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UTX, a histone H3-lysine 27 demethylase, acts as a critical switch to activate the cardiac developmental program

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SUMMARY

The removal of histone H3 lysine27 (H3K27) trimethylation mark is important for the robust induction of many cell type-specific genes during differentiation. Here we show that UTX, a H3K27 demethylase, acts as a critical switch to promote a cardiac-specific gene program. UTX-deficient ESCs failed to develop heart-like rhythmic contractions under a cardiac differentiation condition. UTX deficient mice show severe defects in heart development and embryonic lethality. We found that UTX is recruited to cardiac-specific enhancers by associating with core cardiac transcription factors and demethylates H3K27 residues in cardiac genes. In addition, UTX facilitates the recruitment of Brg1 to the cardiac-specific enhancers. Together, our data reveal key roles for UTX in a timely transition from poised to active chromatin in cardiac genes during heart development and a fundamental mechanism by which a H3K27 demethylase triggers tissue-specific chromatin changes.

Keywords

UTX; KDM6A; histone demethylase; H3K27; histone; chromatin; transcription; heart; development

INTRODUCTION

In embryonic stem cells (ESCs), many developmental genes exhibit ‘poised status’ marked by bivalent trimethylation of both histone H3-lysine 27 (H3K27me3) and H3-lysine 4 (H3K4me3) (Bernstein et al., 2006; Pan et al., 2007; Rada-Iglesias et al., 2011; Zhao et al., 2007). Given that the H3K27me3 mark is important to repress many developmental genes in self-renewing ESCs (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006; Lee et al., 2006b; Pan et al., 2007; Zhao et al., 2007), the removal of H3K27me3 in a cohort of tissue-specific genes is likely an important step in organogenesis. Despite recent progress in

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understanding the changes of histone post-translational modifications during ESC differentiation, how histone modifying enzymes make specific changes in a battery of lineage-specific genes on the right time and space during development are poorly understood. In particular, the identity of the histone H3K27 demethylases that control cardiac gene expression and the mechanisms by which they are recruited to cardiac-specific enhancers remain unclear. Addressing these questions would resolve a fundamental issue of how poised developmental genes exit the transcriptionally purgatory state to achieve tissue-specific gene expression during organogenesis.

Recent studies have identified UTX (also known as KDM6A), UTY and JMJD3 (also known as KDM6B) as H3K27 demethylases belonging to a family of JmjC-domain-containing proteins (Agger et al., 2007; De Santa et al., 2007; Hong et al., 2007; Jepsen et al., 2007; Lan et al., 2007; Lee et al., 2007). UTX and JMJD3 displayed specific catalytic activity in demethylating H3K27me3/me2, whereas UTY was inactive under the same assay conditions (Hong et al., 2007; Lan et al., 2007). Interestingly, UTX and UTY constitute a large complex that contains multiple subunits including H3K4 methyltransferase MLL3 or MLL4 (Cho et al., 2007; Goo et al., 2003; Issaeva et al., 2007; Lee et al., 2007; Lee et al., 2006a). It is unclear whether JMJD3 forms a steady state complex like UTX. The *UTX* gene is encoded on the X chromosome, but escapes X-chromosome inactivation (Greenfield et al., 1998). The *UTY* gene, the male counterpart of *UTX*, is localized on the Y-chromosome. Unlike *UTX* and *UTY*, the *JMJD3* gene is encoded in an autosome. Notably, UTX is required for *Hox* gene activation and posterior development in *zebrafish* embryos (Agger et al., 2007; Lan et al., 2007; Lee et al., 2007), suggesting roles of UTX in development. However, the function of H3K27 demethylases during cell fate determination and organogenesis remains unknown.

To gain insight into the developmental roles of UTX, we generated UTX-deficient ESCs and mice, and found that UTX is essential for heart development. Our study also uncovered the molecular basis underlying the action of UTX in inducing cardiac genes during heart development. Overall, our results provide important insights into how the histone modifying enzymes orchestrate the establishment of transcriptionally active chromatin in appropriate lineage-specific genes during organogenesis.

RESULTS

UTX is highly expressed in the developing embryos

To understand the role of UTX in embryo development, we generated mice bearing $UTX^{F\text{-}loxP}$ allele, in which *β-galactosidase* gene was inserted into the *UTX* locus and exon 3 of *UTX* was flanked by *loxP* sequences (Fig. 1A). To examine the expression pattern of UTX in embryos, we performed X-gal staining in $UTX^{F\text{-}loxP/+}$ embryos, in which β-galactosidase expression is controlled by *UTX* regulatory elements, and found that UTX is broadly expressed but is enriched in several tissues. At E8.5, UTX was highly expressed in developing heart, anterior region of the neural tube, ventricular zone of the caudal neural tube, neural crest cells and somites (Fig. 1B, C). At E11.5, UTX was enriched in heart, myotome of somites, limb buds, brachial arches, isthmus, cortex and eyes (Fig. 1D). Cross-sections of X-gal stained embryos revealed a high level of UTX expression in

cardiomyocytes (Fig. 1C, E, F). Consistently, UTX transcripts are highly expressed in cardiomyocytes of E10.5 hearts, as shown by in situ hybridization (Fig. S1). UTY is also expressed in E10.5 hearts (Fig. S1). The expression pattern of UTX suggests possible roles of UTX in organogenesis of several tissues including heart.

UTX is required for ESCs to differentiate to a cardiac lineage

To address the role of UTX during developmental transitions from ESCs to a cardiac cell lineage, we used a well-established protocol to differentiate ESCs to cardiac cell types, which closely mimic in vivo development and provide cells at developmental transitions (Boheler et al., 2002). We first generated $UTX^{F\text{-}\Delta/y}$ ESCs, in which exon 3 of *UTX* is deleted, by inducing a Cre-mediated recombination in $UTX^{F\text{-}loxP/y}$ ESCs (Fig. 1A). Neither UTX mRNA nor UTX protein expression was detected in $UTX^{F\text{-}\Delta/y}$ ESCs (Fig. 2A, data not shown), establishing that $UTX^{F\text{-}\Delta/y}$ ESCs are *UTX*-null. $UTX^{F\text{-}loxP/y}$ ESCs showed reduced levels of UTX mRNA and protein (Fig. 2A, data not shown), indicating that $UTX^{F\text{-}loxP}$ is a *UTX* hypomorphic allele.

UTX-null ESCs were indistinguishable from wild-type ESCs in cell proliferation and survival when cultured with leukemia inhibitory factor (LIF), as monitored by MTT assay and western blotting assays with α-cleaved Caspase3 antibody (Fig. 2B, C). Alkaline phosphatase staining revealed that, like wild-type ESCs, *UTX*-null ESCs self-renew in the presence of LIF, and readily differentiate upon withdrawal of LIF and addition of retinoic acid (RA), which trigger ESC differentiation (Fig. 2D). Consistently, Oct4, a transcription factor essential for self-renewal of ESCs, was downregulated and the expression of markers for the three germ layers were induced in *UTX*-null ESCs under an ESC differentiation condition (Fig. S2A-D). These results indicate that UTX is not required for self-renewal, proliferation, survival and differentiation of ESCs.

Wild-type and *UTX*-null ESCs were subjected to a well-established protocol for differentiating ESCs into a cardiac lineage (Fig. S2E). The expression of UTX and UTY was nearly doubled during cardiac differentiation in ESCs (Fig. 2E, F). Notably, the expression of UTY as well as UTX was abolished in *UTX*-null ESCs (Fig. 2E, F), suggesting that UTY expression in male ESCs is dependent on UTX. We then monitored the differentiation of ESCs into cardiac lineage-committed embryoid bodies (cEBs) that show heart beating-like rhythmic contractions, a unique functional property of pulsating cardiac muscle cells. Almost 100% of EBs from wild-type ESCs exhibited prominent beating (Fig. 2G). In the parallel condition, however, EBs derived from *UTX*-null ESCs failed to develop spontaneous contraction (Fig. 2G). Only ~26% of $UTX^{F\text{-}LoxP/y}$ EBs displayed spontaneous beating (Fig. 2G). *UTX*-null EBs did not undergo more cell death than wild-type EBs during cardiac differentiation, as shown by detection of activated Caspase3 and TUNEL staining (Fig. 2C, H), indicating that a failed formation of contracting EBs from *UTX*-null ESCs was not due to excessive cell death. Next, we examined the induction of cardiac-specific genes during cardiomyocyte differentiation in wild-type and *UTX*-null ESCs. Atrial natriuretic factor (ANF), the ventricular isoform of the myosin regulatory light chain (MLC2v), α-cardiac actin (α-CA), cardiac muscle myosin heavy chain 6 (Myh6) and cardiac-specific troponin T2 were markedly induced in wild-type ESCs during differentiation (Fig. 2I-L, data

not shown). However, under the same conditions, these cardiac-specific genes were not upregulated in *UTX*-null ESCs, consistent with the morphological defects in developing contraction (Fig. 2G, I-L, data not shown). Together, these data establish that UTX is required for cardiac lineage differentiation of ESCs and robust induction of the cardiac genes during cardiac differentiation.

