)X\(^{Utx\text{-GT2}}\) embryos have identical phenotypes to homozygotes (A-viii). (B) At E10.5, homozygous X\(^{Utx\text{-GT1}}\)X\(^{Utx\text{-GT1}}\) female embryos exhibit either normal yolk sac vasculature with a reduction in red blood cells (B-ii) or have a completely pale yolk sac with unremodeled vascular plexus (B-iii). doi:10.1371/journal.pgen.1002964.g003
To determine whether UTY can compensate for the loss of UTX, we obtained the Welcome Trust Sanger Institute gene trap line Uty\textsuperscript{Gt(XS0378)Wtsi}, designated as Y\textsuperscript{UTyGT} (Figure 4A). This line, inserted in intron 4, traps the Uty transcript in a similar position of the coding sequence as the Utx alleles (compare to Figure 1A). This gene trap line was verified by RT-PCR in ES cells and subsequent mice (Figures S1 and S2G), and it achieved a 99% reduction in Uty expression from X\textsuperscript{TX}Y\textsuperscript{UTyGT} mouse tail RNA (Figure 4B). Hemizygous Uty mutant males, X\textsuperscript{TX}Y\textsuperscript{UTyGT}, were viable and fertile (Table 1). However, no compound hemizygous X\textsuperscript{TX}Y\textsuperscript{UTyGT} and X\textsuperscript{TX}\textsuperscript{TX}Y\textsuperscript{UTyGT} embryos were recovered at E18.5 (Table 1). At E10.5, expected genotype frequencies of X\textsuperscript{TX}Y\textsuperscript{UTyGT} and X\textsuperscript{TX}\textsuperscript{TX}Y\textsuperscript{UTyGT} males were observed, but these embryos phenocopied the developmental delay, neural tube closure, cardiac, and yolk sac defects observed in Utx homozygous embryos (Figure 4C-iii, iv).

UTX and UTY redundancy is essential for progression of cardiac development

We performed a more detailed phenotypic assessment of Utx and Uty mutant hearts to scrutinize the extent of phenotypic overlap between X\textsuperscript{TX}Y\textsuperscript{UTx+}, X\textsuperscript{TX}X\textsuperscript{TX}, and X\textsuperscript{TX}Y\textsuperscript{UTy-} embryos. Analysis of cardiac development in similar sized E10.5 embryos (Figure 5A-i, ii, iii, iv) revealed that Utx homozygotes and Utx/UTy compound hemizygotes failed to complete heart looping (Figure 5A-vii), whereas Utx heterozygotes and hemizygotes were phenotypically normal (Figure 5A-vi, viii). Additionally, homozygotes and compound hemizygotes had smaller hearts with a lack of constriction between the left and right ventricles. Sectioning of E10.5 hearts confirmed that Utx homozygotes and Utx/UTy compound hemizygotes have small hearts with a reduction in ventricular myocardial trabeculation and little or no initiation of interventricular septum formation (Figure 5B-ii, iv). The outer ventricular wall of these embryos is much thinner, and the overall number of cardiomyocytes and myocardial structure is severely deficient (Figure 5C-ii, iv). In summary, while mid-gestational hearts appear normal in X\textsuperscript{TX}Y\textsuperscript{UTy+} hemizygous males, X\textsuperscript{TX}- X\textsuperscript{TX}-homozygous females and X\textsuperscript{TX}- Y\textsuperscript{UTy-} compound hemizygous males display identical deficiencies in cardiac development. Therefore, UTY compensates for the loss of UTX in hemizygous Utx mutant males, rescuing mid-gestational cardiac phenotypes.

![Figure 4. UTX and UTY have essential, redundant functions in embryonic development.](https://www.plosgenetics.org/doi/10.1371/journal.pgen.1002964.g004)
Mouse and human UTY are incapable of H3K27 demethylation in vivo

UTX and UTY have redundant function in embryonic development, but it is not known whether mouse UTY is capable of H3K27 demethylation. Two independent publications demonstrated that human UTY has no catalytic activity in H3K27 demethylation in vitro [26,29]. It is possible that human UTY (and not mouse UTY) has accumulated a specific polymorphism rendering it demethylase deficient. Additionally, in vitro assays remove UTY from its natural cellular context and may lack cofactors required to promote H3K27 demethylation. Therefore, we utilized an intracellular, in vivo demethylation assay, whereby HEK293T cells transiently over-expressing the UTX carboxyterminus (encoding the JmjC and surrounding domains essential...
For proper structure and function, mRNA and proteins of H3K27me3 demethylase activity. (A) HEK293T cells were transfected with Flag-tagged C-terminal human (H) and mouse (M) UTX and UTY constructs. The C-terminal fragments span AA 880–1401 in human UTX (Figure S6) and include the corresponding region in mouse UTX. Transfected cells (white arrows) over-expressing H-UTX and M-UTY (Flag immunofluorescence, red pseudo-color) exhibited global loss of H3K27me3 immunofluorescence (red pseudo-color). Cells transfected with H-UTY and M-UTY C-terminal constructs did not demethylate H3K27me3. 

