X-linked H3K27me3 demethylase Utx is required for embryonic development in a sex-specific manner

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X-linked H3K27me3 demethylase Utx is required for embryonic development in a sex-specific manner

G. Grant Welstead, Menno P. Creyghton, Steve Bilodeau, Albert W. Cheng, Styliani Markoulaki, