UTX regulates mesoderm differentiation of embryonic stem cells independent of H3K27 demethylase activity

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To investigate the role of histone H3K27 demethylase UTX in embryonic stem (ES) cell differentiation, we have generated *UTX* knockout (KO) and enzyme-dead knock-in male ES cells. Deletion of the X-chromosome-encoded *UTX* gene in male ES cells markedly decreases expression of the paralogous *UTY* gene encoded by Y chromosome, but has no effect on global H3K27me3 level, *Hox* gene expression, or ES cell self-renewal. However, *UTX* KO cells show severe defects in mesoderm differentiation and induction of *Brachyury*, a transcription factor essential for mesoderm development. Surprisingly, UTX regulates mesoderm differentiation and *Brachyury* expression independent of its enzymatic activity. UTY, which lacks detectable demethylase activity, compensates for the loss of UTX in regulating *Brachyury* expression. UTX and UTY bind directly to *Brachyury* promoter and are required for Wnt/β-catenin signaling-induced *Brachyury* expression in ES cells. Interestingly, male *UTX* KO embryos express normal levels of *UTY* and survive until birth. In contrast, female *UTX* KO mice, which lack the *UTY* gene, show embryonic lethality before embryonic day 11.5. Female *UTX* KO embryos show severe defects in both *Brachyury* expression and embryonic development of mesoderm-derived posterior notochord, cardiac, and hematopoietic tissues. These results indicate that UTX controls mesoderm differentiation and *Brachyury* expression independent of H3K27 demethylase activity, and suggest that UTX and UTY are functionally redundant in ES cell differentiation and early embryonic development.

In vitro, embryonic stem (ES) cells are capable of differentiating into three germ layers, ectoderm, endoderm, and mesoderm, which mimics the early stage of embryonic development in vivo (1). Transcription factor Brachyury (T) is highly expressed in the primitive streak during gastrulation and is required for mesoderm formation (2). Mutation of *Brachyury* gene in mice causes defective formation of posterior mesoderm, failure of notochord morphogenesis, and embryonic death around 10 d of gestation (2). *Brachyury* expression is directly regulated by Wnt/β-catenin signaling in mesoderm and in ES cells. Wnt/β-catenin signaling promotes de-phosphorylation of β-catenin, which enters nucleus and binds transcription factor LEF1/TCF1 to activate *Brachyury* expression (3, 4). Brachyury also positively regulates Wnt/β-catenin signaling. Such a positive auto-regulatory loop between Brachyury and Wnt/β-catenin signaling maintains the mesodermal progenitor cells and is essential for posterior mesoderm development in vertebrates (5).

The Polycomb Repressive Complex 2 (PRC2) is critical for the proper differentiation of ES cells. PRC2 is localized on a large number of developmental regulator genes in ES cells. Disruption of PRC2 in ES cells markedly decreases the global levels of H3K27 di- and trimethylation (H3K27me2 and H3K27me3) and leads to up-regulation of many developmental regulator genes (6, 7).

UTX belongs to a subfamily of JmjC domain-containing proteins that also includes UTY and JMJD3. Previously, we and others identified UTX and Jmjd3 as histone H3K27 demethylases (8–12). *UTX* gene is located on the X chromosome, whereas *UTY* gene is on the Y chromosome and thus only expressed in male cells. UTY is a paralog of the X-linked UTX and shares 88% sequence homology with UTX protein. Unlike UTX, UTY lacks detectable histone demethlase activity in vitro (8, 12). The viability data from male and female *UTX* KO mice indicate a largely functional redundancy between UTX and UTY during male embryonic development (13).

UTX has been shown to regulate myocyte differentiation, heart development, and T-box transcription factor target gene expression (13–15). However, UTX functions in ES cell differentiation and early embryonic development are unclear. Using *UTX* KO, conditional KO, and enzyme-dead knock-in ES cells, here we show that UTX, but not its H3K27 demethylase activity, is required for ES cell differentiation into mesoderm. Mechanistically, UTX and UTY are redundant and they directly control the induction of *Brachyury*, a master regulator of mesoderm differentiation. Consistently, female *UTX* KO ($UTX^{-/-}$) embryos, but not male *UTX* KO ($UTX^{-/Y}$) ones that express *UTY* normally, show severe defects in *Brachyury* expression and mesoderm development.

Results

Generation of *UTX* KO, Conditional KO, and Enzyme-Dead Knock-In Male ES Cell Lines. Most ES cell lines including the ones used here are male and carry one allele of *UTX* and one allele of *UTY*. To investigate the role of UTX in ES cell differentiation and animal development, we established male *UTX* conditional KO (flox) ES cell lines by gene targeting (Fig. 1*A*). Exon 24 was flanked by two *lox*P sites in the flox allele. Deletion of exon 24 from the flox allele by Cre recombinase resulted in the KO allele. His1146 (H1146) and Glu1148 (E1148) encoded by exon 24 are essential for UTX demethylase activity (11, 16). To identify UTX functions that depend on its demethylase activity, we also generated the enzyme-dead knock-in (KI) allele by mutating H1146 and E1148 of endogenous UTX to Ala (Fig. 1*A*). The genotypes of flox ($UTX^{flox/Y}$; Y stands for UTY), KI ($UTX^{KI/Y}$), and KO ($UTX^{-/Y}$) cell lines were confirmed by genomic PCR (Fig. S1 *A* and *B*). The H1146A and E1148A mutations in the KI allele were confirmed by sequencing (Fig. S1*C*). UTX was expressed at similar levels in flox and KI cells but absent in KO cells (Fig. 1*B*). Histone demethylase assay in vitro confirmed that H1146 and E1148 were essential for UTX demethylase activity (Fig. S1*D*). Endogenous UTX from the KI ES cells was also deficient in