(B) H3K27me3 demethylase assay of UTX and UTY mutant constructs. H-UTX H1146A contains a point mutation in a residue that was previously reported as defective in H3K27 demethylation. Cells expressing H-UTX H1146A had no loss of H3K27me3. Mouse UTY has a Y to C amino acid change that corresponds to position 1135 in human UTX. This UTX residue is predicted to regulate H3K27me3 binding and demethylation. Expression of H-UTX Y1135C failed to demethylate H3K27me3. Mouse UTY also has a T to I amino acid change that corresponds to position 1143 in human UTX that is predicted to regulate binding of ketoglutarate in the demethylase reaction. Expression of H-UTX T1143I failed to demethylate H3K27me3. Correction of these two altered residues in mouse Uty (M-UTY C947Y, I955T) failed to recover H3K27me3 demethylation activity. (C) Alignment of the JmjC domain of human/mouse UTX human UTY, mouse UTY, and human/mouse JMJD3. UTY non-conservative substitutions are indicated by white boxes and residues of interest are labeled with red asterisks. The UTX mutations that were analyzed are listed above the alignment, while JMJD3 mutations are listed below the alignment. (D) HEK293T cells were transfected with C-terminal UTX and UTY constructs or full-length mouse UTX JMJD3 constructs carrying various AA substitutions. Medium-high expressing cells (N=100 cells scored for each experiment) were scored for any visible reduction in H3K27me3 levels relative to nearby untransfected cells. 100% of WT H-UTX, M-UTY and M-JMJD3 expressing cells had observable H3K27me3 demethylation. The negative controls of H-UTX H1146A, M-JMJD3 H1388A, and M-JMJD3 with deletion of the JmjC domain had no visible H3K27me3 demethylation (0% of cells). Wild type H-UTY and M-UTY had 0% of cells with detectable demethylation.

expression of Flag-tagged human and mouse UTX demethylated H3K27me3 and H3K27me2, while a mutation known to disrupt activity (H1146A) was unable to demethylate H3K27 (Figure 6A and Figure S5A). Human UTX expression had no effect on other histone modifications we tested, such as H3K4me2 (Figure S5B). In contrast, neither human nor mouse UTY were capable of demethylating H3K27me3 and H3K27me2, with the exception of the UTY mutation Y1135C (Figure 6A and 6B, Figure S5A). Human UTX expression had no effect on other histone modifications we tested, such as H3K4me2 (Figure S5B). In contrast, neither human nor mouse UTY were capable of demethylating H3K27me3 and H3K27me2, while a mutation known to disrupt activity (H1146A) was unable to demethylate H3K27me3 and H3K27me2. Correction of these two altered residues in mouse Uty (M-UTY C947Y, I955T) failed to recover H3K27me3 demethylation activity. (C) Alignment of the JmjC domain of human/mouse UTX human UTY, mouse UTY, and human/mouse JMJD3. UTY non-conservative substitutions are indicated by white boxes and residues of interest are labeled with red asterisks. The UTX mutations that were analyzed are listed above the alignment, while JMJD3 mutations are listed below the alignment. (D) HEK293T cells were transfected with C-terminal UTX and UTY constructs or full-length mouse UTX JMJD3 constructs carrying various AA substitutions. Medium-high expressing cells (N=100 cells scored for each experiment) were scored for any visible reduction in H3K27me3 levels relative to nearby untransfected cells. 100% of WT H-UTX, M-UTY and M-JMJD3 expressing cells had observable H3K27me3 demethylation. The negative controls of H-UTX H1146A, M-JMJD3 H1388A, and M-JMJD3 with deletion of the JmjC domain had no visible H3K27me3 demethylation (0% of cells). Wild type H-UTY and M-UTY had 0% of cells with detectable demethylation.

Of the point mutations in UTY predicted to affect H3K27me3, only mutation of H-UTX Y1135C and T1143I with corresponding M-JMJD3 Y1377C and T1385I had no detectable H3K27 demethylation (0%). (E) Stereo view of the active site of human UTX (PDB ID: 3AVR). The corresponding residues in mouse UTY are also indicated in parentheses. The figure was prepared with the program Pymol (Schrödinger LLC).

doi:10.1371/journal.pgen.1002964.g006

for human UTX is catalytically inactive. Notably, restoring the 2 crucial mouse UTY polymorphisms (M-UTY C947Y, I955T) failed to recover H3K27 demethylase activity (Figure 6B). These data suggest that unidentified structural elements in the UTY C-terminal region are also responsible for the lack of H3K27 demethylase activity.

UTX and UTY associate in common protein complexes and are capable of H3K27 demethylase independent gene regulation

Although human and mouse UTY have lost the ability to demethylate H3K27, they retain considerable sequence similarity with UTX, suggesting a conserved function. To gain more insight into the overlap in UTX and UTY activities, we performed a biochemical analysis of tagged constructs to determine if UTX and UTY can associate in common protein complexes. Co-transfection of Flag tagged UTX or UTY with HA-UTX followed by immunoprecipitation demonstrates that UTX can form a multimeric complex with itself and UTY (Figure 7A). UTX associates with a H3K4 methyl-transferase complex containing MLL3, MLL4, PTIP, ASH2L, RBBP5, PA-1, and WDR5 [23,44]. To examine incorporation into this complex, we performed immunoprecipitations with Flag tagged UTX and UTY constructs. Both UTX and UTY were capable of associating with RBBP5 (Figure 7B). Thus, UTX and UTY are incorporated into common protein complexes.

