Histone demethylase dUTX antagonizes JAK-STAT signaling to maintain proper gene expression and architecture of the Drosophila testis niche

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SUMMARY

Adult stem cells reside in microenvironments called niches, where they are regulated by both extrinsic cues, such as signaling from neighboring cells, and intrinsic factors, such as chromatin structure. Here we report that in the Drosophila testis niche an H3K27me3-specific histone demethylase encoded by Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (dUTX) maintains active transcription of the Suppressor of cytokine signaling at 36E (Socs36E) gene by removing the repressive H3K27me3 modification near its transcription start site. Socs36E encodes an inhibitor of the Janus kinase signal transducer and activator of transcription (JAK-STAT) signaling pathway. Whereas much is known about niche-to-stem cell signaling, such as the JAK-STAT signaling that is crucial for stem cell identity and activity, comparatively little is known about signaling from stem cells to the niche. Our results reveal that stem cells send feedback to niche cells to maintain the proper gene expression and architecture of the niche. We found that dUTX acts in cyst stem cells to maintain gene expression in hub cells through activating Socs36E transcription and preventing hyperactivation of JAK-STAT signaling. dUTX also acts in germline stem cells to maintain hub structure through regulating DE-Cadherin levels. Therefore, our findings provide new insights into how an epigenetic factor regulates crosstalk among different cell types within an endogenous stem cell niche, and shed light on the biological functions of a histone demethylase in vivo.

KEY WORDS: Germline, Cyst stem cell, Niche, Epigenetics, Histone demethylase, Drosophila

INTRODUCTION

Extrinsic signals are important to maintain appropriate interaction between stem cells and their niches (Morrison and Spradling, 2008). In addition, epigenetic regulation that changes chromatin structure without altering the associated DNA sequence acts intrinsically to regulate proper gene expression in stem cells (Clapier and Cairns, 2009). Both mechanisms are essential for regulating stem cell identity and activity (Cherry and Matunis, 2010; Eliazer et al., 2011). However, the crosstalk between them is not fully understood.

The Drosophila male germline stem cell (GSC) lineage is a paradigmatic system with which to investigate the molecular mechanisms that govern adult stem cell activity in their physiological environment (Kiger et al., 2001; Tulina and Matunis, 2001; Yamashita et al., 2003; Yamashita et al., 2007). Drosophila male GSCs reside in a microenvironment composed of two types of somatic cells: postmitotic hub cells located at the tip of the testis and cyst stem cells (CySCs), two of which encapsulate each GSC (Fig. 1A). Hub cells and CySCs contribute to the niche of GSCs by providing crucial signals to preserve GSC identity and activity (Kiger et al., 2001; Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001; Yamashita et al., 2003; Yamashita et al., 2007; Lim and Fuller, 2012). The Janus kinase signal transducer and activator of transcription (JAK-STAT) and bone morphogenetic gene (BMP) signaling pathways are the two major pathways that maintain the activity of GSCs and CySCs. The JAK-STAT pathway is activated by the cytokine Unpaired (Upd; Outstretched – FlyBase) secreted from the hub cells, which initiates the downstream cascade to activate the Stat92E transcription factor in GSCs and CySCs (reviewed by de Cuevas and Matunis, 2011). Activation of Stat92E in CySCs initiates BMP signaling required for GSC self-renewal, and activation of Stat92E in GSCs enhances their adhesion to the hub cells (Leatherman and Dinardo, 2008; Leatherman and Dinardo, 2010). Suppressor of cytokine signaling at 36E (Socs36E), which is expressed in hub cells and CySCs, attenuates JAK-STAT signaling (Terry et al., 2006) to maintain an appropriate balance between CySCs and GSCs in the testis niche (Issigonis et al., 2009).

In addition to signaling pathways, epigenetic mechanisms can profoundly influence decisions of stem cell maintenance versus differentiation (Buszczak and Spradling, 2006; Li and Zhao, 2008). DNA wraps around four core histones (H3, H4, H2A and H2B) to form nucleosomes, the repeating basic units of chromatin. In Drosophila, there are two major epigenetic regulators: chromatin remodeling factors that use ATP hydrolysis to drive histone repositioning and histone-modifying enzymes that covalently modify histones (Becker and Hörz, 2002). Both mechanisms have been shown to act intrinsically to maintain GSCs in the testis niche (Buszczak et al., 2009; Cherry and Matunis, 2010).

Among the histone-modifying enzymes, histone demethylases have been identified as ‘epigenetic erasers’ that remove methyl groups from methylated lysine residues of histones (Klose et al., 2006). Among the 14 demethylases in Drosophila (Klose et al., 2006; Metzger et al., 2005; Shi et al., 2004), Ubiquitously transcribed tetratricopeptide repeat gene on the X chromosome (dUTX; also known as Utx – FlyBase) encodes the sole demethylase that specifically removes the repressive trimethylation on lysine 27 of histone H3 (H3K27me3) (Smith et al., 2008). H3K27me3 is generated by a member of the Polycomb group (PcG) family of
proteins and has been shown to associate with silent regions of chromatin (Cao et al., 2002; Müller et al., 2002). Increased H3K27me3 levels have been reported to cause certain human cancers (Bracken et al., 2003; Kleer et al., 2003; Kondo et al., 2008; Varambally et al., 2002). Consistently, mutations that inactivate UTX (also known as KDM6A), the mammalian homolog of dUTX, cause an increase in H3K27me3 and lead to human cancers (van Haften et al., 2009). In Drosophila, dUTX has been reported to act as a suppressor of Notch- and Retinoblastoma-dependent tumors (Herz et al., 2010).