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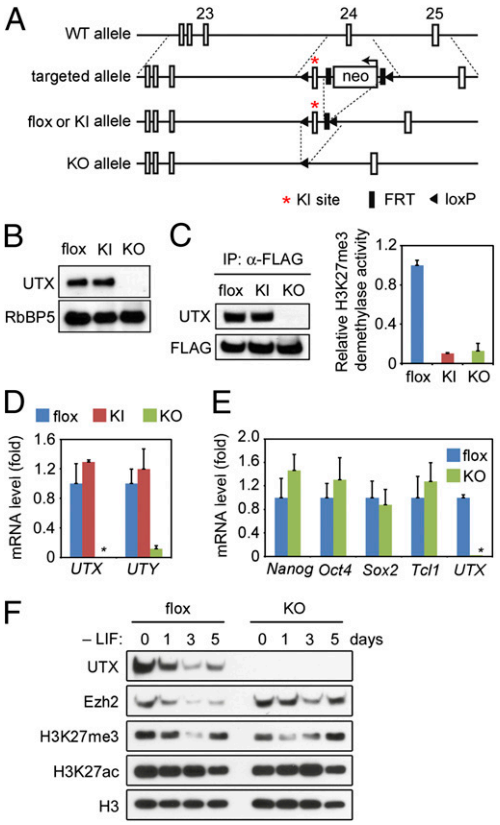

Fig. 1. Characterization of *UTX* KO, conditional KO, and knock-in male ES cells. (*A*) Schematic representation of mouse *UTX* wild-type (WT) allele, targeted allele, conditional KO (flox) allele, enzyme-dead knock-in (KI) allele, and KO allele. Deletion of neo selection cassette from the targeted allele by FLP recombinase generates the flox allele. Deletion of exon 24 from the flox allele by Cre generates the KO allele. The KI allele carries H1146A and E1148A mutations (represented by the red asterisk) in exon 24. (*B*) Immunoblotting of nuclear extracts prepared from flox ($UTX^{flox/Y}$; Y stands for UTY), KI ($UTX^{KI/Y}$) and KO ($UTX^{-/Y}$) ES cells using indicated antibodies. (*C*) Endogenous UTX in KI ES cells is deficient for H3K27 demethylase activity. Nuclear extracts were prepared from flox, KI and KO cells stably expressing FLAG-tagged PA1, a unique subunit of the MLL3/4 histone methyltransferase complex that strongly associates with UTX (11, 32). Endogenous UTX was pulled down from the nuclear extracts by affinity purification of MLL3/4 complex using anti-FLAG antibody as described (32), followed by a demethylase assay using the JMJD3/UTX demethylase activity assay kit (Epigenase no. P-3084). (*Left*) Immunoblotting of immunoprecipitated endogenous UTX and FLAG-PA1. (*Right*) Demethylase assay. (*D*) UTX, but not its demethylase activity, controls *UTY* expression in male ES cells. *UTX* and *UTY* mRNA levels were analyzed by qRT-PCR. The absence of *UTX* expression in KO cells was indicated by an asterisk. (*E*) UTX is dispensable for self-renewal of ES cells. Expression of markers for undifferentiated ES cells was analyzed by qRT-PCR. (*F*) Deletion of *UTX* has little effect on the global H3K27me3 level during ES cell differentiation. flox and KO ES cells were cultured without LIF for 0, 1, 3, or 5 d. Nuclear extracts were analyzed by immunoblotting. Quantitative PCR data in all figures are presented as means ± SD.

H3K27 demethylase activity (Fig. 1*C*). Quantitative reverse-transcriptase PCR (qRT-PCR) confirmed deletion of exon 24 in KO cells. Interestingly, *UTY* mRNA levels were similar in flox and KI cells but decreased markedly in *UTX* KO cells, indicating that in male ES cells, UTX protein, but not its enzymatic activity, controls *UTY* expression (Fig. 1*D*).

Deletion of *UTX* and the subsequent loss of *UTY* did not affect expression of transcription factors critical for ES cell self-renewal, including Nanog, Oct4, Sox2, and Tcl1 (17) (Fig. 1*E*). The absence of UTX and UTY in ES cells had little effects on the population doubling time and colony-formation ability either (Fig. S2 *A* and *B*). Moreover, alkaline phosphatase, a marker for undifferentiated ES cells, was expressed at similar levels in flox and KO cells (Fig. S2*C*). Removing the cytokine LIF from medium would initiate spontaneous differentiation of ES cells cultured in the absence of fibroblast feeder cells (18). Alkaline phosphatase staining showed that, compared with flox cells, KO cells formed similar amount of differentiated colonies after LIF removal (Fig. S2*C*). These results indicate that UTX and UTY are dispensable for ES cell self-renewal and initial differentiation potential.