To identify common gene targets of UTX and UTY mediated regulation we generated E10.5 mouse embryonic fibroblast (MEF) cell lines containing mutations in Utx and Uty (alleles X(Utx2)D and Y(Uty2)X). The gene traps in these MEFs efficiently trapped Utx and Uty transcripts (Figure 7C). These MEFs did not demonstrate differences in levels of global H3K27me3 (Figure S9A). Genome-wide UTX promoter occupancy has been mapped in fibroblasts [30]. Therefore, we screened our mutant MEFs for misregulated transcripts (Figure 7C). These MEFs did not demonstrate differences in levels of global H3K27me3 (Figure S9A). Genome-wide UTX promoter occupancy has been mapped in fibroblasts [30]. Therefore, we screened our mutant MEFs for misregulated genes affected by the loss of both Utx and Uty that had been documented as direct UTX targets. The Fnbp1 promoter is bound by UTX [30]. We verified UTX and UTY binding to the Fnbp1 promoter by ChIP (Figure S9B and S9C). Fnbp1 expression was reduced to 68% of WT levels in X(Utx2)D and Y(Uty2)X MEFs, but was further compromised to 42% in Y(Uty2)X Utx2 and 48% in X(Utx2)D Y(Uty2)X MEFs in which all Utx and Uty activity was lost (Figure 7C). Analysis of E12.5 MEFs of a secondary allele (X(Utx2)F) also demonstrated diminished Fnbp1 expression in both
Figure 7. UTX and UTY associate in common protein complexes and are capable of H3K27 demethylase independent gene regulation. (A) Co-transfection of HA-UTX with Flag-UTX or Flag-UTY demonstrates that HA-UTX can immunoprecipitate with both Flag-UTX and Flag-UTY. (B) Immunoprecipitation of Flag-UTX and Flag-UTY reveal interaction with RBBP5, a component of the H3K4 methyl-transferase complex. Flag vector transfection was used as a negative control for immunoprecipitation. (C) Fnbp1, a gene targeted directly by UTX, has intermediate downregulation in X^{Utx-} Y^{Uty+} MEFS (68% of WT, t-test p-value = 0.002), but was further compromised in X^{Utx-} X^{Utx-} (42% of WT, t-test p-value relative to X^{Utx-} Y^{Uty+} = 0.001) and X^{Utx-} Y^{Uty-} (48% of WT, t-test p-value relative to X^{Utx-} Y^{Uty+} = 0.02, N>4 independent MEF lines per genotype) MEFS.
were generated from the X<sup>UtgGT2fl</sup> and Y<sup>UtgGT</sup> alleles. (D) Fnbp1 is similarly mis-expressed in X<sup>UtgGT2fl</sup> allelic combinations of E12.5 MEFs. X<sup>UtgGT2fl</sup>–X<sup>UtgG</sup> and X<sup>UtgG</sup>–Y<sup>Utg</sup> MEFs significantly differ from X<sup>UtgG</sup>–Y<sup>Utg</sup> MEFs (t-test p-value = 0.05 and 0.02 respectively, N = 4 independent MEF lines per genotype). (E) H3K27me3 ChIP was performed over a negative control region (an intergenic region) as well as a positive control (observed in X<sup>xFnb1</sup>). Alternatively, H3K4me3 significantly accumulated at the 52% and 57% level of expression respective to X<sup>xFnb1</sup>. (F) H3K4me3 ChIP was performed over a negative control region (intergenic region) as well as a positive control (HoxB1). Fnbp1 failed to accumulate H3K27me3 in X<sup>xFnb1</sup>–X<sup>UtgG</sup> MEFs (t-test p-value = 0.5, N = 4 independent MEF lines per genotype). (F) H3K4me3 ChIP was performed on E12.5 X<sup>UtgG</sup>–Y<sup>Utg</sup> control (green) and X<sup>xFnb1</sup>–Y<sup>Utg</sup> (red) MEFs. An IgG antibody control is indicated in grey. Quantitative PCR for the ChIP was performed over a negative control region (intergenic region) as well as a positive control (Npm1). The WT Fnbp1 promoter exhibited significant H3K4me3 accumulation, which was reduced in X<sup>xFnb1</sup>–X<sup>UtgG</sup> MEFs (t-test p-value = 0.005, N = 3 independent MEF lines per genotype).

doi:10.1371/journal.pgen.1002964.g007

**Discussion**

We have undertaken a rigorous genetic analysis contrasting UTX and UTY function in mouse embryonic development. In alignment with current literature, Utx homozygous females are lethal in mid-gestation with a block in cardiac development [39]. We now demonstrate that Utx hemizygous mutant males are viable at late embryonic timepoints in expected Mendelian frequencies. In fact, approximately 25% are capable of reaching adulthood. Our comprehensive phenotypic analysis of Utx hemizygous males illustrates that these embryos are phenotypically normal at mid-gestation and lack the cardiovascular dysfunction of Utx homozygous females. This stark phenotypic disparity suggests that UTY may compensate for the loss of UT in the male embryo. Compound hemizygous Utx/Uty mutant male embryos phenocopy the cardiovascular and gross developmental delay of homozygous females, proving that UTX and UTY have redundant function in embryonic development. As we have demonstrated that mouse UTX lacks H3K27 demethylase activity in vivo, the overlap in embryonic UTX and UTY function is due to H3K27 demethylase independent activity. Given the widespread developmental delay and pleiotropy, it is difficult to assess the primary defect and tissue(s) responsible for UTX and UTY redundancy. The presence of functional UTY in Utx hemizygous males is not capable of preventing peri-natal runting and lethality, suggesting that UTX and UTY are not completely overlapping in activity. These later phenotypes could be due to H3K27 demethylase dependent activity of UTX. Furthermore, the lack of phenotype in Uty hemizygotes demonstrates the absence of any essential UTY specific function in mouse development.