Mammalian species have multiple H3K27me3-specific demethylases. Therefore, studying the functions of dUTX in Drosophila greatly reduces the complications that might result from gene redundancy. The UTX protein is evolutionarily conserved and contains several tetratricopeptide (TRP) repeats, as well as the catalytic Junmøni C (JmjC) domain (Klose et al., 2006). dUTX has been shown to physically associate with RNA polymerase II (Pol II) in vivo, suggesting its involvement in transcriptional activation (Smith et al., 2008). To date, much of the knowledge about the epigenetic regulation of histone demethylases comes from biochemical studies undertaken in vitro or in cell culture, and their in vivo functions are not well understood. Therefore, to better understand the biological roles of dUTX, we have examined its role in the Drosophila testis niche.

MATERIALS AND METHODS
Fly stocks
Flies were raised on standard yeast/molasses medium at 25°C. The following stocks were used: dUTX¹ FRT40A (from A. Shilatifard, Stowers Institute for Medical Research, Kansas City, MO, USA), w¹¹¹B, Df(2L) BSC144 (Bloomington Stock Center, BL-9504), UAS-dUTX shRNA (TRIP.HMS00575 from Bloomington Stock Center), upd-Gal4 (from D. Harrison, University of Kentucky, Lexington, KY, USA), nanos-Gal4 (from M. Van Doren, Johns Hopkins University, Baltimore, MD, USA), c587-Gal4 (from A. Spradling, Carnegie Institution Department of Embryology, Baltimore, MD, USA), y,w; Ubi-GFP, Ubi-GFP, FRT40A (Bloomington Stock Center, BL-5189), hs-FLP¹²² (Bloomington Stock Center, BL-33216), Arm-lacZ, FRT40A (Bloomington Stock Center, BL-7371), UAS-dUTX and UAS-dUTX¹⁰⁰c [from A. Shilatifard, refer to Materials and methods in Herz et al. (Herz et al., 2010)], UAS-Socs36E-45 (from B. Callus, University of Western Australia, Perth, WA, Australia), Stat92E06346 (from N. Perrimon, Harvard Medical School, Boston, MA, USA), UAS-DE-Cad¹⁰⁰c⁸⁸⁸ and UAS-DE-Cad¹⁰⁰c⁸⁸⁹ (from Y. Yamashita, University of Michigan, Ann Arbor, MI, USA), and hs-FLP, UAS-GFP, UAS-GFP, tub-Gal4/FM7; tub-Gal80 FRT40A/CyO (from E. Bach, New York University School of Medicine, New York, NY, USA).

Clonal induction
dUTX¹ clones that are negative for the GFP or β-Gal marker were generated using the FLP/FRT recombination system. The flies used were of the following genotypes: hs-FLP¹²²; Arm-lacZ, FRT40A/dUTX¹ FRT40A or hs-FLP¹²²; Ubi-UTX, Ubi-GFP, FRT40A/dUTX¹ FRT40A. The clones were induced by heat shocking pupae on days 8 and 9 for 2 hours at 37°C. After the second heat shock, flies were placed at 25°C and dissected and stained 3 days after clone induction. Mosaic analysis with a repressible cell marker (MARCM) clones were generated using flies of genotype hs-FLP, UAS-GFP, tub-Gal4/Y, tub-Gal80 FRT40A/dUTX¹ FRT40A. The clones were induced by heat shocking pupae on days 8 and 9 for 2 hours at 37°C. After the second heat shock, flies were placed at 25°C and dissected and stained 1 day after clone induction.

Immunofluorescence staining
Immunofluorescence staining was performed as previously described (Cheng et al., 2008). The primary antibodies used were: rabbit anti-Zfh1 (1:500; from Ruth Lehmann, Skirball Institute of Biomolecular Medicine, NY, USA); mouse anti-Armadillo [1:100; developed by Eric Wieschaus (Princeton University, Princeton, NJ, USA) and obtained from Developmental Studies Hybridoma Bank (DSHB)]; rat anti-Vasa (1:100; developed by Allan Spradling and Dianne Williams and obtained from DSHB); rabbit anti-dUTX (1:2000; from Ali Shilatifard); rabbit anti-trimethyl-histone H3 (Lys27) (1:200; Millipore, #07-449); chicken anti-GFP (1:1000; Abcam, #13970); rabbit anti-Stat92E (1:800; from Denise Montell, Johns Hopkins School of Medicine, Baltimore, MD, USA); guinea pig anti-Traffic jam (1:3000; from Mark Van Doren); rabbit anti-phospho-histone H3 (Ser10) (1:2000; Millipore, #06-570); and rabbit anti-Caspase 3 (1:100; BD Biosciences, #610322).