We also examined global H3K27me3 and H3K27ac levels during spontaneous differentiation of *UTX* KO cells. Consistent with a previous report (19), both Ezh2 and H3K27me3 levels decreased during spontaneous differentiation of ES cells. However, deletion of *UTX* did not lead to obvious changes of the global levels of H3K27me3 and H3K27ac (Fig. 1*F*). Interestingly, UTX protein level decreased along with that of Ezh2, suggesting that the reduced H3K27me3 level in differentiated cells is due to reduced Ezh2 level.

UTX has been reported to regulate *Hox* genes as well as genes encoding Rb-binding proteins (10–12, 20). However, we found that the loss of both UTX and UTY in ES cells did not affect retinoic acid (RA)-induced *Hoxb1* expression, constitutive expression of *Hoxb7*, or expression of genes encoding Rb-binding proteins such as HBP1 and RbBP6 (Fig. S3 *A*–*C* and *E*). Similar results were obtained from mouse embryonic fibroblasts (MEFs) (Fig. S3 *D* and *F*). Thus, UTX and UTY are dispensable for expression of *Hox* genes and genes encoding Rb-binding proteins in ES cells and MEFs.

UTX Controls Mesoderm Differentiation of ES Cells Independent of H3K27 Demethylase Activity. Next, we investigated whether UTX was required for ES cell differentiation using well established protocols (7, 19, 21–23). Cells were cultured on Petri dishes in the absence of LIF to form 3D multicellular aggregates called embryoid bodies (EBs). Differentiation of EBs recapitulates the generation of all three primary germ layers, ectoderm, mesoderm, and endoderm, during embryonic gastrulation (1). EBs were treated with RA to promote ES cells to differentiate along the neural ectoderm lineage and become neuron precursor (NP) cells expressing Nestin and Tubb3 (β-III Tubulin) (21). Immunostaining revealed robust expression of Tubb3 in both flox and KO cells (Fig. S4*A*). By examining gene expression before and after differentiation, we observed marked decreases of ES cell markers *Nanog* and *Oct4* and marked increases of NP markers *Nestin* and *Tubb3* in both flox and KO cells (Fig. S4*B*). These results indicate that UTX is dispensable for RA-induced ES cell differentiation toward NP cells.

To investigate whether UTX was required for mesoderm and endoderm differentiation of ES cells, EBs were cultured for 12 d in the absence of RA (23), because RA strongly inhibits mesoderm differentiation (5). As expected, flox cells formed cystic EBs starting from day 8. In contrast, KO cells formed much smaller EBs with no obvious cystic morphology, indicating a severe differentiation defect (Fig. 2*A*). Consistent with the morphologies, during differentiation of the flox cells, expression of ES cell markers *Nanog* and *Oct4* decreased markedly while expression of mesoderm markers *Brachyury* and *Wnt3* increased markedly and reached the peak levels at day 4 and then dropped. In contrast, KO EBs retained *Nanog* and *Oct4* expression and failed to induce *Brachyury* and *Wnt3* during differentiation. The induction of endoderm markers *Gata4* and *Gata6* was largely unaffected by *UTX* deletion (Fig. 2*B*). The same sets of experiments were repeated using the KI cells carrying the enzyme-dead *UTX* allele. Surprisingly, the KI cells pheno-copied the flox ones, suggesting that the enzymatic activity of UTX is dispensable for mesoderm differentiation of ES cells (Fig. 2 *A* and *B*). Together, these results indicate that UTX controls mesodermal differentiation of ES cells independent of its H3K27 demethylase activity.