UTX-null mice are defective in cardiac development

To address the role of UTX in vivo, we created mice with UTX^{Δ} allele by crossing the $UTX^{F\text{-}loxP/+}$ mice with *Rosa-Flp* and *EIIa-Cre* mice, which constitutively express Flp and Cre recombinase respectively, in succession (Fig. 1A) (Farley et al., 2000; Lakso et al., 1996). $UTX^{\Delta/\Delta}$ female and $UTX^{\Delta/y}$ male mice did not express detectable levels of *UTX* transcripts (Fig. S3A). UTY is expressed in both wild-type $UTX^{+/y}$ and *UTX*-null $UTX^{\Delta/y}$ male embryos at E8.5 (Fig. S3B), suggesting that UTY expression in developing male embryos is independent on UTX, unlike male ESCs. UTY expression was not detected in female embryos, as expected (Fig. S3B). $UTX^{\Delta/\Delta}$ female mice, which do not have the *UTY* gene, did not survive past E10.5, whereas $UTX^{\Delta/+}$ heterozygote female mice were viable and fertile. $UTX^{\Delta/y}$ male mice displayed embryonic lethality and severe developmental retardation, but some $UTX^{\Delta/y}$ mice that escaped embryonic lethality lived into adulthood. Thus, UTX is required for the survival of female embryos, and UTY partially compensates for the loss of UTX in males.

$UTX^{\Delta/\Delta}$ female embryos exhibited severe cardiac malformation and neural tube closure defects, while their allantois appeared to develop normally (Fig. 3A-F, data not shown). At E9, $UTX^{\Delta/+}$ and $UTX^{+/y}$ sibling embryos displayed cardiac looping morphogenesis, which marks chamber formation and results in a physical separation between the primitive atrial and ventricular regions, and completed neural tube closure in the anterior region (Fig. 3A-B). In contrast, $UTX^{\Delta/\Delta}$ embryonic hearts largely retained a tubular heart structure and their neural tubes remained open in the anterior portion (Fig. 3C-D, Fig. S3C). We further examined the gross structure of hearts by performing hematoxylin and eosin staining on the transverse sections of E9 embryos. As predicted, hearts of $UTX^{\Delta/+}$ and $UTX^{+/y}$ embryos were highly looped and consisted of separate chambers, whereas $UTX^{\Delta/\Delta}$ embryos were largely linear throughout the heart tube and lacked chamber development (Fig. 3E, F). In addition, the myocardium of *UTX*-null embryos appeared thinner than that of littermate control embryos (Fig. 3E, F). At E10.5, $UTX^{\Delta/\Delta}$ embryos were markedly growth retarded as compared to wild-type littermates, and were completely reabsorbed by E11.5 (data not shown). These results indicate that UTX is needed for early heart development and neural tube closure.

Impaired cardiac differentiation in *UTX*-null embryos

To understand the basis for the cardiac defects in E9 *UTX*-null embryos, we analyzed cell proliferation and survival in E8.5 embryos using Ki67 and TUNEL staining and found little difference between *UTX*-null and $UTX^{\Delta/+}$ heterozygote embryonic hearts (Fig. 3G). Next, we monitored expression levels of core cardiac transcription factors using quantitative RT-PCR with E8.5 littermate whole embryos. The expression of the four core cardiac factors, Nkx2.5, Tbx5, GATA4, and SRF, was comparable among embryos of various genotypes at

E8.5 (Fig. S3D-F). Immunohistochemical analyses revealed that both $Nkx2.5^{+}Isl1^{-}$ and $Nkx2.5^{+}Isl1^{+}$ myocardial lineage cells are specified in E8.5 *UTX*-null embryos (Fig. 3H). UTX is expressed in both myocardial lineages (Fig. S4). Together, these results suggest that proliferation, survival, and initial fate specification are not overtly disturbed in myocardial lineage cells of *UTX*-null embryos at E8.5.

Next, we examined expression of Nkx2.5 and Tbx5 in E9 *UTX*-null and control littermate embryos using whole mount in situ hybridization analyses. Nkx2.5 and Tbx5 were highly expressed in the sinoatrial structures and heart tube of $UTX^{\Delta/\Delta}$ embryos, but the overall levels were reduced compared to those in $UTX^{\Delta/+}$ littermates (Fig. 3I-P), suggesting that UTX plays a role in maintaining high levels of Nkx2.5 and Tbx5 in the developing heart. To monitor the expression of heart-specific genes that are regulated by the core cardiac transcription factors, we performed whole mount in situ hybridization analyses in littermate embryos. ANF, an atrial natriuretic peptide, is a marker of the differentiating chamber myocardium. SRF, Tbx5, Nkx2.5 and GATA4, alone or in combination, bind to their cognate sites in the *ANF* enhancer and activate its transcription (Bruneau et al., 2001; Charron and Nemer, 1999; Durocher and Nemer, 1998; Garg et al., 2003; Hiroi et al., 2001; McBride and Nemer, 2001; Small and Krieg, 2003, 2004; Tanaka et al., 1999; Thuerauf et al., 1994). In addition, Nkx2.5 and Tbx5 also activate expression of the ventricle-specific contractile protein MLC2v (Bruneau et al., 2001; Lyons et al., 1995; Tanaka et al., 1999). Whole mount in situ hybridization revealed that ANF expression was greatly reduced in $UTX^{\Delta/\Delta}$ embryonic heart (Fig. 3Q-T). In addition, MLC2v was also substantially decreased and the expression pattern of MLC2v was severely altered in $UTX^{\Delta/\Delta}$ embryos (Fig. 3U-Z). Together, our data establish that UTX plays a crucial role in maintaining high levels of Nxk2.5 and Tbx5 expression and marked upregulation of heart-specific genes during embryonic development.

UTX functions as a coactivator of the core cardiac transcription factors

Given that *UTX*-null mice exhibit severely arrested heart development and down-regulation of ANF and MLC2v, target genes of core cardiac transcription factors, we asked whether UTX functions as a coactivator of any of the cardiac transcription factors. To test this idea, we first examined the association between UTX and Serum response factor (SRF), a MADS box factor that functions as a strong activator to induce cardiac-specific gene transcription during development (Miano et al., 2004; Niu et al., 2005; Parlakian et al., 2004; Shore and Sharrocks, 1995). We performed co-immunoprecipitation (CoIP) assays in HEK293T cells transfected with Myc-tagged UTX and HA-tagged SRF, and found that UTX associates with SRF in cells (Fig. 4A). Furthermore, we detected the interaction between endogenous UTX and SRF by CoIP assays in the developing heart tissues (Fig. 4B, S5A). Neither UTX nor SRF associated with Ezh2, a histone H3K27 methyltransferase, in the hearts (Fig. S5A), confirming the specificity of interaction between UTX and SRF (Fig. S5A).

To test whether UTX affects the transcriptional activity of SRF, we performed cell-based reporter assays using luciferase reporters linked to synthetic serum response elements (SREs) or enhancer elements of *ANF*, *SM22*, or *α-CA* gene, each of which contains essential SRF binding sites (Chen and Schwartz, 1996; Li et al., 1997; Small and Krieg, 2004;

Strobeck et al., 2001). UTX enhanced SRF-mediated transcriptional activation in all SRF-responsive reporters in a dose-dependent manner, while UTX alone had little effect without SRF (Fig. 4C, D, Fig. S5B, D). Conversely, shRNA-mediated knocking down of endogenous UTX strongly suppressed the SRF-mediated activation, which was rescued by expression of shRNA-insensitive form of UTX (Fig. 4E, F, Fig. S5C, E). These data demonstrate that UTX is important for the SRF-mediated transcriptional activation.

Next, we tested whether UTX cooperates with other core cardiac transcription factors. CoIP assays demonstrated that UTX associates with Tbx5, Nkx2.5, and GATA4 in HEK293T cells (Fig. 4G-I). Likewise, CoIP assays in E12.5 hearts revealed the interaction of UTX with endogenous Tbx5, Nkx2.5, and GATA4 (Fig. 4J). In contrast, UTX did not interact with Pax6 homeodomain transcription factor in HEK293T cells (Fig. S5F), confirming the specificity of association between UTX and core cardiac transcription factors.

The proximal enhancer of *ANF* bears the critical response elements for Tbx5, Nkx2.5, and GATA4 in addition to SRF (Bruneau, 2002; Bruneau et al., 2001; Charron and Nemer, 1999; Durocher and Nemer, 1998; Garg et al., 2003; Hiroi et al., 2001; McBride and Nemer, 2001; Small and Krieg, 2003, 2004; Tanaka et al., 1999; Thuerauf et al., 1994). Consistent with the observed interactions between UTX and the cardiac transcription factors, UTX expression further potentiated Tbx5, Nkx2.5 and GATA4-dependent activation of the *ANF* enhancer, while UTX-shRNA inhibited this activation (Fig. 4K, L, Fig. S5G-J). Interestingly, we found that Tbx5 and SRF synergize in activating the *ANF* enhancer (Fig. 4M, N). UTX strengthened this combinatorial action of Tbx5 and SRF, and UTX-shRNA suppressed this action (Fig. 4M, N). Similarly, UTX also supported the cooperative actions of Nkx2.5 and GATA4 in activating the *ANF* enhancer, and UTX-shRNA inhibited this action (Fig. S5K, L). Combined with the phenotypic analyses of *UTX*-null hearts, our results suggest that UTX potentiates the induction of heart-specific genes, at least in part, by functioning as an essential common coactivator for the core cardiac transcription factors, SRF, Tbx5, Nkx2.5 and GATA4.