Isolation of total RNA and quantitative reverse transcription PCR (qRT-PCR)
Total RNA was isolated from wild-type (wt) and dUTX three instar larval testes using TRIzol reagent (Invitrogen, #15956-018) according to the manufacturer’s instructions. Yield and quality of RNA were determined with a NanoDrop spectrometer (NanoDrop Technology, San Diego, CA, USA). Reverse transcription was performed using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, #K1621). Transcript levels were measured using SYBR Green PCR Master Mix (Fermentas, #K0221) and normalized to fringe. Primers used for qRT-PCR are listed in supplementary material Table S1.

Chromatin immunoprecipitation (Chip)
Chip was performed as described (Gan et al., 2010). For each biological replicate we dissected 200 pairs of dUTX testes and 200 pairs of wt testes. Primers used for qPCR are listed in supplementary material Table S1.

Statistical analysis
Statistical significance was calculated using two-tailed Student’s t-test or Fisher’s test. P-values are indicated in figures or in figure legends. Error bars indicate s.d.

RESULTS
dUTX prevents overpopulation of Zfh1-expressing cells around the hub
dUTX encodes a histone demethylase that has been shown to remove H3K27me3 in somatic cells (Smith et al., 2008). To study the effect of dUTX loss on the level of H3K27me3 in testes, we used a strong loss-of-function allele of dUTX (dUTX¹) (Herz et al., 2010). The dUTX¹/Df hemizygous flies (referred to hereafter as dUTX) are adult lethal, but survive up to the early pupal stage. Because of the adult lethality, analysis of H3K27me3 levels in adult dUTX testes required the FLP/FRT recombination system (Xu and Rubin, 1993). Immunoreactivity with a dUTX-specific antibody raised against the N-terminal 153 residues (Smith et al., 2008) was absent in dUTX¹ germline clones (supplementary material Fig. S1A,A*)*, suggesting that dUTX¹ is a strong loss-of-function allele. Consistent with the H3K27me3-specific demethylase activity (Herz et al., 2010), dUTX homozygous germline clones showed an increase of the H3K27me3 signal using an H3K27me3-specific antibody (Chen et al., 2011) when compared with the neighboring heterozygous germ cells (supplementary material Fig. S1B,B*), demonstrating that dUTX acts as an H3K27me3 demethylase in germ cells. Using the MARCM system, we generated dUTX mutant cyst cell clones that are positively labeled by GFP (Lee and Luo, 1999), which showed increased H3K27me3 signal compared with a neighboring wild-type (wt) cyst cell (supplementary material Fig. S1C-C*). These data demonstrate that dUTX also acts as an H3K27me3 demethylase in cyst cells in the testis.

To determine the function(s) of dUTX in the male GSC niche, we analyzed testes isolated from the third instar larvae of dUTX mutant males. Using antibodies against Armadillo (Arm) to label hub cells and zinc finger homeodomain 1 (Zfh1) to label CySCs and early cyst cells (Leatherman and Dinardo, 2008), we detected niche
architectural defects in dUTX tests. In wt testes, Zfh1-expressing CySCs surround GSCs and extend thin protrusions toward the hub, while their nuclei remain one cell diameter away from the hub (Fig. 1B, B'). However, 48% of dUTX testes had three or more Zfh1-expressing cells with their nuclei directly contacting the hub (Fig. 1C, C'; arrows; Fig. 2D, compare the first and second columns). These Zfh1-expressing cells with nuclei that directly contact hub cells stabilized for Traffic jam (TJ), a transcription factor expressed in early cyst cell nuclei (Li et al., 2003), suggesting that they retain their identity as early cyst cells (data not shown). Overpopulation of Zfh1-expressing cells was not, however, accompanied by an increase in the overall number of Zfh1-expressing cells surrounding the hub [30.6±6.6 in wt testes (n=27) versus 31±9.5 in dUTX testes (n=30), P>0.05]. These results suggest that loss of dUTX does not affect Zfh1-expressing cell number but rather their behavior, which causes the Zfh1-expressing cells to overpopulate around the hub area.

**dUTX acts in CySCs and early cyst cells to prevent overpopulation of Zfh1-expressing cells around the hub**

To determine in which cell type dUTX acts to prevent overpopulation of Zfh1-expressing cells around the hub, different cell type-specific Gal4 drivers were used in combination with a UAS-dUTX small hairpin microRNA (shmiRNA) (Ni et al., 2011) to knockdown dUTX in a cell type-specific manner. Knockdown of dUTX exclusively in germ cells using nanos (nos)-Gal4 (Van Doren et al., 1998) (supplementary material Fig. S2A, A'), or in hub cells using upd-Gal4 (Boyle et al., 2007) (supplementary material Fig. S2B, B'), did not lead to overpopulation of Zfh1-expressing cells around the hub. By contrast, knockdown of dUTX using the cyst cell driver c587-Gal4 (Manseau et al., 1997) led to a 45% increase in testes with an overpopulation of Zfh1-expressing cells around the hub (Fig. 2A, A', arrows; 2D, compare the third and fourth columns). There was also an overpopulation of Zfh1-expressing cells around the hub in 30% of c587-Gal4 control males, which was probably due to Gal4 expression in cyst cells, as a similar phenotype was observed in 35% of testes carrying another cyst cell-specific driver,eya-Gal4 (Leatherman and Dinardo, 2008).