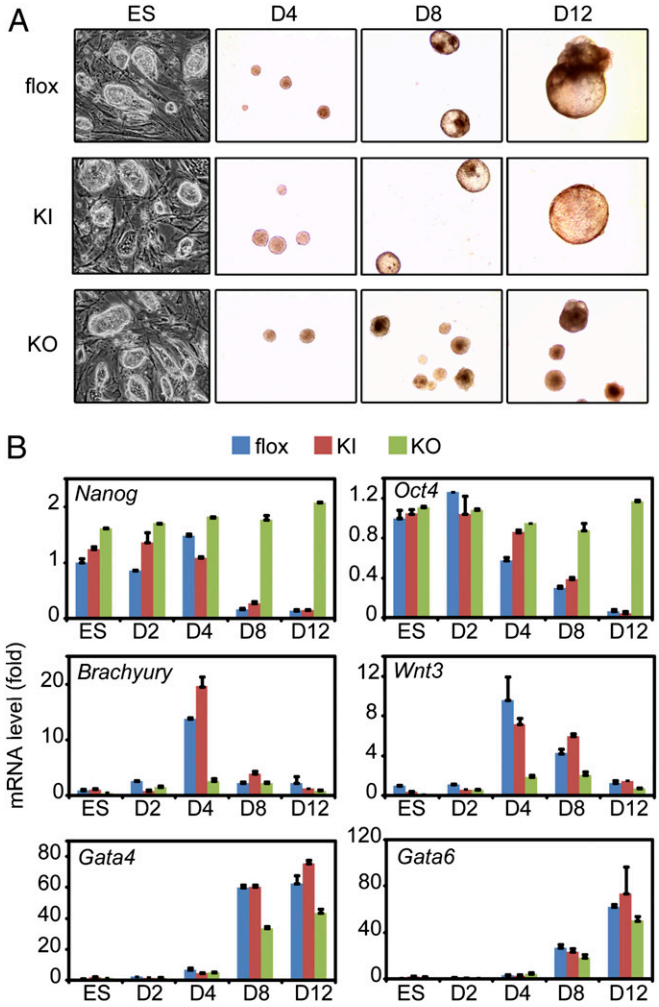

Fig. 2. UTX controls mesodermal differentiation of ES cells independent of H3K27 demethylase activity. (*A*) Microscopic pictures of flox, KI, and KO ES cells before differentiation and EBs at day 4, day 8, and day 12 of differentiation. (*B*) qRT-PCR analysis of ES cell markers (*Nanog*, *Oct4*), mesoderm markers (*Brachyury*, *Wnt3*) and endoderm markers (*Gata4*, *Gata6*) at indicated time points of differentiation. D2, D4, D8, and D12 refer to day 2, 4, 8, and 12, respectively.

***Brachyury* Is a Direct UTX Target Gene in ES Cells.** To understand the mechanism underlying the defective mesoderm differentiation in *UTX* KO cells, we compared gene expression profiles between flox and KO cells. Microarray analysis revealed that deletion of *UTX* in ES cells led to over 2.5-fold decreased expression of 141 genes (Table S1). Gene ontology analysis revealed that 52 genes were associated with developmental process, which was the most significant functional category (Fig. 3*A*). qRT-PCR confirmed that deletion of *UTX* decreased expression of developmental genes such as *Brachyury*, *Fgf5*, *Hmga2*, *Pitx2*, *Wnt3*, and *Wt1* in undifferentiated ES cells. However, expression of ES cell markers *Nanog* and *Oct4* and endoderm markers *Gata6* and *Hnf4a* was largely unaffected (Fig. 3*B*). Although expression of *Brachyury* and *Wnt3* decreased over threefold in the KO cells, expression of *Nodal*, another key regulator of mesoderm differentiation, was not affected by *UTX* deletion. By chromatin immunoprecipitation (ChIP) assays in flox and KO cells, we observed specific enrichment of endogenous UTX on the proximal promoter of *Brachyury* but not any other genes examined (Fig. 3*C*). Thus, UTX directly and selectively regulates *Brachyury* gene in ES cells. Because Brachyury is essential for mesoderm formation, the identification of *Brachyury* as a direct target gene of UTX provides a possible mechanism by which UTX controls mesoderm differentiation of ES cells.

UTX and UTY Are Required for Wnt/β-Catenin Signaling-Induced *Brachyury* Expression. Because *Brachyury* is a direct target gene of Wnt/β-catenin signaling (3, 4), we treated ES cells with BIO, a small molecule inhibitor of GSK3β kinase and a robust activator of Wnt/β-catenin signaling, to induce *Brachyury* expression (24). Deletion of *UTX* in ES cells severely impaired BIO-induced *Brachyury* expression. In contrast, BIO-induced *Brachyury* expression was normal in the KI cells (Fig. 4*A*), indicating that UTX protein, but not its demethylase activity, is required for Wnt/β-catenin signaling-induced *Brachyury* expression in ES cells.

ChIP assays revealed UTX enrichment in the region surrounding the Wnt/β-catenin-responsive TCF1/LEF1 binding site on *Brachyury* promoter (Fig. 4 *B* and *C*). BIO treatment increased UTX