The UTY Junonji-C domain has maintained high conservation in the absence of catalytic H3K27 demethylase activity. JMJD3 mediated regulation of lymphocyte Th1 response requires an intact Junonji-C domain, but is also not dependent on H3K27 demethylylation [37]. Therefore, this domain may be an essential structural protein component, a protein binding domain, or a domain that may demethylate non-histone substrates. UTX and UTY can associate in a common protein complex and can both interact with RBBP5 of the H3K4 methyl-transferase complex. UTX, UTY, and JMJD3 all associate with H3K4 methyl-transferase complexes from multiple mouse and human cell types [23,25,44,45]. The Fabp1 promoter is bound by UTX, and gene expression is positively regulated by both UTX and UTY in MEFs. Based on our histone profiling at this locus, UTX and UTY affect the deposition of H3K4 methylation, not H3K27me3 demethylation. Therefore, the common UTX/UTY pathway in embryonic development may involve gene activation rather than removal of gene repression. JMJD3 has been linked more directly to transcriptional activation as the protein complexes with and facilitates factors involved in transcriptional elongation [46]. One cardiac target of UTX regulation, atrial natriuretic factor (ANF), was misregulated in E5 cell differentiation [39]. Cell culture experiments suggest that ANF may be a target of both H3K27 demethylase dependent and demethylase independent regulation; however, this study could not distinguish UTX versus UTY.

UTX/UTY Histone Demethylase-Independent Function

UTX and UTY can both associate with heart transcription factors to regulate downstream target genes

It has been documented that UTX can associate with heart transcription factors and with the SWI/SNF chromatin remodeler, BRG1 [39]. It has been hypothesized that UTX association with these factors mediates H3K27 demethylase dependent and demethylase independent induction of the cardiomycocyte specification program. As UTX and UTY have redundant demethylase independent function in embryonic development, we examined whether UTX can also associate with these proteins. Co-transfection of Myc-UTY with Flag-BRG1 followed by immunoprecipitation demonstrated that UTY associates with BRG1 (Figure 8A). Myc-UTY also co-immunoprecipitated with Flag-NKX2-5, Flag-TBX5, and Flag-SRF (Figure 8B and Figure 81A). Thus, UTX can form the same protein complexes as UTX with respect to BRG1 and heart transcription factors.

To examine function of UTY in direct activation of downstream heart transcription factor targets, we assessed the regulation of one previously characterized target, atrial natriuretic factor (ANF) [39]. Co-transfection of NKX2-5 with a ANF promoter-Luciferase reporter construct demonstrated a significant upregulation in expression off the ANF promoter (Figure 8C). The reporter expression was significantly enhanced when NKX2-5 was co-transfected with UTY (Figure 8C). The level of ANF reporter transcriptional enhancement was relatively weaker with UTY as compared to UTX, but this is most likely due to a reduction in the transfection efficiency of full-length UTY relative to UTX (as demonstrated in Figure 7A and 7B). UTY also significantly enhanced the ANF reporter response to TBX5 (Figure 81B). Finally, ANF expression was significantly affected in the hearts of only X<sup>xFnb1</sup>–Y<sup>Utg</sup> embryos (with 52% and 57% level of expression respective to X<sup>xFnb1</sup>–Y<sup>Utg</sup> controls, Figure 8D). X<sup>xFnb1</sup>–Y<sup>Utg</sup> hemizygotes only had a moderate loss of ANF expression (76% expression level respective to X<sup>xFnb1</sup>–Y<sup>Utg</sup>) that was not statistically significant from wild type controls due to the variability in ANF expression. In summary, both UTX and UTY can associate with heart transcription factors to modulate expression of downstream targets.
Figure 8. UTY associates with BRG1 and heart transcription factors, and regulates downstream ANF gene expression. (A) Myc-UTY was co-transfected with a Flag vector control or Flag BRG1 and immunoprecipitated with Flag-Agarose beads. Myc-UTY specifically immunoprecipitates with Flag-BRG1. (B) Myc-UTY was co-transfected with the Flag vector control or Flag-NKX2–5. Myc-UTY was co-immunoprecipitated by Flag-NKX2–5. (C) ANF:Luciferase reporter assay. HEK293T were transfected with the reporter ANF:Luciferase construct alone (−), with NKX2–5, with NKX2–5 and UTY, or with NKX2–5 and UTX. Reporter activity was significantly enhanced with the addition of UTY (t-test p-value = 0.01). Right panel illustrates the comparison of UTY versus UTX enhancement of NKX2–5 driven ANF expression. N = 3 independent transfections per group. (D) Anf expression was analyzed from E10.5 heart RT-PCR of various X<sup>Utx<sup>G72.1</sup></sup> and Y<sup>Uty</sup> allelic combinations. A moderate, but
not significant downregulation of ANF was observed in X^{Utx\text{-}}\ Y^{Uty\text{-}} hearts (76% of WT, t-test p-value = 0.06), but was significantly compromised in X^{Utx\text{-}}\ X^{Utx\text{-}} (52% of WT, t-test p-value relative to X^{Utx\text{-}}\ Y^{Uty\text{-}} = 0.005) and X^{Utx\text{-}}\ Y^{Uty\text{-}} (57% of WT, t-test p-value relative to X^{Utx\text{-}}\ Y^{Uty\text{-}} = 0.02, N>4 per genotype) hearts.