UTX is recruited to cardiac-specific enhancers

Our results predict that UTX is recruited to cardiac-specific enhancers to control cardiac gene expression in the developing heart. To test this prediction, we monitored the binding of endogenous UTX to cardiac enhancers using chromatin immunoprecipitation (ChIP) assays with an α-UTX antibody in dissected hearts, limbs and brains from E12.5 mouse embryos. UTX was strongly recruited to the *ANF* enhancer that contains the key responsive elements for SRF, Tbx5, Nkx2.5 and GATA4 in embryonic hears, whereas its binding to the *ANF* enhancer in embryonic limbs and brains was much weaker (Fig. 5A, B). Similarly, UTX bound to the cardiac-specific enhancer of a Brg1-associated factor *Baf60c*, which was co-occupied by SRF, Nkx2.5 and GATA4 (He et al., 2011), in hearts, but UTX did not bind to the *Baf60c* enhancer efficiently in limbs and brains (Fig. 5C). These data underline a heart-specific recruitment of endogenous UTX to the cardiac enhancers, which are co-regulated by core cardiac transcription factors, in embryos.

To test whether UTX binding to the cardiac enhancers is developmentally regulated, we performed ChIP assays in self-renewing pluripotent ESCs and differentiated cEBs that

display spontaneous heart-like beating. As expected, the expression of Tbx5, Nkx2.5, SRF and GATA4 was induced as ESCs differentiate to cEBs (Fig. S6A-D). Interestingly, UTX was recruited to the *ANF* enhancer in cEBs that express cardiac transcription factors, while it did not bind to the enhancer in self-renewing ESCs (Fig. 5D). Likewise, ESC differentiation into cEBs induced UTX to bind to the *Baf60c* enhancer (Fig. 5E). These data suggest that the recruitment of UTX to the cardiac-specific genes is facilitated during the developmental transition of ESCs into the cardiac lineage, likely due to upregulation of the cardiac transcription factors and UTX and their subsequent association. Together, these data show that the occupancy of UTX to the cardiac lineage genes is controlled in a developmental stage- and tissue-specific manner.

UTX mediates demethylation of H3K27 residues in cardiac-specific genes

The recruitment of UTX to the cardiac genes led us to ask whether UTX functions as a H3K27 demethylase enzyme that removes a repressive transcription mark H3K27me3 in cardiac genes upon the activation and/or expression of the cardiac transcription factors. To this end, we monitored the H3K27me3 levels in the cardiac enhancers with or without serum shock, which promotes the transcriptional activity of SRF, in P19 mouse embryonic cells. The H3K27me3 levels in the *ANF* and *Baf60c* enhancers were markedly suppressed when SRF was activated by serum shock (Fig. 5F, G). This SRF-mediated demethylation of H3K27 in the cardiac enhancers was blocked by shRNA-mediated knockdown of endogenous UTX (Fig. 5F, G), suggesting that UTX is a demethylase that eliminates the H3K27me3 mark. Further supporting this idea, re-expression of UTX wild-type repressed the elevated H3K27me3 levels in the cardiac enhancers in UTX knockdown conditions, while a catalytically inactive UTX mutant failed to rescue the UTX knockdown phenotype (Fig. 5F, G). The expression levels of UTX wild-type and mutant were comparable (data not shown). These data demonstrate that UTX enzymatic activity is needed for the activated SRF to remove a repressive mark H3K27me3 in the cardiac genes.

UTX facilitates the recruitment of Brg1 to cardiac-specific genes

The ATP-dependent chromatin remodeling Swi/Snf complex containing Brg1, an ATPase subunit, plays crucial roles in heart development and potentiates the transcription activity of core cardiac transcription factors (Hang et al., 2010; Lickert et al., 2004). Interestingly, it was reported that H3K27 demethylases mediate a functional interaction between a T-box transcription factor T-bet and the Brg1-complex (Miller et al., 2010). Our findings that UTX functions as a coactivator of cardiac transcription factors, along with the known roles of Brg1 in heart development, raise the question of whether UTX also contributes to heart-specific gene expression by recruiting the Brg1-containing Swi/Snf chromatin remodeling complex to the cardiac genes. We found that UTX associates with Brg1 in HEK293T cells (Fig. 6A). We then tested whether UTX facilitates the interaction between Tbx5 and Brg1 using CoIP assays in HEK293 cells transfected HA-tagged Tbx5 with or without UTX. Expression of UTX greatly promoted the interaction between Tbx5 and Brg1 (Fig. 6B).

Next, to test whether UTX plays a role in recruiting Brg1 to the heart-specific genes, we monitored the recruitment of Brg1 to the *ANF* and *Baf60c* enhancers in P19 cells treated with serum shock. Brg1 binding to the cardiac enhancers was substantially reduced by UTX

knockdown (Fig. 6C, D), suggesting that endogenous UTX facilitates the recruitment of Brg1 to the cardiac enhancers. Interestingly, enzymatically inactive UTX, as well as wild-type UTX, enhanced Brg1-binding to the cardiac enhancers (Fig. 6C, D). These data suggest a demethylase activity-independent function of UTX in facilitating the recruitment of the Brg1-containing chromatin remodeling complex to the cardiac-specific genes.

UTX is required to trigger transcriptionally active chromatin in cardiac-specific genes

Considering the recruitment of UTX to the cardiac enhancers during cardiac lineage differentiation, we asked whether UTX is important to shift the chromatin signature of the cardiac-specific genes from a poised state in self-renewing ESCs to an active state in cEBs. The H3K27me3 levels in the *ANF* enhancer were high in ESCs, and substantially reduced as wild-type ESCs differentiated into cEBs (Fig. 7A), as levels of ANF transcript increased (Fig. 2I). In sharp contrast, in *UTX*-null ESCs, the *ANF*-H3K27me3 levels were not suppressed under a cardiac differentiation condition (Fig. 7A), suggesting that UTX is required to remove the H3K27me3 repressive mark in the *ANF* enhancer during cardiac development. These changes in the H3K27me3 levels were not specific to the *ANF* gene. *UTX*-null ESCs also failed to suppress the H3K27me3 levels efficiently during cardiac differentiation in the *Baf60c* enhancer and the enhancer of *α-CA*, which contains the binding sites for SRF, Nkx2.5 and GATA4 (Chen and Schwartz, 1996; Sepulveda et al., 1998), while their H3K27me3 levels declined in wild-type cEBs (data not shown). These results suggest that UTX is important to eliminate the H3K27me3 mark in cardiac genes during cardiac lineage specification.

Interestingly, the H3K4me3 levels in the *ANF* gene remained unchanged and relatively low during cardiac differentiation in *UTX*-null ESCs, while the *ANF*-H3K4me3 levels were highly induced under parallel differentiation conditions in wild-type ESCs (Fig. 7B). We also obtained the similar data with the *Baf60c* enhancer (data not shown). These data indicate that in the absence of UTX, cardiac-specific genes fail to undergo removal of H3K27me3 and deposition of H3K4me3, thus maintaining the repressive chromatin in cardiac genes (Fig. 7C). This failure of establishment of active chromatin in cardiac genes likely contributes to various defects in *UTX*-null embryonic hearts.

DISCUSSION

Cardiac lineage decisions and the subsequent morphogenesis of the early developing heart are orchestrated by a set of transcription factors, including SRF, Tbx5, Nkx2.5 and GATA4, which support each other's expression and act in combination to control the overlapping target genes (Bruneau, 2002; Garg et al., 2003; Hiroi et al., 2001; McBride and Nemer, 2001; Olson, 2006; Searcy et al., 1998; Sepulveda et al., 1998; Small and Krieg, 2004; Tanaka et al., 1999). In spite of crucial roles of histone methylation in gene regulation and development (Bhaumik et al., 2007; Cloos et al., 2008), the identity of enzymes that control histone methylation levels for cardiac lineage-specific genes was unclear. We found that UTX is recruited to the cardiac genes by associating with core cardiac transcription factors Nkx2.5, Tbx5, GATA4 and SRF, thereby activating heart-specific genes in cardiac lineage cells, but not in other cell types that do not express the cardiac transcription factors (Fig.

7C). Considering broad expression of UTX in multiple tissues, the association of UTX with the cardiac transcription factors likely plays an important role for specifically targeting UTX to cardiac genes only in cells committed to a cardiac lineage. The adjacent locations of SRF, Tbx5, Nkx2.5 and GATA4 binding sites in the enhancer regions of many developmentally regulated cardiac genes (Bruneau, 2002; He et al., 2011; McBride and Nemer, 2001; Small and Krieg, 2004) are likely crucial to recruit UTX more efficiently to cardiac genes, as UTX associates with multiple transcription factors that co-occupy the enhancers.

Our data support two mechanisms by which UTX activates transcription of cardiac genes after UTX is recruited to the genes via association with cardiac transcription factors. First, UTX, a histone H3K27 demethylase, eliminates the H3K27me3 repressive mark in heart-specific genes during cardiac differentiation to allow transcriptional activation. UTX knockdown blocks the activated SRF from reducing the H3K27me3 levels in the cardiac genes, which was rescued by UTX wild-type but not by enzymatically inactive UTX. Consistently, in the absence of UTX, cardiac genes are still marked by the H3K27me3 even under a cardiac differentiation condition. These data suggest that the enzymatic activity of UTX is crucial in establishing a transcriptionally active chromatin in cardiac genes. Second, UTX associates with Brg1 and facilitates the recruitment of the Brg1-containing ATPase-dependent Swi/Snf chromatin-remodeling complex to the cardiac genes. Interestingly, this function of UTX appears independent of its demethylase activity. Consistently, a catalytically inactive UTX mutant enhanced the activity of the cardiac transcription factors in luciferase assays and partially restored the transcription of some UTX target genes inhibited by UTX knockdown (data not shown). Our data indicate that UTX strongly promotes the association between Brg1 and Tbx5. JMJD3, another histone H3K27 demethylase, has also been shown to mediate the association between Brg1 and a T-box factor T-bet in T helper cells (Miller et al., 2010). Thus, H3K27 demethylases may act to bridge the Brg1-containing Swi/Snf complex and developmental transcription factors in multiple cell types. It is worth noting that Baf60c, a heart-specific component of the Brg1-complex, mediates the association between Brg1 and multiple cardiac transcription factors, such as Tbx5, Nkx2.5, and GATA4, and potentiates cardiac gene expression (Lickert et al., 2004). In the future, it will be interesting to investigate the relationship between UTX and Baf60c in the Brg1-dependent transcriptional activation of cardiac genes.

Our studies revealed that loss of UTX disrupts the coordination of H3K4 and H3K27 methylations on poised cardiac genes during cardiac differentiation. These observations suggest that H3K27 demethylation is intricately linked to H3K4 methylation and that UTX is essential in orchestrating the chromatin signature of the cardiac genes from a poised to active state in the developing heart. UTX may be important to bring the H3K4 methyltransferases MLL3/4 to the cardiac genes during development (Fig. 7C), given they form a steady-state complex with MLL3 or MLL4 (Cho et al., 2007; Goo et al., 2003; Issaeva et al., 2007; Lee et al., 2007; Lee et al., 2006a). Investigating a role of UTX in recruiting MLL3/4 methyltransferases to cardiac genes will help understand how histone codes are tightly coordinated in cell lineage-specific genes during organogenesis.

Altogether, our findings establish UTX as a central player in heart development and point to an intriguing possibility that cardiac-specific gene and signaling networks converge on UTX

to trigger a wide range of cardiac gene transcription during development. Our findings may contribute to development of novel strategies to efficiently produce cardiomyocytes from stem cells for therapeutic purpose.

The removal of the transcriptionally repressive chromatin mark H3K27me3 is likely important for the gene expression for multiple lineages. Relatively broad expression of UTX suggests its possible role beyond cardiac development. Indeed, *UTX*-null embryos exhibit neural tube closure impairment as well as cardiac defects, consistent with an excencephaly phenotype of mutant mice with a *UTX* gene trap allele (Cox et al., 2010). Interestingly, our data show that *UTX*-null ESCs are not defective in cellular differentiation in general. Under a differentiation condition with RA, *UTX*-null ESCs stop self-renewal and readily differentiate. In addition, *UTX*-null embryos develop until E9.5. We also found that *UTX*-null ESCs differentiate into neurons, similarly to wild-type ESCs (data not shown). It is possible that UTX is dispensable for differentiation of certain cell lineages and/or that JMJD3, another H3K27 demethylase, functions redundantly with UTX. Analyses of JMJD3-deficient mice and mutant mice lacking both JMJD3 and UTX may be employed to address specific and redundant functions of JMJD3 and UTX in future.

A single allele mutation in *UTX* gene in males is sufficient to inactivate *UTX* gene as it is located on the X-chromosome. In light of this, it is noteworthy that somatic mutations of *UTX* were found in various human cancers (van Haaften et al., 2009). $UTX^{\Delta/y}$ male mice displayed a wide range of phenotypes from embryonic lethality with severe cardiac malformation to tumor formation in adults, while $UTX^{\Delta/+}$ heterozygote female mice were viable and fertile. $UTX^{\Delta/\Delta}$ female mice invariably show embryonic lethality. These analyses of *UTX*-deficient mice suggest that UTY compensates for the loss of UTX but only partially. Given previous studies that UTY lacks the H3K27 demethylase enzymatic activity in vitro (Hong et al., 2007; Lan et al., 2007), compensation of UTX deficiency by UTY might be attributed to its demethylase activity-independent functions such as recruitment of Brg1 to the developmental transcription factors. However, it is still possible that UTY exhibits demethylase activity under certain in vivo contexts. Further investigation into the redundant and unique actions of UTX and UTY will provide important insights into not only the developmental roles of histone demethylases but also their anti-tumorigenic functions. Our *UTX*-deficient mice will serve as crucial tools for future investigation.

EXPERIMENTAL PROCEDURES

Generation of *UTX*-null ESCs

$UTX^{F\text{-}loxP}$ ESCs were obtained from the European Conditional Mouse Mutagenesis Program (EUCOMM). $UTX^{F\text{-}loxP}$ ESCs and their wild-type counterparts were maintained on top of irradiated mouse embryonic fibroblast (MEF) feeder cells in propagation medium consisting of Knockout-DMEM (Dulbecco's modified Eagle's medium), 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, and leukemia inhibitory factor (LIF, 1000 units/ml, Chemicon). To generate $UTX^{F\text{-}\Delta/y}$ ESCs which are deficient in exon 3 of the *UTX* gene, 2000 $UTX^{F\text{-}loxP}$ ESCs were plated in 35 mm dish with MEF feeder cells for 24 hours and transfected with pOG231-Cre plasmid (Addgene) using Lipofectamine 2000 (Invitrogen). Three days later, single colonies were

picked, further amplified, and verified for the loss of UTX expression by Western blot and RT-PCR analyses. PCR was used to determine the genotype of ESCs with the primers 5'-GTTGTGGTTTGTCCAAACTCA and 5'-CGAGTGATTGGTCTAATTTGG. The amplified product from the $UTX^{F\text{-}\Delta}$ allele is 209 bp, whereas that from the $UTX^{F\text{-}loxP}$ allele is 996 bp. Additional set of primers was also used to determine the $UTX^{F\text{-}loxP}$ allele, which produces 264 bp PCR product; 5'-GTTGTGGTTTGTCCAAACTCA and 5'-CTGTAATCTCTGCTGATACAA.

Generation of UTX-deficient mice

$UTX^{F\text{-}loxP}$ ESCs were introduced into mouse blastocysts. Chimeric mice were mated with wild-type C57BL6 mice. $UTX^{F\text{-}loxP/+}$ heterozygous F1 mice were mated to *Rosa*-Flp mice to delete the sequences between FRT sites (Farley et al., 2000). To delete exon 3 of *UTX* constitutively, *EIIa*-Cre mice (Lakso et al., 1996) were crossed with mice bearing *UTX*-floxed allele. Genotyping was performed by PCR using primers 5'-TCTGGTATAGATTTTGGCTAA and 5'-CGAGTGATTGGTCTAATTTGG. PCR reaction results in 932 bp product from wild-type allele and 286 bp product from *UTX*-null allele.

Differentiation ESCs into a cardiac lineage

Cardiac differentiation of ESCs was performed as previously described (Boheler et al., 2002). Briefly, 500 ESCs in 20 μl of differentiation medium (ESC growth medium without LIF) were placed on the lids of 150 mm petri dishes for two days. The aggregated ESCs, now termed embryoid bodies (EBs), were floated in bacteriological low attach dishes for five days and medium was changed every other day. At day 7, EBs were plated onto tissue culture dishes coated with 0.1% gelatin. Spontaneous beating areas could be detected as early as day 8 (day 0 means the day when hanging drops were seeded).

Alkaline phosphatase (AP) staining

To measure AP activity, 3000 cells/well were seeded on MEF feeder cells in 24-well plate in propagation medium. For ESC differentiation, 1 μM of retinoic acid (RA) was added in the absence of LIF. At day4, cells were fixed in 4% paraformaldehyde/PBS and AP activity was measured using alkaline phosphatase detection kit (Chemicon #SCR004).

MTT cell proliferation assays

500 cells/well were seeded on MEF feeder cells in 96-well plate in propagation medium. 10 μl of MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, 5 mg/ml, Sigma) solution was added to each well containing cells and incubated at 37°C. Six hours later, medium was removed and 200 μl of Dimethyl Sulfoxide (DMSO) was added and absorbance was measured at 550 nm using spectrophotometer.

TUNEL assays

To monitor cell death, differentiated cEBs and E8.5 embryos were analyzed with *In situ* cell death detection kit, TMR red (Roche). Briefly, tissues were fixed in 4% paraformaldehyde for 20 minutes at room temperature, washed with PBS twice, permeabilized (0.1% Triton

X-100 in 0.1% sodium citrate), washed with PBS twice and TUNEL reaction mixture was added and incubated for 1 hour at 37 °C.

Immunohistochemistry and in situ hybridization assays

For immunohistochemistry and in situ hybridization analyses, the embryos were harvested at indicated stage, fixed in 4% paraformaldehyde, embedded in OCT and cryosectioned at 12-18 μm. Immunohistochemistry was performed as described (Lee et al., 2009) using the following antibodies and reagents: α-Ki67 (Novocastra), α-Nkx2.5 (H-114, Santa Cruz), α-Isl1 (DSHB) and α-UTX (a gift from K. Helin) antibodies and 4,6-diamidino-2-phenylindole (DAPI) (Sigma).