To confirm that the upd-Gal4 driving dUTX shmiRNA did reduce dUTX levels in hub cells, we stained testes from upd>dUTX shmiRNA males with the H3K27me3 antibody. As a control, the c587>dUTX shmiRNA testes were stained with the same antibody. The H3K27me3 signal in hub cells from upd>dUTX shmiRNA testes was higher than that in neighboring germ cells, which had normal levels of dUTX (supplementary material Fig. S2C, C'). By contrast, the H3K27me3 signal in hub cells from c587>dUTX shmiRNA testes was similar to that in the neighboring germ cells; in this genotype, both hub cells and germ cells have normal dUTX levels (supplementary material Fig. S2D, D'). These results demonstrate that normal function of dUTX is required in CySCs and/or early cyst cells, but not in hub cells, to prevent the overpopulation of Zfh1-expressing cells around the hub.

**The function of dUTX in CySCs and early cyst cells depends on its demethylase activity**

dUTX was reported to demethylate H3K27me3 via its catalytic JmJc domain (Smith et al., 2008). To determine whether the demethylase activity of dUTX is required for its function in CySCs and early cyst cells, dUTX<sup>Δtm1c</sup> (Herz et al., 2010) was driven by the c587-Gal4 driver in the dUTX mutant background. As a control, wild-type dUTX was expressed using the same driver. The overpopulation of Zfh1-expressing cells in dUTX testes was rescued significantly by the wild-type dUTX transgene (Fig. 2B, B' and 2D, compare the fifth and sixth columns), but not by the dUTX<sup>Δtm1c</sup> transgene (Fig. 2C, C' and 2D, compare the fifth and seventh columns). However, even the wild-type transgene did not completely rescue overpopulation of Zfh1-expressing cells around the hub. This could be due to insufficient expression or inappropriate expression timing using cDNA transgenes. In summary, these data demonstrate that the demethylase activity of dUTX is required to maintain proper niche architecture.

**dUTX demethylates H3K27me3 at the Socs36E genomic locus for its active transcription**

Because the overpopulation of Zfh1-expressing cells around the hub in dUTX testes resembled the reported loss-of-function phenotype of the Socs36E gene (Issigonis et al., 2009), we used qRT-PCR to measure the Socs36E transcript level in dUTX testes. Since Socs36E is expressed specifically in hub cells and CySCs (Terry et al., 2006), we used the constitutively expressed somatic gene eya-Gal4 as an internal control. Indeed, we found that the Socs36E transcript level in dUTX testes decreased to ~65% of the level in the wt control (Fig. 3A). However, using the entire testes might underestimate the change in Socs36E transcript level.

Previously, chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) data revealed that both the active histone modification H3K4me3 and RNA Pol II are enriched near the transcription start site (TSS) of Socs36E (Gan et al., 2010) (supplementary material Fig. S3A). By contrast, the repressive
histone modification H3K27me3 was depleted at the same region around the TSS of Socs36E (Gan et al., 2010) (supplementary material Fig. S3A). Because dUTX is an H3K27me3-specific demethylase (supplementary material Fig. S1B-C′′′), we examined whether dUTX is required to remove H3K27me3 from the Socs36E TSS region, using anti-H3K27me3 ChIP followed by qPCR analysis. To generate high-resolution ChIP data, a 2 kb genomic region around the Socs36E TSS was divided into 400 bp intervals and tested for H3K27me3 binding using a series of primer sets (p1-p5 in Fig. 3B; supplementary material Fig. S3A). Control and tested for H3K27me3 binding using a series of primer sets (p1-p5 in Fig. 3B; supplementary material Fig. S3A). Consistent with decreased transcription of Socs36E in dUTX testes (Fig. 3A), there was a ~4-fold enrichment of the repressive H3K27me3 mark at the p2 region in the dUTX testes compared with the wt control (Fig. 3B). The H3K27me3 binding profile at the Socs36E locus was consistent with the published ChIP-seq results (Gan et al., 2010), which showed a peak enrichment of H3K27me3 at ~200-400 bp downstream of the TSSs of target genes. By contrast, the control regions showed similar H3K27me3 binding between dUTX and wt testes (supplementary material Fig. S3A,B, see numbers underneath control regions p6-p8). Furthermore, recently published ChIP-chip data using anti-dUTX antibody showed enrichment of dUTX around the Socs36E TSS region (supplementary material Fig. S3C) (Tie et al., 2012). Taken together, these results demonstrate that dUTX directly regulates Socs36E transcription by removing the repressive H3K27me3 histone modification from its TSS region.

We next examined whether overexpression of Socs36E independent of its genomic context is sufficient to rescue the niche architectural defects in dUTX testes. To achieve this, a UAS-Socs36E cDNA transgene (Callus and Matthey-Prevot, 2002) was driven by either the upd-Gal4 (Callus and Matthey-Prevot, 2002) or the c587-Gal4 driver. Whereas upd-Socs36E failed to suppress the dUTX phenotype (Fig. 3C, arrows point to Zfh1-positive cells around the hub), c587>Socs36E reduced the overpopulation of Zfh1-expressing cells around the hub in 93% of dUTX testes (Fig. 3D), further suggesting that Socs36E is a critical target gene of dUTX in CySCs. In summary, dUTX acts in CySCs and/or early cyst cells to directly regulate the chromatin state of the Socs36E gene locus.