doi:10.1371/journal.pgen.1002964.g008

function in ES cell differentiation. Both UTX and UTY affect the transcriptional response of an exogenous ANF reporter in the presence of heart specific transcription factors, suggesting that UTX and UTY can operate more directly by aiding in transcriptional activation of this gene rather than altering chromatin structure. Consistently, ANF expression was affected in X^{Utx\text{-}}\ X^{Utx\text{-}} and X^{Utx\text{-}}\ Y^{Uty\text{-}} embryonic hearts. UTX and UTY can both associate with the SWI/SNF chromatin remodeler BRG1, which has been hypothesized to mediate histone demethylase independent gene regulation, but the relevance and mechanism of this interaction is not known. Drosophila UTX associates with BRM (orthologous to BRG1) and CBP (a H3K27 acetyl-transferase), and the coupling of H3K27 demethylation with H3K4 methylation. The developmental activation of "bivalent" PRC2 targets by coordinating H3K27 demethylation with H3K4 methylation in X mice are not well characterized, but have been reported to be perinatal lethal with distinct features in comparison to Utx hemizygotes [32]. Therefore, it is likely thatJMJD3 has distinct targets in development. Overall, the earliest H3K27 demethylation dependent phenotypes for all members of this gene family do not manifest until late embryonic development. This timepoint is much later than the converse early embryonic phenotypes from mutations in the H3K27 methyl-transferase complex [7,21,22]. Thus, there appears to be a lack of interplay between H3K27 methylation and demethylation in gene regulation, and the early embryonic removal of H3K27me3 from PRC2 mediated processes (such as ES cell differentiation, reactivation of the inactive X-chromosome, or establishing autosomal imprinting) may involve other mechanisms such as histone turnover or chromatin remodeling. H3K27 demethylases may certainly have crucial roles in the specification of progenitor cell populations of organ systems essential in peri-natal or postnatal viability, and genetic model systems will best assess the functional impact that H3K27 demethylation plays in these processes.

Methods

Cell culture and constructs

HEK293T were maintained in DMEM supplemented with Glutamine, Pen-Strep, and 10%FBS. Flag-Human UTX (Plasmid #17438) and UTY (Plasmid #17439) were obtained through Addgene [29]. The N-terminus of H-UTX and H-UTY were deleted with QuickChange Lightning (Agilent) as directed producing H-UTX C-terminus 380–1401 (Genbank: NP_066963.3) and H-UTY C-terminus 827–1343 (Genbank: NP_009056.3, an N-terminal His tag was also incorporated into both constructs). Site directed mutagenesis was performed via QuickChange Lightning (Agilent) as directed to produce point mutations. The mouse UTX C-terminus (880–1401) deviated from Human UTX at 2 residues, R1073K and S1263N (According to the Sanger Vega server, the primary Utx transcript Kdm6a-001 encodes for Genbank: CAM27157, we also detected this transcript in E14 ES cell RT-PCR). These changes were created in the H-UTX C-terminus to generate the M-UTX construct. The Flag-tagged mouse UTY C-terminus (692–1212, Genbank: NP_033510.2) was subcloned by RT-PCR of E14 ES cell RNA and introduced into the same vector as the other UTX constructs (PGS2+MT backbone). HA tagged H-UTX was obtained through Addgene [24]. Flag tagged mouse JMJD5 was generously provided by Burgold et al. [27]. Flag tagged BRG1 was obtained through Addgene (Plasmid #19143). Flag tagged NKKx2-5 (Plasmid #32969), TBX5 (Plasmid #32968), and SRF (Plasmid #32971) were obtained through Addgene and recombined into DEST26 (Invitrogen). Flag tagged NKKx2-5 was also generously provided by Benoit Bruneau [64].

Transfections, immunofluorescence, Western blotting, antibodies

Transfection of HEK293T was accomplished with Lipofectamine 2000 as directed (Invitrogen). Lipid complexes were removed 24 hours post-transfection, and analysis was performed after 48 hours total. Fixation, extraction, and immunofluorescence were performed as described [65]. Immunofluorescence antibodies include anti-Flag (Sigma F3165, 1:500), anti-H3K27me3 (Millipore 07-449, 1:500), anti-H3K27me2 (Millipore 07-452, 1:500), and anti-H3K4me3 (Millipore 07-030, 1:300). Cells were imaged with Zeiss axiovision software. Image stacks were deconvolved and z-projected. Quantification of H3K27me3 immunofluorescence was performed on deconvolved z-projected stacks (with no pixel...
saturation in images) using ImageJ software (NIH). The average mean H3K27me3 signal was calculated for untransfected and transfected cells in a given image. For each image, the relative % H3K27me3 was determined, and the average relative % H3K27me3 was calculated for >15 images per construct. For western blotting, nuclear lysates were prepared according to Invitrogen's nuclear extraction protocol. Immunoprecipitations were carried out with 50 μl Flag beads (Sigma A2220) in buffer A as described, using 500 μg (UTY-UTX, RBP9, BRG1, TBX5, SRF associations) or 1 mg (UTY-NKX2-5 association) of lysate [44]. Immunoprecipitation reactions were boiled off beads and run with 10% input on an 8% SDS-PAGE gel. Histone extractions were prepared as described [66]. Western blotting was performed as described [67] with anti-Flag (Cell Signaling 3236, 1:4000), anti-RBBPS (Bethyl Labs A-300-109A, 1:5000), anti-HA (Roche 11867423001, 1:10000), anti-Myc (Abcam ab9132, 1:5000), anti-RBBP5, BRG1, TBX5, SRF associations) or 1 mg (UTY-UTX, UtyGT2fl) Flag beads (Sigma ab1012, 5 μl) Flag beads (Sigma ab1012, 5 μl), or Rabbit IgG (Sigma, I5006) and qPCR was performed as described above.