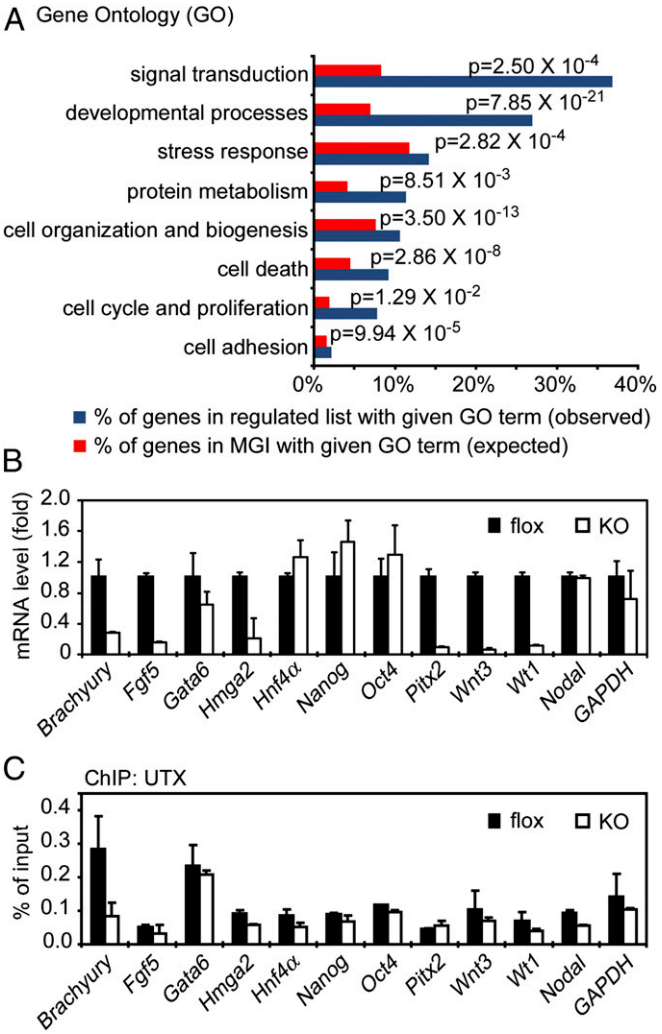

Fig. 3. *Brachyury* is a direct UTX target gene in ES cells. (*A*) Gene ontology (GO) analysis of down-regulated genes identified in microarray. Genes with over 2.5-fold decrease in *UTX* KO cells compared with flox cells were subjected to GO analysis. Dark blue bars represent the observed percentage of down-regulated genes in a particular GO category. Red bars represent the expected percentage in all GO-annotated genes among the entire mouse genome recorded in the MGI database. *P* value of each term was determined using Fisher's exact test. (*B*) Confirmation of microarray data by qRT-PCR. (*C*) ChIP assays of UTX on proximal promoters of the indicated genes in flox and KO ES cells.

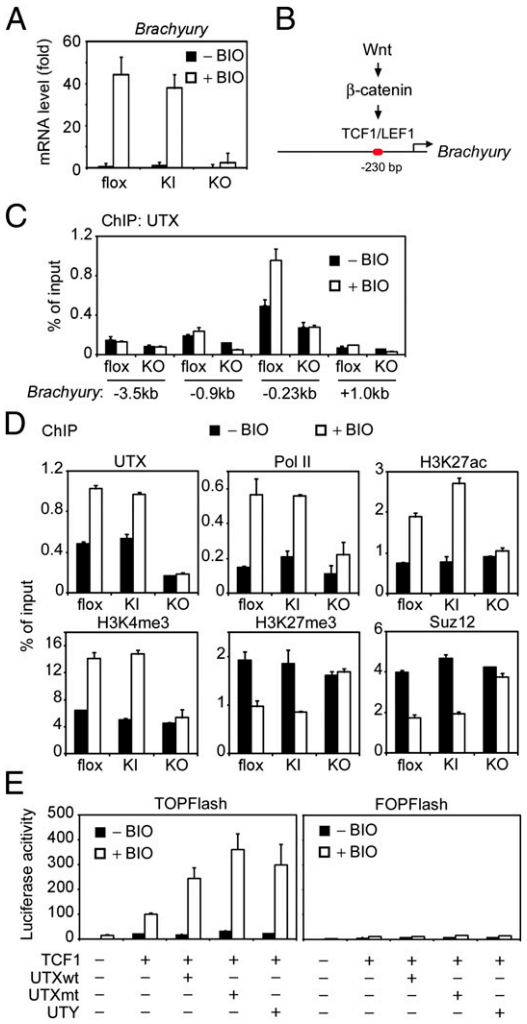

Fig. 4. UTX is required for Wnt/β-catenin signaling-induced *Brachyury* expression. (*A–D*) flox, KI, and KO ES cells were passaged twice on 0.1% gelatin-coated dishes to remove feeder cells and then cultured on gelatin-coated dishes. Cells were treated with 2 μM BIO for 48 h in the absence of LIF. (*A*) UTX, but not its H3K27 demethylase activity, is required for BIO-induced *Brachyury* expression in ES cells. *Brachyury* expression was determined by qRT-PCR. (*B*) Schematic representation of *Brachyury* proximal promoter containing the Wnt/β-catenin signaling-responsive TCF1/LEF1 binding site. (*C*) ChIP assays of UTX on the indicated regions of *Brachyury* gene. (*D*) ChIP assays of levels of UTX, RNA polymerase II (Pol II), indicated histone modifications, and the Suz12 subunit of PRC2, around the TCF1/LEF1 binding site on *Brachyury* proximal promoter. (*E*) UTX and UTY function as TCF1 coactivators independent of demethylase activity. HeLa cells were transfected with 30 ng of TCF1 plasmid, 30 ng of TOPFlash or FOPFlash, and 150 ng of pCS2 plasmids expressing wild-type (UTXwt) or enzyme-dead (H1146A and E1148A) mutant UTX (UTXmt) or UTY. Twenty-four hours later, cells were treated with 2 μM BIO for 24 h, followed by luciferase assays using dual-luciferase assay kit (Promega).

level on *Brachyury* promoter in flox and KI cells but not in KO cells (Fig. 4*D*). Consistent with the BIO-induced *Brachyury* expression in Fig. 4*A*, BIO induced enrichment of RNA polymerase II (Pol II) as well as positive histone marks H3K27ac and H3K4me3 on the *Brachyury* promoter in flox and KI cells but not in KO cells. Interestingly, BIO attenuated H3K27me3 level on *Brachyury* promoter in flox and KI cells but not in KO cells, which was likely due to the loss of PRC2 (represented by the Suz12 subunit) on the same promoter (Fig. 4*D*). These results suggest that UTX directly regulates Wnt/β-catenin signaling-induced *Brachyury* expression in ES cells independent of H3K27 demethylase activity.