**dUTX activates Socs36E transcription to control JAK-STAT signaling activity in the testis niche**

Because Socs36E acts as a negative regulator of the JAK-STAT pathway (Terry et al., 2006), we assessed JAK-STAT signaling activity in the presence and absence of dUTX. In wt testes, Stat92E is enriched in GSCs and in some of their immediate daughter cells, called gonialblasts (GBs), but rapidly declines in further differentiated cells. Stat92E is also present in CySCs but is absent in hub cells (Leatherman and Dinardo, 2008) (Fig. 4A,A′). By contrast, Stat92E was ectopically turned on in dUTX testes (Fig. 4B,B′), including hub cells and further differentiated somatic cells. Using a 2×Stat-GFP reporter, which reflects Stat92E activity in CySCs (Bach et al., 2007), we found that the GFP reporter was ectopically turned on in further differentiated cyst cells in dUTX mutant testes (supplementary material Fig. S4B,B′), but not in the heterozygous control (supplementary material Fig. S4A,A′).

By qRT-PCR, we detected a ~1.5-fold increase of the Stat92E transcript in dUTX testes compared with the wt control (Fig. 4C). However, because we used whole testes for this analysis, the change in Stat92E transcript levels might be underestimated. Furthermore, knockdown of dUTX using the cyst cell driver c587-Gal4 (Fig. 4E,E′), but not the germ cell driver nos-Gal4 (supplementary material Fig. S4C,C′ versus S4D,D′) nor the hub cell driver upd-Gal4 (supplementary material Fig. S4E,E′ versus S4F,F′), led to ectopic Stat92E in further differentiated cells, similar to the phenotype observed in dUTX mutant testes. In addition, removing one copy of Stat92E using a strong loss-of-function allele (Hou et
al., 1996) suppressed the overpopulation of Zfh1-expressing cells around the hub in 90% of dUTX testes (Fig. 4F,F′), suggesting that hyperactivation of JAK-STAT signaling causes the niche architectural defects in dUTX testes. Together, these results indicate that dUTX acts in CySCs and early cyst cells to prevent ectopic JAK-STAT signaling activity.

**dUTX acts in CySCs to maintain proper gene expression in hub cells**

Our results also revealed dynamic communication among different cell types within the testis niche, where CySCs can send feedback to hub cells to maintain proper gene expression. We found that zfh1, a target gene of the Stat92E transcription factor (Leatherman and Dinardo, 2008; Terry et al., 2006), was ectopically expressed in hub cells in 92% of dUTX testes (Fig. 1C,C′; Fig. 2E, compare the first and second columns). However, the total number of hub cells did not change in dUTX testes [13.6±2.3 hub cells for dUTX third instar testes (n=15) versus 13.6±2.0 hub cells for wt third instar testes (n=18), P>0.05]. In addition, no hub cells underwent cell death in dUTX testes as determined by immunostaining with anti-Caspase 3, an apoptotic marker (n=30), and none underwent mitosis as determined by immunostaining with anti-phospho-histone H3 (H3S10P; n=96), suggesting that hub cells maintain their number but turn on Zfh1 expression ectopically.

Because both the hub cells and cyst cells in adult testes originate from the same group of somatic gonadal precursors (SGPs) in embryonic testes (Le Bras and Van Doren, 2006), one possibility for ectopic Zfh1 expression in hub cells from adult testes is that Zfh1 becomes misexpressed in hub precursor cells in dUTX embryonic testes. In order to test this possibility, we induced dUTXm mutant mitotic clones in adult testes and found it to be sufficient to cause Zfh1 misexpression in hub cells (supplementary material Fig. S5). These results suggest that ectopic Zfh1 expression in hub cells is due to loss of dUTX in CySCs and/or GSCs, the two cell types capable of forming mitotic clones next to the hub cells. Furthermore, we found that shmiRNA knockdown of dUTX in CySCs and/or early cyst cells using c587-Gal4 (Fig. 2A,A′ and 2E, compare the third and fourth columns) is sufficient to turn on Zfh1 expression ectopically in hub cells. By contrast, neither nos-Gal4 (supplementary material Fig. S2A,A′) nor upd-Gal4 (supplementary material Fig. S2B,B′) driving dUTX shmiRNA resulted in a similar phenotype. Together, these data demonstrate that loss of dUTX in CySCs leads to ectopic Zfh1 expression in hub cells.

In addition, we found that the catalytic domain of dUTX is required to prevent ectopic Zfh1 expression in hub cells, as expression of the wild-type dUTX transgene rescued this phenotype (Fig. 2B,B′ and 2E, compare the fifth and sixth columns). We also observed partial rescue upon expression of the dUTXmC transgene (Fig. 2C,C′ and 2E, compare the fifth and seventh columns), suggesting a demethylase-independent role of dUTX in regulating proper gene expression in hub cells. Finally, restoring Socs36E expression in CySCs and early cyst cells (Fig. 3D) or removing one copy of Stat92E (Fig. 4F,F′) reduced ectopic Zfh1 expression in the hub cells of dUTX testes from 92% to 43-45%. Together, these data demonstrate that dUTX acts primarily as a histone demethylase in CySCs to prevent ectopic Zfh1 expression in hub cells by maintaining proper JAK-STAT signaling activity.