For in situ hybridization analysis, embryos were processed with digoxigenin-labeled Nkx2.5, Tbx5, MLC2v (gifts from J. Martin), ANF (gift from B. Bruneau), UTX and UTY riboprobes, as described previously (Wilkinson and Nieto, 1993). cDNA for mouse UTX and UTY were cloned to pBluescript vector to generate riboprobes.

For H&E staining, transverse sections of E9 embryos were stained with hematoxylin and eosin according to standard techniques.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNAs were extracted using the Trizol (Invitrogen) and reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). The levels of mRNA were determined using quantitative RT-PCR (Mx3000P, Stratagene). The levels of mRNA were normalized against the levels of cyclophilin A. The primers used were as follows: ANF, forward 5'-GAAAAGGCAGTCGATTCTGC, reverse 5'-CAGAGTGGGAGAGGCAAGAC; MLC2v, forward 5'-ACAGAGACGGCTTCATCGAC, reverse 5'-TGAATGCGTTGAGAATGGTC; Myh6, forward 5'-CACTCAATGAGACGGTGGTG, reverse 5'-GTGGGTGGTCTTCAGGTTTG; SRF, forward 5'-CCTTCAGCAAGAGGAAGACG, reverse 5'-GAGAGTCTGGCGAGTTGAGG; Nkx2.5, forward 5'-CTTGAACACCGTGCAGAGTC, reverse 5'-GGTGGGTGTGAAATCTGAGG; UTX, forward 5'-CTGAAGGGAAAGTGGAGTCTG, reverse 5'-TCGACATAAAGCACCTCCTG; UTY, forward 5'-ATAGTGTCCAGACAGCTTCA, reverse 5'-GAGGTAGGAATACGTAAGAA; Tbx5, forward 5'- GTGGCTGAAGTTCCACGAAG, reverse 5'-GGCTCTGCTTTGCCAGTTAC; *α-CA*, forward 5'-TGCTTCTGGAAGAACCACAG, reverse 5'-TCACGGACAATTTCACGTTC; GATA4, forward 5'-CCCCTCATTAAGCCTCAGC, reverse 5'-ATTCAGGTTCTTGGGCTTCC; cyclophilin A, forward 5'-GTCTCCTTCGAGCTGTTTGC, reverse 5'-GATGCCAGGACCTGTATGCT

Whole mount X-gal staining

Embryos were harvested, fixed in 4% paraformaldehyde for 10 min at 4°C, and washed in Rinse solution (100 mM sodium phosphate, pH7.5, 2 mM $MgCl_2$ and 0.02% NP-40) three times for 20 min each at 4 °C. Then, embryos were stained with X-gal staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in Rinse

solution) at 37 °C overnight. Embryos were post-fixed in 4% paraformaldehyde for 1 hour at room temperature and washed in Rinse buffer. To make the embryos transparent, embryos were incubated in serial dilution of glycerol (25, 50, 80% in PBS) for at least five hours in each step.

DNA constructs

ANF:Luc, Flag-Nkx2.5, Flag-GATA4, HA-Tbx5 (gifts from B. Bruneau), Myc-UTX (a gift from K. Gei), shRNA-human UTX (a gift from K. Helin), SM22:Luc (a gift from J. Epstein), αCA:Luc (a gift from M. Parmacek), SRE:luc, and HA-SRF vectors were described previously (Agger et al., 2007; Bruneau et al., 2001; Chen et al., 2002; Hong et al., 2007; Lee et al., 2000). Mouse Pax6 full-length was cloned to pcDNA3HA vector. shRNA-mouse UTX target sequence is AAGTTAGCAGTGGAACGTTAT.

Luciferase reporter assays

HEK293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum. Cells were plated in 48-well plate and incubated for 24 hours, and transient transfections were performed using Superfect (QIAGEN). An actin-β-galactosidase plasmid was co-transfected for normalization of transfection efficiency, and empty vectors were used to equalize the total amount of DNA. Cells were harvested 48 hours after transfection. Cell extracts were assayed for luciferase activity and the values were normalized with β-galactosidase activity. All transfections were repeated independently at least three times. Data are represented as the mean of triplicate values obtained from representative experiments. Error bars represent standard deviation.

Co-immunoprecipitation (CoIP) and western blotting assays

HEK293T cells were seeded on 10 cm tissue cultures dishes and expression vectors were transfected as described above. Forty eight hours later, cells were harvested and lysed in IP buffer (20mM Tris-HCl, pH 8.0, 0.5 % NP-40, 1 mM EDTA, 150 mM NaCl, 2 mM PMSF, 10% Glycerol, 4 mM Na_3VO_4, 200 mM NaF, 20 mM Na-pyroPO_4, and protease inhibitor cocktail). In these studies, precipitations were performed with α-Myc (Abcam), α-HA (Covance) and α-Brg1 (Millipore) antibodies. The interactions were monitored by western blotting assays using α-Flag (Sigma), α-Myc (Abcam), α-Brg1 (Millipore) and α-UTX (a gift from K. Helin) antibodies.

E12.5 mouse embryonic heart tissues were lysed in IP buffer, immunoprecipitated with α-UTX (a gift from K. Helin), α-SRF (G-20), α-Tbx5 (H-70), α-Nkx2.5 (H-114) and α-Gata4 (H-112) antibodies from Santa Cruz, and then the presence of UTX in immunopurified complex was monitored by immunoblotting with α-UTX antibody.

Western blotting assays with α-cleaved Caspase3 (Asp175) (Cell Signaling) antibody was used to monitor apoptotic cells in ESCs and cEBs. α-Oct34 (N-19, Santa Cruz) antibody was used to monitor the self-renewal and differentiation of ESCs. α-tubulin (Santa Cruz) antibody was used as loading control.

Chromatin immunoprecipitation (ChIP) assays

P19 cells were transfected with shRNA against mouse UTX, knockdown insensitive form of human UTX wild-type or catalytically inactive UTX mutant UTX-H1146A using Lipofectamine2000 (Invitrogen). Forty eight hours later, cells were washed with PBS and growth media (Minimum Essential Medium α, 7.5% bovine calf serum and 2.5% fetal bovine serum) was replaced with MEMα supplemented with 0.5% fetal bovine serum and incubated for 17 hours. Serum shock was applied by changing the low serum to 20% fetal bovine serum for 2 hours. E12.5 mouse embryonic heart, limbs and brain were used for ChIP experiments. ChIP experiments were performed as previously described (Lee and Pfaff, 2003). Briefly, cells were crosslinked with formaldehyde and sonicated. Protein-DNA complexes were precipitated with α-UTX (a gift from K. Helin), α-Brg1 (Millipore), α-H3K27me3 and α-H3K4me3 (Millipore) antibodies. The purified DNA was amplified using PCR and the SYBR green kit (Invitrogen) for quantitation. Quantitative results were normalized to the total input control. Primers used were *ANF*-enhancer, forward 5'-TGAATCAGGTGTGAAGCTAGCTCC, reverse 5'-GGAGCAGAAAAGAGTCCTTGGTTA; *ANF*-exon1, forward 5'-GGGAGATGCTGGCAGCTAGGAGAC, reverse 5'-CTTGAAATCCATCAGATCTGTGTT; *Baf60c*-enhancer, forward 5'-TTAAGCAGTCAGAAGATCTCCTTG, reverse 5' –GGCTCTCCACCTCAATAGGTTCCT. All experiments were repeated independently at least three times. Data are represented as the mean of triplicate values obtained from representative experiments. Error bars represent standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- UTX is required for the activation of cardiac genes and heart development.
- UTX associates with core cardiac transcription factors.
- UTX binds to cardiac-specific enhancers and demethylates histone H3 on K27.
- UTX facilitates the recruitment of Brg1 to the cardiac-specific enhancers.