Luciferase assay

We received the ANF promoter-Luciferase reporter construct from Benoit Bruneau [69]. This construct was co-transfected in the presence of NKX2-5 or TBX5 with or without UTX or UTY. Luciferase activity was measured using the Promega Dual Luciferase Reporter Assay System on the Promega Glomax Multi Detection System. All readings were normalized to a Renilla Luciferase control that was co-transfected with all samples.

Mouse crosses

All mouse experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Utx homozygous data was generated either by crosses between Utx hemizygous males and Utx heterozygous females, or by crosses between $X^{UtxGT1} Y^{Utx+}$ and VasaCre males and $X^{UtxGT2} Y^{Utx+}$ heterozygous females. $X^{UtxGT1} Y^{Utx+}$ VasaCre males were utilized because of an initial difficulty in generating $X^{UtxGT2} Y^{Utx+}$ males and due to the efficient and specific activity of VasaCre in the male germline [70]. Utx hemizygous phenotypic data was developed from the previously mentioned homozygous crosses or through crosses between a WT male and heterozygous Utx female. Compound hemizygous Utx/UTy embryos were generated by crossing heterozygous Utx females with hemizygous Uty males. Embryos were PCR genotyped from yolk sac samples for Utx and were sexed by a PCR genotyping scheme to distinguish Utx from Uty. All primer sequences are available upon request.

Histology, in situ hybridization, and LacZ staining

Histology samples, in situ hybridization, and LacZ staining were performed as described [71]. In situ hybridization probes were generated to be identical to previous literature [72].

Supporting Information

Figure S1 Schematic of genotyping strategies for Utx and Uty alleles. (A) The $X^{UtxGT1}$ allele was genotyped with a three-primer scheme spanning the insertion site in intron 3. (B) The $X^{UtxGT2}$ allele was verified by Southern blotting with an HpaI restriction digest. HpaI sites are noted as “H”, and the 5’ probe location is marked as a red box. The introduction of a novel HpaI site within the targeting cassette reduces the HpaI product from 17-Kb to 10-Kb. A three-primer scheme was designed for genotyping. Due to a deletion of intron 3 within the targeting vector, the product size of primers 1-2 will be larger in WT than in $X^{UtxGT2}$, even with the introduction of the loxP site. Primers 3-2 will only amplify if Cre recombination takes place to delete exon 3. (C) The $Y^{UtxGT}$ insertion site was not mapped because intron 4 is approximately 25-Kb. The allele was verified by a RT-PCR three-primer genotyping scheme. (TIF)

Figure S2 Verification of Utx and Uty alleles. (A-C) Verification of the $X^{UtxGT1}$ allele. (A) Trap specific primers between Utx exon 2 and the B-Gal reporter amplify the expected band in $X^{UtxGT1}$ Y* ES cells. WT male E14 ES cells were used as a control. (B) The gene trap DNA location was mapped within Utx intron 3, and primers were designed to distinguish wild type (WT) and gene trap (GT) alleles in mice generated from these cells. (C) Quantitative RT-PCR downstream of the gene trap (exons 23-25) from tail RNA of $X^{UtxGT1}$ Y* mice demonstrate the gene trap effectiveness. (D-F) Verification of the $X^{UtxGT2}$ allele. (D) Southern blotting of WT and $X^{UtxGT2}$ Y* ES cells using a 5’ probe and HpaI digest demonstrated the expected shift in banding due to a novel restriction site. (E) A PCR genotyping scheme was designed to distinguish WT (X*) and $X^{UtxGT2}$, and Y* ES cells. Chromosomes were crossed to CD1 to assess germline transmission, and were backcrossed to 129/SvJ or C57BL/6J. $S/o2 Cre$ and Rod$^{b}$ transgenes were obtained from The Jackson Laboratory [40]. The VasaCre transgene was developed by Gallardo et al. [70].
ii, iii) and $X^{UtxGT1}$ $X^{UtxGT2}$ (A-iv) E10.5 embryos. Embryos were cleared in A-iii, iv, (B) In situ hybridization of Utx sense control (B-i, iv), Utx antisense (B-ii, v), and Uty antisense (B-iii, vi) probes on E10.5 sagittal sections of WT male embryos.