**dUTX maintains hub architecture by regulating DE-Cadherin levels in GSCs**

We found that dUTX also acts in germ cells to maintain proper hub size. Whereas dUTX directly regulates Socs36E transcription, unlike Socs36E testes (Issigonis et al., 2009) dUTX testes do not have decreased GSC numbers [12.8±3.0 for dUTX third instar testes (n=98) versus 12.6±3.0 for wt third instar testes (n=80), P>0.05]. This was due to a significant increase in hub area (Fig. 5A-C) in dUTX testes, which accommodated the overpopulation of Zfh1-expressing cells around the hub without affecting GSC number. As mentioned previously, the increase in hub area in dUTX testes could not be attributed to an increase in hub cell number. However, we did observe an increase in individual hub cell size in dUTX testes compared with the wt control (Fig. 5D). In addition, knockdown of dUTX using the germ cell driver nos-Gal4, but not the cyst cell driver c587-Gal4 nor the hub cell driver upd-Gal4, led to an increased hub area (Fig. 5E).

In wt testes, GSCs are attached to the hub via DE-Cadherin-mediated adherens junctions (Jenkins et al., 2003; Yamashita et al., 2003), resulting in a rosette-like structure (Fig. 5A,A′). The GSC-hub interface in wt testes averaged 4.3 μm (Fig. 6C, first column).

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**Fig. 3. dUTX removes the repressive H3K27me3 histone modification at the Socs36E genomic locus and allows active transcription of Socs36E.** (A) Socs36E mRNA measured by qRT-PCR in three independent biological replicates, normalized by fringe. (B) Anti-H3K27me3 ChiPed DNA analyzed by qPCR, normalized to input (percentage input) and then compared between dUTX testes and wt controls, based on three independent biological replicates. *P*-value calculated using Student’s t-test. Error bars represent s.d.

(CD) Immunostaining using antibodies against Arm (blue), Vasa (green) and Zfh1 (red). Hub area is outlined (white dotted line). (C) upd-Gal4; UAS-Socs36E-cDNA transgene in a dUTX background; arrows point to overpopulating Zfh1-expressing cells with nuclei that directly contact the hub. (D) c587-Gal4; UAS-Socs36E-cDNA transgene in a dUTX background. Scale bars: 10 μm.
By contrast, the GSC-hub interface in dUTX testes was disrupted (Fig. 5B,B', arrows). GSCs appeared to intrude into the hub area, leading to an increase of the GSC-hub interface to an average of 5.9 μm (Fig. 6C, second column). We examined whether this defect in the dUTX mutant niche is due to misregulation of DE-Cadherin. Using qRT-PCR we detected a ~2-fold increase in the DE-Cadherin (shotgun – FlyBase) transcript level in dUTX testes compared with that in the wt control (Fig. 6D). Additionally, we found that expression of a dominant-negative form of DE-Cadherin (UAS-DE-CadDEFL) (Inaba et al., 2010) in germ cells suppressed the dUTX hub size defect (Fig. 6A,A',E) and resulted in a decrease of the GSC-hub interface (Fig. 6C, third column). By contrast, overexpression of the wild-type DE-Cadherin (UAS-DE-CadCRkh) (Inaba et al., 2010) in germ cells enhanced the dUTX hub size defect (Fig. 6B,B',E) and led to an increase of the GSC-hub interface (Fig. 6B, arrows, and 6C, fourth column). As a control, when both forms of DE-Cadherin were expressed in germ cells in the wt background, no obvious defect was detected (Fig. 6C,E). DE-Cadherin is unlikely to be the only target gene of dUTX in germ cells. Therefore, although mutations in dUTX led to upregulated DE-Cadherin transcript levels, overexpression of DE-Cadherin itself in germ cells is not sufficient to recapitulate the dUTX loss-of-function phenotype. In summary, our data demonstrate that dUTX acts in germ cells to maintain the proper GSC-hub interface and hub size by regulating DE-Cadherin transcription.

DISCUSSION

In this study, we identify a new epigenetic mechanism that negatively regulates the JAK-STAT signaling pathway in the Drosophila testis niche (Fig. 6F): the H3K27me3-specific demethylase dUTX acts in CySCs to remove the repressive H3K27me3 histone modification near the TSS of Socs36E to allow its active transcription. Socs36E acts upstream to suppress Stat92E activity and to restrict CySCs from overpopulating the testis niche. In addition, dUTX acts in CySCs to prevent hyperactivation of Stat92E in hub cells, which would otherwise ectopically turn on Zhf1 expression. When we ectopically drove zhf1 CDNA in hub cells using the upd-Gal4 driver, no obvious defect could be identified. Therefore, the biological consequence of ectopic Zhf1 expression in hub cells remains unclear. However, ectopic Zhf1 expression in hub cells and the overpopulation of Zhf1-expressing cells around the hub are two connected phenomena because both phenotypes are caused by loss of dUTX in CySCs.

dUTX also acts in GSCs to regulate DE-Cadherin levels to maintain proper GSC-hub interaction and normal morphology of the hub. It has been reported that differential expression of different cadherins causes cells with similar cadherin types and levels to aggregate (Friedlander et al., 1989; Steinberg and Takeichi, 1994). In wt testes, hub cells express higher levels of DE-Cadherin and therefore tightly associate with each other (Le Bras and Van Doren, 2006). Loss of dUTX in germ cells leads to higher levels of DE-Cadherin in GSCs, which probably allows them to intermingle with hub cells and causes disrupted hub architecture. It has also been demonstrated that the major role of JAK-STAT in GSCs is for GSC-hub adhesion (Leatherman and Dinardo, 2010), suggesting that the expression and/or activity of cell-cell adhesion molecules, such as DE-Cadherin, depends on JAK-STAT signaling. Therefore, the abnormal DE-Cadherin activity in GSCs in dUTX testes could also result from misregulated JAK-STAT signaling in the testis niche.