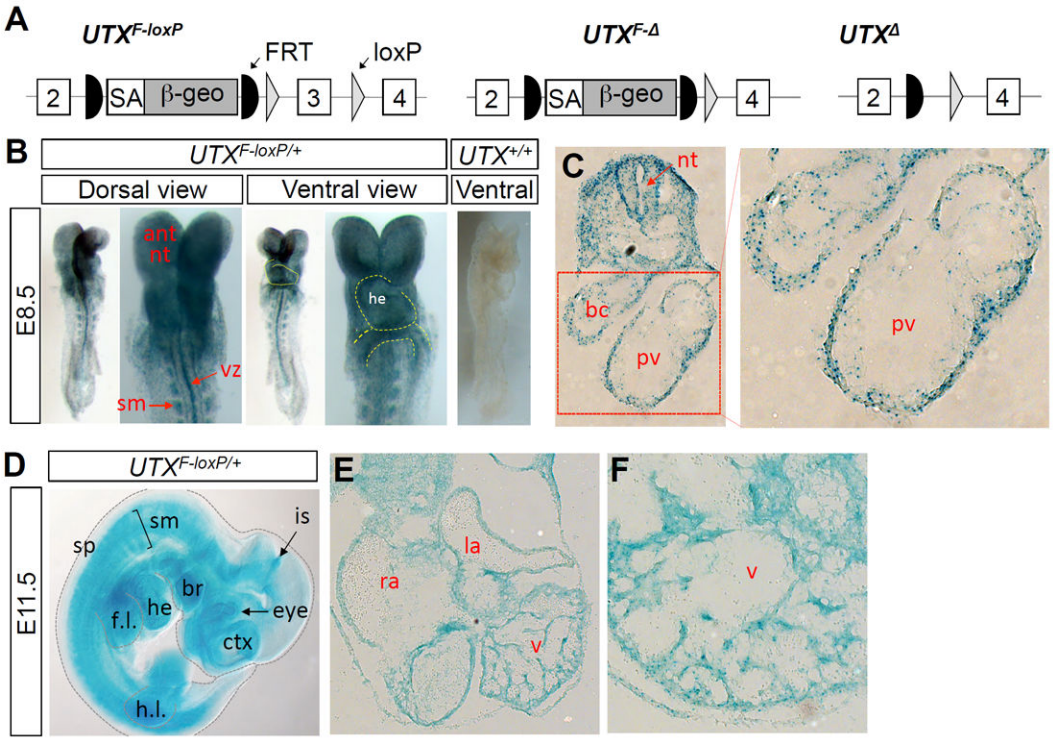

Figure 1. UTX expression pattern in the developing embryos

(A) Schematic representation of three mutant alleles of *UTX*. **(B-F)** Whole mount X-gal staining in E8.5 (B, C) and E11.5 (D-F) $UTX^{F\text{-}loxP/+}$ embryos. X-gal stained areas in $UTX^{F\text{-}loxP/+}$ embryos depict the embryonic tissues expressing UTX. sm, somites; ant nt, anterior region of the neural tube; vz, ventricular zone of the neural tube; he, heart; nt, neural tube; bc, bulbus cordis; pv, primitive ventricle; ctx, cortex; is, isthmus; br, brachial arches; sp, spinal cord; f.l., forelimb; h.l., hindlimb; ra, right atrium, la, left atrium; v, ventricle. See also Figure S1.

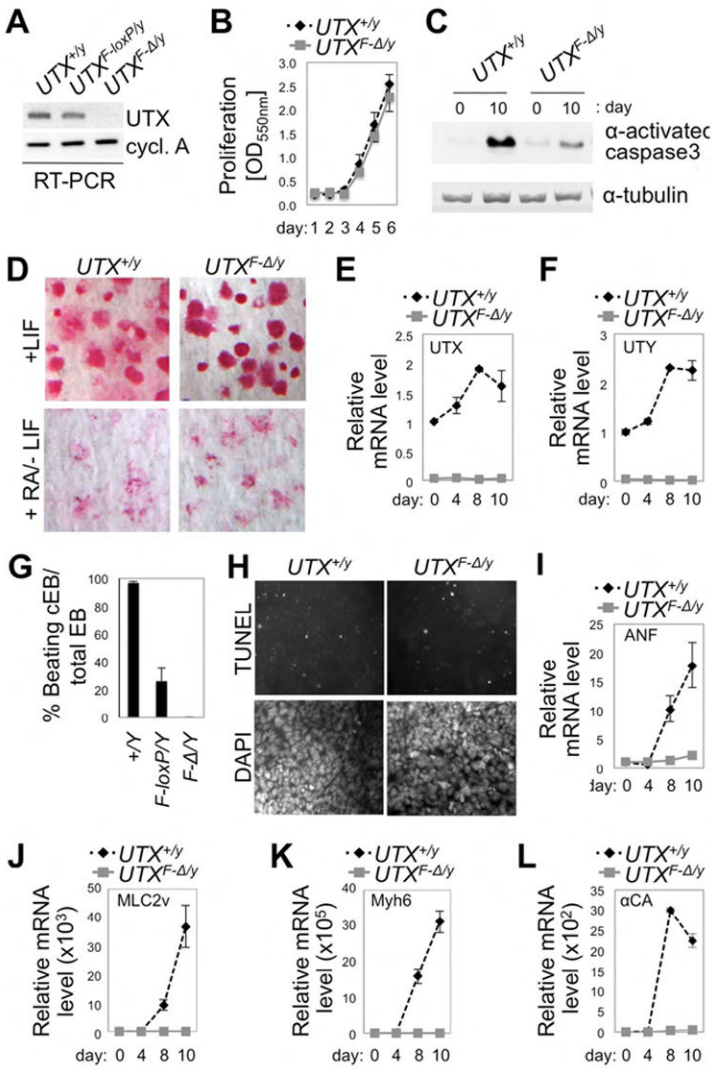

Figure 2. UTX is required for the differentiation of ESCs into a cardiac lineage

(A) RT-PCR using ESCs of various genotypes as shown above. UTX expression was not detected in *UTX*-null ($UTX^{F\Delta/y}$) ESCs. cycl. A, cyclophilin A. (**B**) MTT assays to monitor proliferation of ESCs. (**C**) Western blot to detect the levels of activated Caspase3 in self-renewing ESCs (day 0) and cardiac differentiated ESCs (day 10). (**D**) Alkaline phosphatase staining of ESC colonies under self-renewal condition (+LIF) and differentiation condition incubated with RA in the absence of LIF (+RA/-LIF). *UTX*-null ESCs self-renew and readily differentiate when stimulated with RA, like wild-type ESCs. **(E, F, I-L)** Quantitative RT-PCR to monitor mRNA levels during cardiac differentiation of wild-type ESCs ($UTX^{+/y}$, black dotted lines) and *UTX*-null ESCs ($UTX^{F\text{-}\Delta/y}$, gray lines). X-axis represents differentiation days. Y-axis represents relative mRNA levels. *UTX*-null ESCs did not induce cardiac-specific genes during cardiac differentiation. The mRNA level in wild-type ESCs at day 0 was calculated as 1. Error bars represent the standard deviation in all graphs (B, E-G, I-L). **(G)** Quantification of EBs that show heart-like spontaneous contraction among all EBs. *UTX*-null ESCs failed to form beating EBs. **(H)** TUNEL assays in cardiac differentiated EBs

(cEBs, day 10). *UTX*-null cEBs do not show more cell death than wild-type cEBs. See also Figure S2.

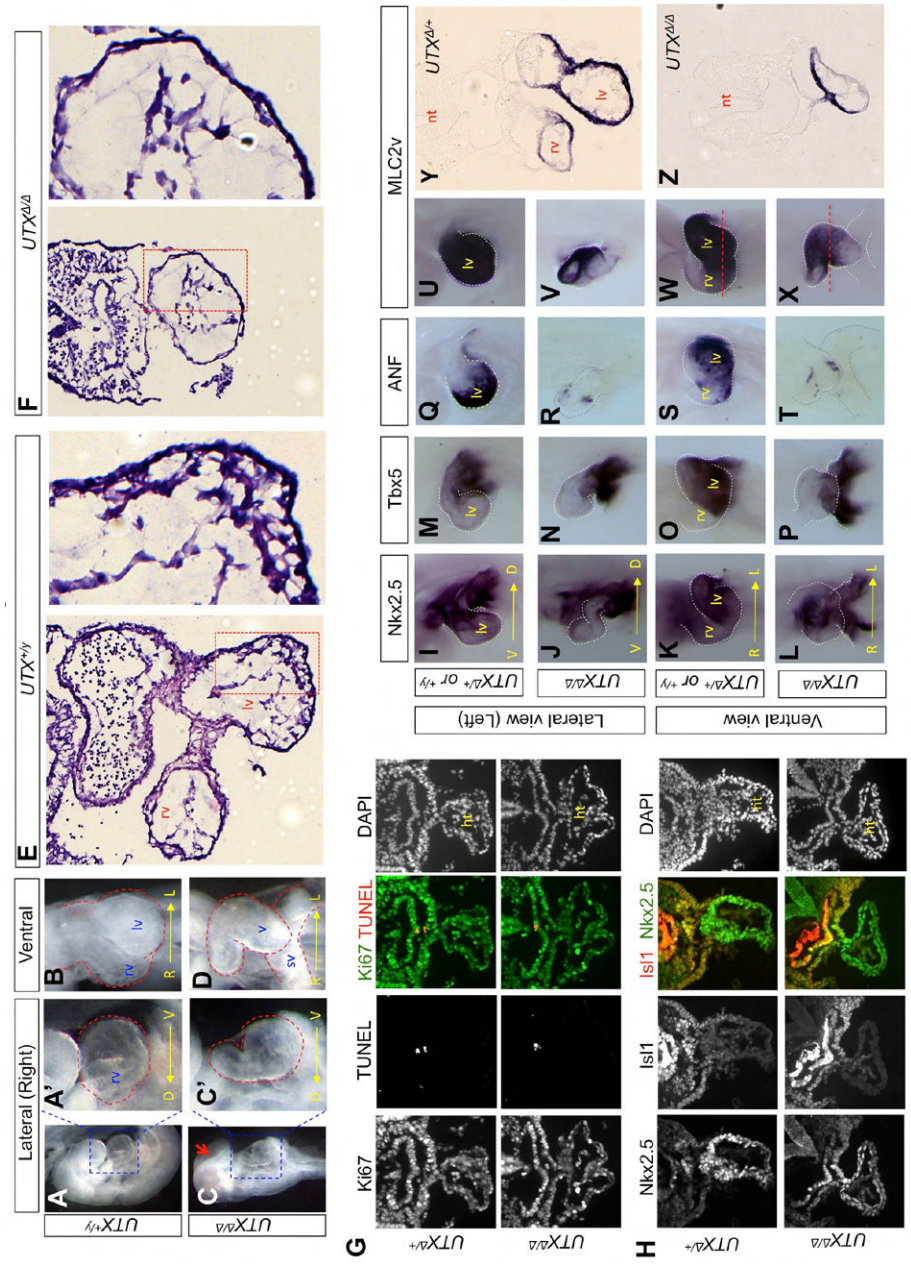

Figure 3. UTX is required for early heart development

(A-F) Images of E9 littermate wild-type embryo ($UTX^{+/y}$, A, A', B, E) and *UTX*-null embryo ($UTX^{\Delta/\Delta}$, C, C', D, F), and H&E staining of E9 embryonic hearts (E, F). At E9, *UTX*-null embryonic hearts displayed no overt looping, whereas wild-type embryos underwent cardiac looping morphogenesis, adopting a spiral shape consisting of cardiac chambers. *UTX*-null embryos also show neural tube closure defects (red arrow in C). **(G, H)** Immunohistochemical analyses with a proliferation marker Ki67, TUNEL labeling, and myocardiac lineage markers Nkx2.5 and Isl1. ht, heart tube. **(I-Z)** Whole mount in situ hybridization analyses of E9 embryos with probes of various cardiac genes, including Nkx2.5 (I-L), Tbx5 (M-P), ANF (Q-T), and MLC2v (U-Z). Lateral view from left side of