Next, we asked whether the enzymatically inactive UTY could compensate for the loss of UTX in ES cells. Because endogenous UTY level is dramatically reduced in *UTX* KO ES cells, we stably expressed human UTY in the KO cells (Fig. S5*A*). Interestingly, ectopic expression of UTY in *UTX* KO cells rescued not only BIO-induced *Brachyury* expression (Fig. S5*B*), but also BIO-induced increases of Pol II, H3K27ac, and H3K4me3 and decreases of H3K27me3 and Suz12, on *Brachyury* promoter (Fig. S5*C*). Furthermore, BIO induced UTY enrichment to the same region that UTX bound on *Brachyury* promoter (Fig. S5*C*).

We next tested whether UTX and UTY could regulate TCF1 transcription activity using β-catenin responsive luciferase reporter TOPFlash containing seven repeats of TCF/LEF binding sites, with FOPFlash as the negative control. We found that not only wild-type and enzyme-dead UTX, but also UTY, were able to enhance BIO-induced TCF1 transcription activity (Fig. 4*E*). Collectively, these results suggest that UTX and UTY function as TCF1 coactivators to promote Wnt/β-catenin signaling-induced *Brachyury* expression in ES cells independent of H3K27 demethylase activity.

UTX Is Required for *Brachyury* Expression and Mesoderm Development in Mice. To verify the ES cell results, we knocked out *UTX* gene in mice. Because of the early embryonic lethality in female *UTX* KO mice (see below), we first generated male KO mice ($UTX^{-/Y}$) by crossing male wild-type ($UTX^{+/Y}$) with female $UTX^{+/-}$ mice (Fig. S6*A*). Male *UTX* KO mice could survive until the end of embryonic development but died around birth, due to neuron tube closure defect and inability to breathe (Fig. S6 *B* and *C*). Thus, deletion of the single *UTX* allele in male mice did not affect mesoderm development. Unlike in the male ES cells, deletion of *UTX* in male embryos did not affect *UTY* expression (Fig. S6*D*), suggesting a functional redundancy between UTX and UTY in regulating mesoderm development in male embryos.

Next, we deleted both alleles of *UTX* in female mice where *UTY* gene is absent. Because of the perinatal lethality of male *UTX* KO mice, female $UTX^{flox/flox}$ mice were crossed with male *Prm1*-Cre mice expressing Cre under the control of sperm-specific *Prm1* promoter. The resulting male $UTX^{flox/Y}$;*Prm1*-Cre mice, which had *UTX* deletion in the sperms, were crossed with female $UTX^{+/-}$ to generate female *UTX* KO ($UTX^{-/-}$) embryos (Fig. 5*A*). Consistent with a recent report (13), $UTX^{-/-}$ female embryos were not viable after embryonic day E10.5, although degenerated dead $UTX^{-/-}$ embryos were found at E11.5 (Fig. 5). The vast majority of E 9.5–10.5 $UTX^{-/-}$ embryos displayed abnormal or truncated posterior bodies (Fig. 5 *B* and *C* and Fig. S6*F*), which pheno-copied homozygous *Brachyury* mutant mice (25). In the remaining two E9.5 $UTX^{-/-}$ embryos with relatively intact posterior body formation, we observed markedly reduced *Brachyury* expression (Fig. 5*B* and Fig. S6*E*).

In E10.5 wild-type embryos, red blood cells were visible in Aorta-Gonad-Mesonephros, where early hematopoietic stem cells arise (26). In contrast, anemia was observed in $UTX^{-/-}$ embryos, suggesting a critical role of UTX in hematopoiesis (Fig. 5*C*). The majority of $UTX^{-/-}$ embryos also displayed severe defects in heart development and neural tube closure (Fig. 5*C*). In contrast, male *UTX* KO littermates ($UTX^{-/Y}$) at E 9.5–10.5 were morphologically normal except for the neural tube closure defect in some of them (Fig. 5*C*), suggesting that UTX and UTY are largely redundant in regulating mesoderm development. Taken together, these results indicate that UTX is required for *Brachyury* expression and mesoderm development in mice.

Discussion

In this paper, we show UTX controls *UTY* expression in ES cells independent of its enzymatic activity. Deletion of *UTX* and the