**dUTX is a new negative epigenetic regulator of the JAK-STAT signaling pathway**

The JAK-STAT signaling pathway plays crucial roles in stem cell maintenance in many different stem cell types across a wide range of species. Here, our studies identify the histone demethylase dUTX as a new upstream regulator of the JAK-STAT pathway, which directly controls the transcription of Socs36E. In addition to acting as an antagonist of JAK-STAT signaling, Socs36E has been reported to be a direct target gene of the Stat92E transcription factor (Terry et al., 2006). Therefore, increased Stat92E would be expected to upregulate Socs36E expression, but this was not observed in dUTX mutant testes. Instead, our data revealed that Socs36E expression decreased, whereas Stat92E expression increased, in dUTX testes, consistent with the hypothesis that Socs36E is a direct target gene of dUTX and acts upstream of Stat92E.
Sustained activity of the JAK-STAT pathway in cyst cells has been reported to activate BMP signaling, which leads to GSC self-renewal outside the niche and gives rise to a tumor-like phenotype in testis (Leatherman and Dinardo, 2010). To examine BMP pathway activity, we performed immunostaining experiments using antibodies against phospho-SMAD (pSMAD), a downstream target of BMP signaling. We did not detect any obvious difference in the pSMAD signal between the downstream target of BMP signaling. We did not detect any using antibodies against phospho-SMAD (pSMAD), a pathway activity, we performed immunostaining experiments in testis (Leatherman and Dinardo, 2010). To examine BMP renewal outside the niche and gives rise to a tumor-like phenotype (marbles (bam)) in GSCs (Chen and McKearin, 2003; López-Onieva et al., 2008). In the Drosophila intestinal stem cell (ISC) niche, the visceral muscle cells underlying the intestine secrete Wingless to activate Wnt signaling and Upd to activate JAK-STAT signaling in ISC, which are required to maintain ISC identity and activity (Beebe et al., 2010; Jiang et al., 2009; Lin et al., 2008; Lin et al., 2010; Xu et al., 2011).

More studies have now revealed the multidirectionality of signaling within the stem cell niche. For example, in the Drosophila female GSC niche, GSCs activate Epidermal growth factor receptor (Egfr) signaling in the neighboring somatic cells, which subsequently represses expression of the glypicanc Dally, a protein required for the stabilization and mobilization of the BMP pathway ligand Dpp. Through this communication between GSCs and the surrounding somatic cells, only GSCs maintain high BMP signaling (Liu et al., 2010). Here, our studies establish another example of the multidimensional cell-cell communications that occur within the testis stem cell niche, where CySCs and GSCs have distinct roles in regulating hub cell identity and morphology.

**Distinct biological functions of histone demethylases**

Our data identified new roles of a histone demethylase in regulating endogenous stem cell niche architecture and proper gene expression. Previous studies have reported in vivo functions of histone demethylases in several model organisms. For example, mammalian UTX has been shown to associate with the H3K4me3 histone methyltransferase MLL2 (Ishaeva et al., 2007), suggesting its potential antagonistic role to the PcG proteins. The PcG proteins play a crucial role in Hox gene silencing in both Drosophila and mammals (Beuchle et al., 2001; Ringrose and Paro, 2007; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). Consistently, mammalian UTX has been reported to directly bind and activate the HOXBI gene locus (Agger et al., 2007). In addition to antagonizing PcG function, H3K27me3 demethylases play crucial roles during development. For example, in zebrafish, inactivating the UTX homolog (kdm6al) using morpholino oligonucleotides leads to defects in posterior development (Lan et al., 2007), and in C. elegans the dUTX homolog (UTX-1) is required for embryonic and postembryonic development (Vandamme et al., 2012), including gonad development (Agger et al., 2007). Furthermore, loss of UTX function in embryonic stem cells leads to defects in mesoderm differentiation (Wang et al., 2012), and somatic cells derived from UTX loss-of-function human or mouse tissue fail to return to the ground state of pluripotency (Mansour et al., 2012). These reports demonstrate that UTX is not only required for proper cellular differentiation but also for successful reprogramming. However,
dUTX regulates fly testis niche.

(A-B′) Immunostaining for Arm (blue) and Vasa (green). Hub area is outlined (white dotted line). (A,A′) dUTX; nos>DE-CadDEFL; control (4.3±0.4 μm); dUTX; nos>DE-CadDEFL; mutant; (4.9±0.5 μm); dUTX; nos>DE-CaddCR4h; control (4.6±0.3 μm); dUTX+/+; nos>DE-CaddCR4h; control (4.5±0.4 μm). (B,B′) DE-Cadherin mRNA measured by qRT-PCR in three independent biological replicates, normalized by Rpl32. (C) Quantification of the average GSC-hub interface in testes from males of the following genotypes: wt (4.3±0.4 μm); dUTX; nos>DE-CadDEFL; control (4.6±0.3 μm); dUTX+/+; nos>DE-CaddCR4h; control (4.5±0.4 μm). Error bars represent s.d. Scale bars: 10 μm.