the embryos or ventral view are shown as indicated. Transverse sections of MLC2v-hybridized embryos corresponding to the dotted lines in W and X are shown in Y and Z, respectively. In E9 *UTX*-null embryos, expression of ANF and MLC2v was markedly downregulated compared to control embryos, and the separate chamber formation did not occur. rv, right ventricle; lv, left ventricle; v, ventricle; nt, neural tube. (A-D, I-Z) Relative position of embryos was marked by V (ventral), D (dorsal), right (R) and left (L) along with arrows. See also Figure S3 and S4.

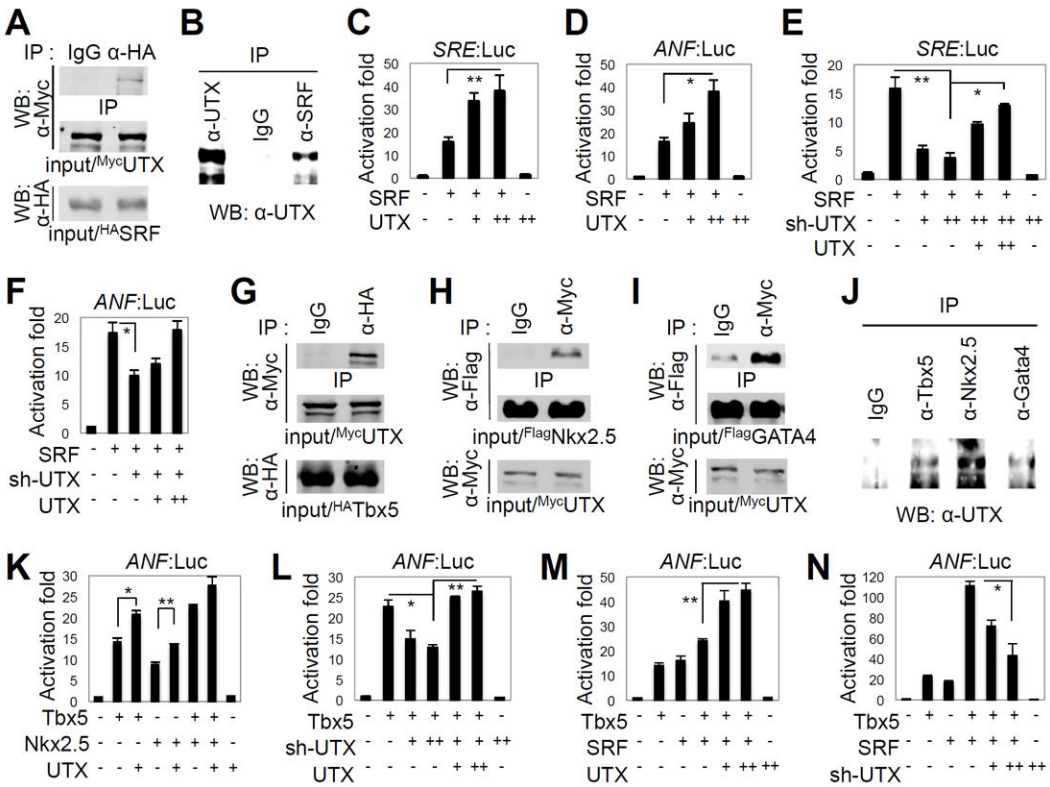

Figure 4. UTX functions as a coactivator of the cardiac transcription factors SRF, Nkx2.5, Tbx5, and GATA4

(A, G-I) CoIP assays in HEK293T cells transfected with Myc-UTX and the cardiac transcription factors. UTX associates with SRF, Nkx2.5, Tbx5 and GATA4 in cells. **(B, J)** CoIP assays using α-UTX antibody for western blotting assays in dissected E12.5 embryonic hearts. The antibodies used for immunopurification were marked on the top. **(C-F, K-N)** Luciferase reporter assays using *SRE*:luciferase (C, E) and *ANF*:luciferase (D, F, K-N) reporters with vectors indicated below each graph. The transcriptional activity of SRF, Tbx5, and Nkx2.5, alone or in combination, was enhanced by UTX, and inhibited by UTX-shRNA (sh-UTX). The basal luciferase activity for each reporter was calculated as 1 in Y-axis. + and ++ represent relative dosage of plasmids used for transfection. Error bars represent the standard deviation in all graphs. *, $p<0.05$; **, $p<0.01$ in two-tailed Student's t-test. See also Figure S5.

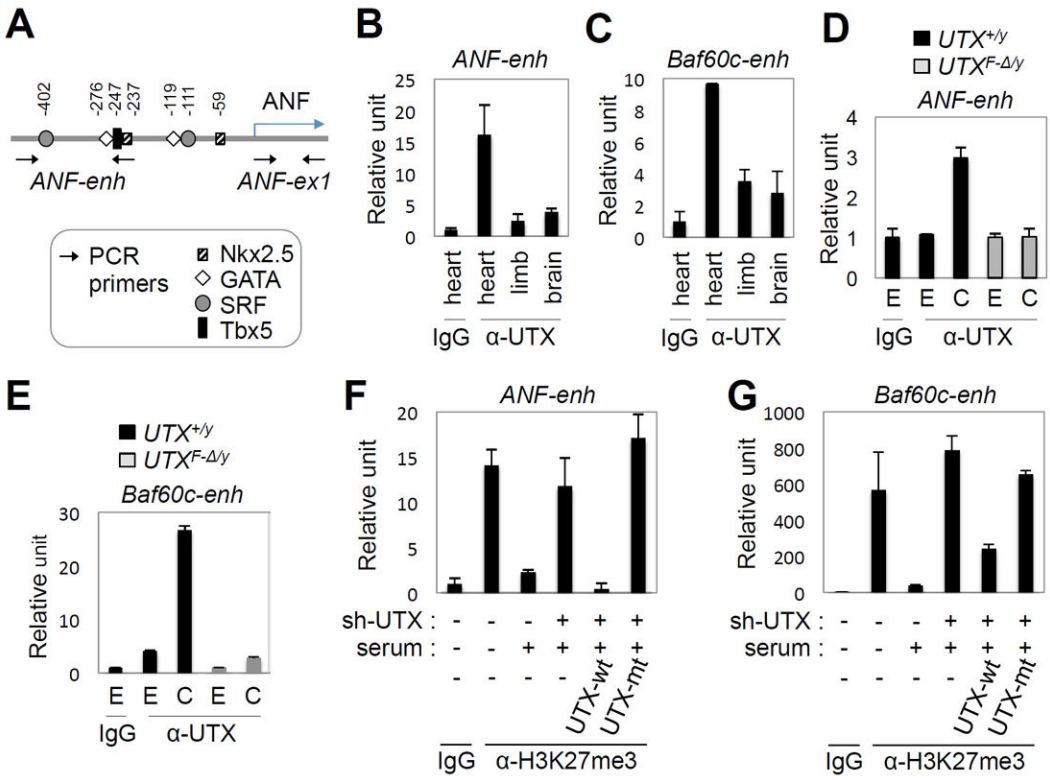

Figure 5. UTX is recruited to the cardiac genes in a cardiac cell-type specific manner and triggers the demethylation of H3K27 resides

(A) Schematic representation of the *ANF* gene. The arrows indicate two sets of primers detecting *ANF*-enhancer (*ANF-enh*) and *ANF*-exon1 (*ANF-ex1*). The specific response elements for the cardiac transcription factors are also indicated. **(B-E)** ChIP assays with α-UTX antibody in dissected tissues from E12.5 embryos (B, C) or ESCs (D, E). **(D, E)** Wild-type ESCs ($UTX^{+/y}$, black bars) and *UTX*-null ESCs ($UTX^{F\text{-}\Delta/y}$, gray bars) were cultured under self-renewing ESC condition (E) or cardiac differentiation condition (C). UTX is efficiently recruited to the *ANF*-enhancer and *Baf60c*-enhancer only in hearts or cEBs, but UTX binding to the cardiac enhancers is very weak in limbs, brains, and self-renewing ESCs. **(F, G)** ChIP assays with α-H3K27me3 in P19 cells treated with serum shock and transfected with sh-UTX and UTX expression vectors as indicated below each graph. UTX-wt, wild-type UTX; UTX-mt, catalytically inactive UTX. (B-G) The quantitative PCR levels in IgG samples were calculated as 1. Error bars represent the standard deviation in all graphs. See also Figure S6.