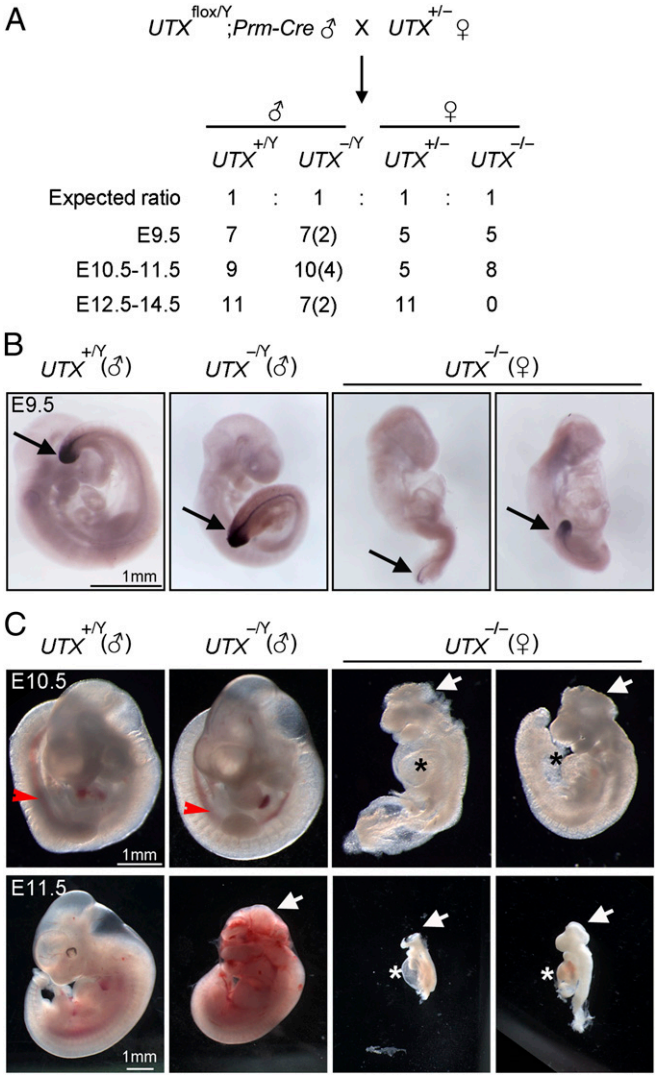

Fig. 5. UTX is required for *Brachyury* expression and mesoderm development in mice. (*A*) The mating strategy for generating female *UTX* KO embryos ($UTX^{-/-}$) and the genotyping result. The numbers of embryos with execenphaly are indicated in the parentheses. (*B*) Decreased *Brachyury* expression in female but not male *UTX* KO E9.5 embryos. *Brachyury* was detected by WISH. Black arrows indicate the tail bud and posterior notochord where *Brachyury* is expected to express. (*C*) $UTX^{-/-}$ female embryos show defects in neural tube closure, cardiac development and hematopoiesis. Representative images of E10.5 and E11.5 embryos with indicated genotypes are shown. Abnormal or truncated posterior notochords were observed in $UTX^{-/-}$ embryos. Red arrowheads indicate red blood cells in Aorta-Gonad-Mesonephros, which were absent in $UTX^{-/-}$ embryos. Asterisks indicate cardiac effusion in $UTX^{-/-}$ embryos, represented by swollen pericardial sacs. White arrows point to the defects in neural tube closure.

subsequent loss of *UTY* do not affect global H3K27me3 level, *Hox* gene expression, or ES cell self-renewal. However, UTX, but not its enzymatic activity, is essential for mesoderm differentiation and *Brachyury* expression in ES cells. Unlike in the ES cells, UTX is dispensable for *UTY* expression in mice. UTX and the enzymatically inactive UTY are functionally redundant in early embryonic development. Female *UTX* KO mice, which lack *UTY* gene, show severe defects in *Brachyury* expression and mesoderm development. Together, these results indicate an essential role of UTX protein, but not its H3K27 demethylase activity, in regulating mesoderm development and *Brachyury* expression.

Regulation of ES Cell Differentiation by UTX. Recently, Lee et al. reported that UTX is dispensable for ES cell self-renewal and that UTX is required for *UTY* expression in ES cells but not in embryos (13), which are consistent with our results. We further demonstrate that UTX controls *UTY* expression independent of its enzymatic activity. However, the essential role of UTX in mesoderm differentiation and *Brachyury* expression in ES cells was not observed by Lee et al. (13). The reason of this discrepancy is that we induce EB differentiation toward mesoderm in the absence of RA (21, 22), but Lee et al. included RA. RA has been shown to strongly inhibit mesoderm differentiation by downregulating *Brachyury* expression (5). Consistently, we found that RA strongly inhibits *Brachyury* expression during ES cell differentiation (Fig. S7). Our ES cell data are highly consistent with our observation on the essential role of UTX in mesoderm development and *Brachyury* expression in mice.

Regulation of *Brachyury* Expression by UTX. Our findings that *Brachyury* is a direct UTX target gene and that UTX is required for Wnt/β-catenin signaling-induced *Brachyury* expression in ES cells provide a mechanism to explain the essential role of UTX for ES cell differentiation toward mesoderm. These results are also consistent with recent reports that Wnt/β-catenin signaling is dispensable for the self-renewal of ES cells cultured under appropriate conditions (i.e., on feeder cells in the presence of LIF) and that Wnt/β-catenin signaling appears to be selectively required for mesoderm formation during ES cell differentiation (27).

We confirmed a previous report that BIO-induced *Brachyury* expression is accompanied by decreases of PRC2 and PRC2-mediated H3K27me3 on *Brachyury* promoter (24). However, BIO treatment decreases H3K27me3 on *Brachyury* promoter in both flox and KI cells but not in KO cells, indicating that UTX regulates Wnt/β-catenin signaling-induced *Brachyury* expression independent of its H3K27 demethylase activity. Because PRC2 and H3K27me3 are dispensable for BIO-induced *Brachyury* expression in ES cells (24), there is no need to get the H3K27 demethylase activity involved to activate *Brachyury* expression. The BIO-induced decrease of H3K27me3 on *Brachyury* promoter could be due to another H3K27 demethylase, Jmjd3, which is recruited to the *Brachyury* promoter upon BIO treatment (24). However, it remains unclear whether Jmjd3 is required for BIO-induced *Brachyury* expression.