**Figure S4** Mouse UTY and corresponding mutation of the UTX catalytic domain abolish H3K27me3 demethylation. (A) Western blot of transfections from the H3K27me3 demethylation assay in Figure 6. Flag tagged UTX and UTY constructs are expressed at similar levels in this assay, Rbhp5 blotting served as a loading control. (B) Quantification of H3K27me3 immunofluorescence assay from Figure 5. In a given image, the average H3K27me3 immunofluorescence for transfected and untransfected cells was quantified. The average of the % H3K27me3 immunofluorescence relative to untransfected cells was graphed (N>15 images per transfection).

**Figure S5** Mouse UTY has no H3K27me2 demethylase activity. (A) HEK293T cells were transfected with Flag tagged C-terminal human (H) and mouse (M) UTX and UTY constructs. Transfected cells (white arrows) over-expressing H-UTX and M-UTX (green channel) exhibited global loss of H3K27me2 immunofluorescence (red, top 2 panels). H-UTX Y1135C and M-UTY had no loss of H3K27me2 (bottom 2 panels). (B) Expression of WT H-UTX had no effect on H3K4me2.

**Figure S6** Alignment of human and mouse UTX, UTY, and JMJD3. Alignment of the C-terminal 880–1401 amino acids of H-UTX and corresponding regions of human and mouse UTX, UTY, and JMJD3. The JmjC domain is boxed in pink. Several residues in H-UTX predicted to be important for H3K27 demethylation are mutated in mouse or human UTY. These residues are boxed in black, and these point mutations were made in H-UTX (listed above the box) or JMJD3 (listed below the box).

**Figure S7** Alignment of the JmjC domain of UTX, UTY, and JMJD3. JmjC domain sequences were aligned from all identified homologs of UTX, UTY, and JMJD3. All species have UTX residues H1146 and E1148 required for Iron binding in the demethylase reaction. Y1135 crucial for H3K27me3 binding and T1143 essential for ketoglutarate binding in the demethylase reaction are conserved throughout all species except for mouse UTY.

**Figure S8** Alignment of the JmjC domain of KDM6, KDM2, KDM7, and KDM3. JmjC domain sequences were aligned from human, mouse, a non-mammalian vertebrate (if protein sequences were available), and an invertebrate (if protein sequences were available) species for identified KDM6, KDM2, KDM7, and KDM3 family members. The UTX T1143 essential for ketoglutarate binding in the demethylase reaction is conserved throughout all species except for mouse UTY.

**Figure S9** UTX mutant MEFs have unaltered levels of H3K27me3 and ENBP1 is bound by UTX and UTY. (A) Western blot of H3K27me3 and total H3 following histone extraction from MEFs of the indicated genotypes. There is no change in the level of global H3K27me3 in lines with loss of UTX. (B) HEK293T cells were transfected with a Myc vector control, Myc-UTX or Myc-UTY. ChIP was performed with Myc antibody and qPCR tested association with a negative control (an intergenic region, grey bars), GAPDH (negative control, red bars), ENBP1 (green bars), or HOXA9 (positive control, yellow bars). Myc-UTX and Myc-UTY associate with the ENBP1 promoter. (C) ChIP was performed on primary MEFs with an IgG control or UTX antibody. ChIP with the UTX antibody was performed in wild-type $X^{Utx \text{mas}}$ $Y^{\text{mas}}$, $X^{UtxGT1}$ $Y^{\text{mas}}$, $X^{UtxGT2}$ $Y^{\text{mas}}$ MEFs and qPCR tested association with the Fabp1 promoter relative to a negative control intergenic region.

**Figure S10** UTX and UTY associate with heart transcription factors and regulate expression of ANF. (A) Myc-UTY was co-transfected with the Flag negative control, Flag-TBX5, or Flag-SRF. Myc-UTY was co-immunoprecipitated with Flag-TBX5 and Flag-SRF. (B) ANF:Luciferase reporter assay. HEK293T were transfected with the reporter ANF:Luciferase construct alone (-), with TBX5, or with TBX5 and UTY. Reporter activity was significantly enhanced with the addition of UTX (t-test p-value = 0.004).

**Table S1** Utx hemizygous genotype on inbred backgrounds. Observed (Obs) and expected (Ex) frequencies of indicated genotypes (Geno) at embryonic (E) or postnatal (P) developmental stages with $\chi^2$ p-values (p-value) for the corresponding crosses to obtain each genotype. At E18.5 on the C57BL/6J background, 5 of the 8 observed $X^{\text{UtxGT1}}$ $Y^{\text{mas}}$ males were on the N8 generation.

**Table S2** Genotype frequencies of Sox2Cre driven Utx mutation. Observed (Obs) and expected (Ex) frequencies of indicated genotypes (Geno) at embryonic (E) or postnatal (P) developmental stages with $\chi^2$ p-values (p-value) for the corresponding crosses to obtain each genotype.

**Acknowledgments**

We would like to thank the Magnuson lab for review of this manuscript, Benoit Bruneau for providing DNA constructs, and Kristian Helin for generous contribution of the UTX antibody.

**Author Contributions**

Conceived and designed the experiments: KBS TM. Performed the experiments: KBS. Analyzed the data: KBS TS SY TM. Contributed reagents/materials/analysis tools: KBS TS SY TM. Wrote the paper: KBS TM.