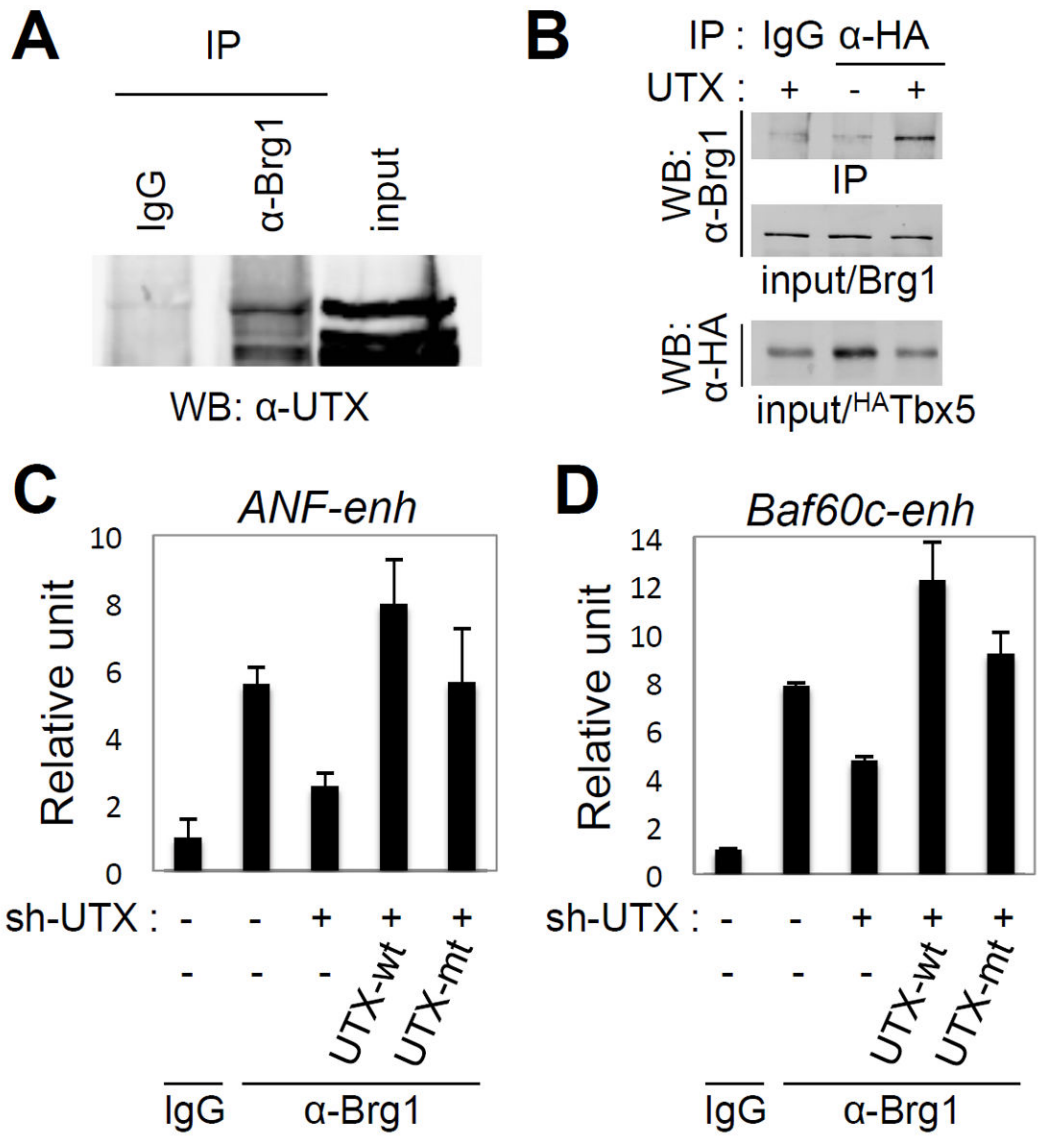

Figure 6. The demethylase activity-independent function of UTX in Brg1 recruitment to the cardiac genes

(A) CoIP assays in HEK293T cells. UTX associates with Brg1. **(B)** CoIP assays in HEK293T cells transfected with HA-Tbx5. UTX mediates the interaction between Tbx5 and Brg1. **(C, D)** ChIP assays with α-Brg1 in P19 cells treated with serum shock and transfected with sh-UTX and UTX expression vectors as indicated below each graph. UTX-wt, wild-type UTX; UTX-mt, catalytically inactive UTX. The quantitative PCR levels in IgG samples were calculated as 1. Error bars represent the standard deviation in all graphs.

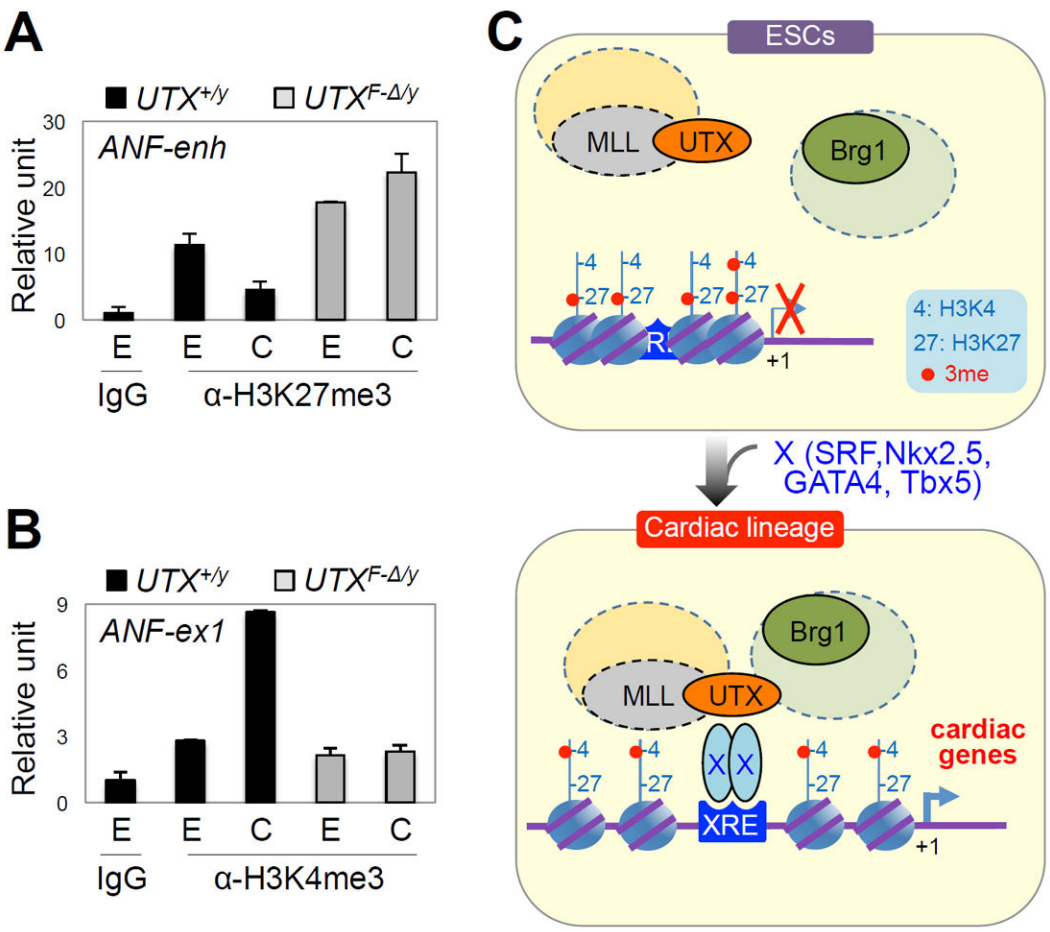

Figure 7. Role of UTX in establishing active chromatin in cardiac-specific genes and model for action of the UTX-complex during developmental transition of ESCs to cardiac cells

(A, B) ChIP assays with α-H3K27me3 (A) and α-H3K4me3 (B) antibodies in self-renewing ESCs (E) and cEBs (C) using wild-type ESCs (*UTX*$^{+/y}$, black bars) and *UTX*-null ESCs (*UTX*$^{F-\Delta/y}$, gray bars). The *ANF* gene loses a repressive mark H3K27me3 and gains an activating mark H3K4me3 during cardiac differentiation in wild-type ESCs, but these chromatin changes do not occur in *UTX*-null ESCs. The quantitative PCR levels in IgG samples were calculated as 1. Error bars represent the standard deviation. **(C)** UTX acts as a critical switch to drive cardiac lineage in development. X represents the cardiac transcription factors SRF, Tbx5, Nkx2.5, and GATA4, and XRE depicts the response elements of these cardiac transcription factors that control expression of a battery of cardiac-specific genes. During cardiac differentiation, the cardiac transcription factors are induced in expression, bind to the XREs of cardiac genes, and recruit the UTX-complex, which removes the H3K27me3 repressive mark. In addition, UTX promotes the recruitment of the Brg1-complex to the cardiac genes in a demethylase activity-independent manner. UTX may also facilitate the recruitment of histone H3K4 methyltransferases MLL3 and MLL4 that form a complex with UTX. These establish active chromatin in the cardiac genes and triggering the transcription.