In summary, our results demonstrate that stem cells send feedback to the niche cells to maintain their proper gene expression and morphology. Furthermore, this feedback is regulated through the JAK-STAT signaling pathway, the activity of which is controlled by a chromatin factor, providing an example of crosstalk between these two regulatory pathways.

Acknowledgements

We thank Drs Ruth Lehmann, Denise Montell and the Developmental Studies Hybridoma Bank for antibodies; Drs Doug Harrison, Mark Van Doren, Erika Matunis, Allan Spradling, Norbert Perrimon, Bernard Callus, Yukiko Yamashita, Erika Bach, the Bloomington Stock Center, and the TRiP at Harvard Medical School for generously providing fly stocks; and Drs Mark Van Doren, Haiqing Zhao and X.C. laboratory members for critical reading and suggestions to this manuscript.


Fig. S1. dUTX acts as an H3K27me3-specific demethylase in germ cells and cyst cells. (A-A") wt testis with dUTX clones generated by conventional FLP/FRT system and immunostained with antibodies against β-Gal (green) and dUTX (red). An outlined dUTX<sup>−/−</sup> clone negative for lacZ signal is absent for dUTX staining. (B-B") wt (dUTX<sup>+</sup>)<sup>+/−</sup> testis with dUTX<sup>−/−</sup> clones generated by conventional FLP/FRT system and immunostained with antibodies against GFP (green) and H3K27me3 (red). dUTX<sup>−/−</sup> clone negative for GFP signal (outlined by white dotted line) has increased H3K27me3 signal. Scale bars: 20 µm. (C-C")<sup>′</sup> wt (dUTX<sup>−/−</sup>) testis with dUTX<sup>−/−</sup> clones generated by MARCM system and immunostained with antibodies against Tj (blue), GFP (green) and H3K27me3 (red). dUTX<sup>−/−</sup> GFP-positive cyst cell (outlined by white dotted line) has increased H3K27me3 signal compared with neighboring heterozygous cyst cell (outlined by yellow dotted line). Scale bar: 10 µm. Hub labeled by asterisks.
Fig. S2. dUTX is not required in germ cells or hub cells to prevent overpopulation of Zfh1-expressing cells around the hub area or ectopic Zfh1 expression in hub cells. (A-B') Immunostaining using antibodies against Arm (blue), Vasa (green) and Zfh1 (red) in testes with the following genotypes: (A) nos-Gal4 control, (A') nos-Gal4; UAS-dUTX shmiRNA, (B) upd-Gal4 control, (B') upd-Gal4; UAS-dUTX shmiRNA. (C-D') Immunostaining using antibodies against H3K27me3 (red) on testes with the following genotypes: (C,C') upd-Gal4; UAS-dUTX shmiRNA, (D,D') c587-Gal4; UAS-dUTX shmiRNA. Hub area outlined with white dotted line. Scale bars: 10 µm.
Fig. S3. Genome browser screenshots show chromatin state of *Socs36E* and *fringe* genomic loci. (A,B) ChIP-seq using antibodies against H3K27me3 (red), H3K4me3 (green) and RNA Pol II (blue) (Gan et al., 2010) at (A) *Socs36E* and (B) *fringe* genomic regions. A 2 kb region around the TSS for each gene is outlined by the brown solid lined box. The five amplicons (p1-p5) used in ChIP-qPCR experiments are labeled in A, which span across the TSS of *Socs36E*. The p6 amplicon is located inside the *Socs36E* gene body. The p7 and p8 amplicons are near the TSS of *fringe*. Numbers underneath p6-p8 denote anti-H3K27me3 ChIPed DNA in *dUTX* testes normalized by input followed by comparison with wt testes, based on three independent biological replicates. (C) ChIP-chip using anti-dUTX shows enrichment of dUTX near the TSS of the *Socs36E* gene (Tie et al., 2012); p1-p5 and p6 are labeled.
Fig. S4. dUTX is required in cyst cells, but not in germ cells or hub cells, to maintain proper Stat92E expression in the niche. (A-B’) Immunostaining using antibodies against Arm (blue), Vasa (red) and GFP (green) in testes with the following genotypes: (A,A’) dUTX/+; 2xStat92E-GFP control, (B,B’) dUTX−/−; 2xStat92E-GFP. (C-F’) Immunostaining using antibodies against Arm (green) and Stat92E (red) in testes with the following genotypes: (C,C’) nos-Gal4 control, (D,D’) nos-Gal4; UAS-dUTX shmiRNA, (E,E’) upd-Gal4 control, (F,F’) upd-Gal4; UAS-dUTX shmiRNA. Hub area outlined with white dotted line. Scale bars: 10 µm.
**Fig. S5.** Zfh1 is ectopically expressed in hub cells from testes with \(dUTX^l\) clones. (A-A‴) Immunostaining using antibodies against GFP (green), Arm (blue) and Zfh1 (red) in a \(dUTX^o\) clone-containing testis. Scale bars: 10 \(\mu\)m.
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