**References**

1. Martin C, Zhang Y (2005) The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol 6: 838–849.
2. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, et al. (2002) Role of histone H3 lysine 27 methylation in Polycombgroup silencing. Science 298: 1039–1043.
3. Muller J, Hart CM, Francis NJ, Vargas ML, Sengupta A, et al. (2002) Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell 111: 197–208.
4. Czermin B, Melfi R, McCabe D, Seitz V, Imhof A, et al. (2002) Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell 111: 185–196.
5. Kuzmichev A, Nishiooka K, Erdjument-Bromage H, Tempst P, Reinberg D (2002) Histone methyltransferase activity associated with a human multi-protein complex containing the Enhancer of Zeste protein. Genes Dev 16: 2893–2905.
6. Montgomery ND, Yee D, Chen A, Kalantry S, Chamberlain SJ, et al. (2005) The murine polycomb group protein Eed is required for global histone H3 lysine-27 methylation. Curr Biol 15: 942–947.

7. Pasini D, Bracken AP, Jensen MR, Lazzerini Denchi E, Helin K (2004) Suz12 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells 22: 1496–1505.

8. Lee TI, Jenner RG, Boyer LA, Guenther MG, Levine SS, et al. (2006) Control of developmental regulators by Polycomb in human embryonic stem cells. Cell 125: 315–326.

9. Ezhkova E, Pasolli HA, Parker JS, Stokes N, Su IH, et al. (2009) Ezh2 and Ezh2 cognate histone H3K27 trimethylation are essential for hair follicle morphogenesis and wound repair. Genes Dev 23: 485–498.

10. Mageraon R, Reinberg D (2011) The Polycomb complex PRC2 and its mark in life. Nature 469: 343–349.

11. Chamberlain SJ, Yee D, Magnuson T (2008) Polycomb repressive complex 2 is dispensable for maintenance of embryonic stem cell pluripotency. Stem Cells 26: 572–577.

12. Boyer LA, Plath K, Zeitlinger J, Brambuka T, Medeiroso LA, et al. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441: 349–355.

13. Agger K, Cloos PA, Rudkjaer L, Williams K, Andersen G, et al. (2009) The H3K27me3 demethylase JmjD3 contributes to the activation of the INK4A-ARF locus in response to oncogene- and stress-induced senescence. Genes Dev 23: 1171–1176.

14. De Santa F, Narang V, Yap ZH, Tsai BK, Burgold T, et al. (2009) JmjD3 contributes to the control of gene expression in LPS-activated macrophages. EMBO J 28: 3341–3352.

15. Miller SA, Mohn SE, Weinmann AS (2010) JmjD3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. Mol Cell 40: 594–605.

16. Jepson D, Forbes S, Lee JW, Lee SK (2012) A proposed path by which genes common to mammalian X and Y chromosomes evolve to become X inactivated. Nature 494: 276–280.

17. Satoth T, Takuchi O, Vandenbon A, Yasuda K, Tanaka Y, et al. (2010) The JmjC domain-containing methyltransferase H3K27 demethylase JmjD3 has histone tail methylation functions. Proc Natl Acad Sci U S A 107: 10034–10039.

18. Yang JR, Ding J, Liu W, Jin E, Mao L, et al. (2010) The histone H3 lysine 27 demethylase JmjD3 regulates animal posterior development. Nature 469: 689–694.

19. De Santa F, Sparaco F, De Santa F, Totaro MG, Prosperi E, et al. (2008) The histone H3 lysine 27-specific demethylase JmjD3 is required for neural polycomb-mediated gene silencing. Cell 130: 1083–1094.
independent of its enzymatic activity. PLoS Genet 8: e1002647. doi:10.1371/journal.pgen.1002647.

64. Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, et al. (2003) GATA4 mutations cause human congenital heart defects and reveal an interaction with TBR5. Nature 421: 443–447.

65. Tucker KE, Berciano MT, Jacobs EY, LePage DF, Shpargel KB, et al. (2001) Residual Cajal bodies in coilin knockout mice fail to recruit Sm snRNPs and SMN, the spinal muscular atrophy gene product. J Cell Biol 154: 293–307.

66. Shechter D, Dormann HL, Allis CD, Hake SB (2007) Extraction, purification and analysis of histones. Nat Protoc 2: 1445–1457.

67. Hebert MD, Szymczyk PW, Shpargel KB, Matera AG (2001) Coilin forms the bridge between Cajal bodies and SMN, the spinal muscular atrophy protein. Genes Dev 15: 2720–2729.

68. Rahl PB, Lin CY, Seila AC, Flynn RA, McGuine S, et al. c-Myc regulates transcriptional pause release. Cell 141: 432–445.

69. Argentin S, Ardati A, Tremblay S, Lihrmann I, Robitaille L, et al. (1994) Developmental stage-specific regulation of atrial natriuretic factor gene transcription in cardiac cells. Mol Cell Biol 14: 777–790.

70. Gallardo T, Shirley L, John GB, Castillo DH (2007) Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre. Genesis 45: 413–417.

71. Chandler RL, Chandler KJ, McFarland KA, Mortlock DP (2007) Bmp2 transcription in osteoblast progenitors is regulated by a distant 3' enhancer located 156.3 kilobases from the promoter. Mol Cell Biol 27: 2934–2951.

72. Xu J, Deng X, Watkins R, Dieste CM (2008) Sex-specific differences in expression of histone demethylases Utx and Uty in mouse brain and neurons. J Neurosci 28: 4521–4527.