UTX has been shown to function as a transcription coactivator and recruit Brg1 chromatin remodeling complex to facilitate promoter accessibility of target genes in T cells and cardiac myocytes (13, 14). Whether UTX recruits Brg1 complex to *Brachyury* promoter remains to be investigated. Consistent with the coactivator role of UTX, UTX is required for Pol II recruitment to *Brachyury* promoter.

Regulation of Early Embryonic Development by UTX and UTY. Mutation of *Brachyury* in mice leads to abnormal posterior notochord organization and posterior truncation (25). Consistent with the requirement of UTX for Wnt/β-catenin signaling-induced *Brachyury* expression in ES cells, $UTX^{-/-}$ embryos show posterior truncation and decreased *Brachyury* expression. Interestingly, knockdown of *UTX* in zebrafish also leads to truncated posterior notochord and abnormal posterior structure, indicating defective dorsal mesoderm formation (12). Together, these results suggest an evolutionally conserved role of UTX in mesoderm development.

In addition to previously reported defects in heart development in female *UTX* KO mice and neural tube closure defects in both female and male *UTX* KO mice (13, 28), we observed anemia in female *UTX* KO mice. Because both hematopoietic cells and cardiac myocytes are derived from mesoderm (1), the defective mesoderm development likely contributes to anemia and impaired heart development in $UTX^{-/-}$ embryos. Whether UTX

plays direct, tissue-specific roles in regulating hematopoiesis and heart development will require deletion of *UTX* in a tissue-specific manner. It is possible that UTX regulates expression of other Wnt target genes, which may also contribute to the regulation of mesoderm development. However, Brachyury is the master regulator of mesoderm development. Regulation of *Brachyury* expression by UTX likely represents the major mechanism for the role of UTX in mesoderm development.

Our data suggest that UTX and UTY are redundant and regulate early embryonic development independent of demethylase activities. Highly consistent with our result, a very recent paper reports that *Caenorhabditis elegans* UTX is essential for normal development in a demethylase activity independent manner (29). However, the fact that female $UTX^{+/-}$ mice appear normal whereas male $UTX^{-/Y}$ mice show perinatal lethality suggests distinct functions of UTX and UTY in peri-/postnatal development. Given UTY lacks detectable demethylase activity (8, 12), it is possible that the H3K27 demethylase activity of UTX may be involved in late embryonic and/or postnatal development. Generating the enzyme-dead *UTX* KI mice will help understand the physiological roles of UTX demethylase activity.

Materials and Methods

Plasmids, Antibodies, Chemicals, Cell Culture, and Differentiation. The details are described in *SI Materials and Methods*. $UTX^{flox/flox}$ primary MEFs were isolated from E12.5 $UTX^{flox/flox}$ female embryos. 3T3 immortalization and retroviral infection of MEFs were done as described (30).

Generation of *UTX* KO, flox, and KI ES Cell Lines. The design of gene targeting constructs and the generation and genotyping of *UTX* KO, flox, and KI ES cell lines are described in *SI Materials and Methods* (also see Fig. 1*A* and Fig. S1*A*). For each of the flox, KO, and KI ES cell lines, three individual colonies were identified by PCR screen and used for characterization and differentiation experiments.

Generation of *UTX* KO and Conditional KO Mice. Generation of UTX conditional KO male ($UTX^{flox/Y}$) and female ($UTX^{flox/flox}$) mice, as well as the mating strategies to generate *UTX* KO male ($UTX^{-/Y}$) and female ($UTX^{-/-}$) mice, are described in *SI Materials and Methods* (also see Fig. S6*A* and Fig. 5*A*). All mouse work was approved by the Animal Care and Use Committee of National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

Microarray, qRT-PCR, ChIP, Immunoblotting, Immunofluorescence, and WISH. For microarray analysis, flox and KO ES cells were passaged twice on 0.1% gelatin-coated dishes to remove feeder cells and then cultured in ES medium on gelatin-coated dishes. Total RNAs were purified and analyzed on Mouse Genome 430 2.0 Array (Affymetrix) as described (31). Data are deposited in National Center for Biotechnology Information GEO database (accession no. GSE35415). Gene ontology (GO) analysis of genes that show decreased expression in KO cells was done using the MGI Slim Chart Tool (http://www.informatics.jax.org/gotools/MGI_GO_Slim_Chart.html). qRT-PCR with 18S rRNA as control and ChIP with PCR quantitation using SYBR Green kit were performed as described (30). Data are presented as means ± SD. The sequences of SYBR Green primers are listed in Table S2. Immunoblotting of nonhistone proteins and immunofluorescence staining were done as described (8). Immunoblotting of histone modifications was done using acid extracted histones (30). Whole-mount in situ hybridization (WISH) of E9.5 mouse embryos was done using a *Brachyury* probe from Chuxia Deng (NIDDK, NIH, Bethesda, MD).

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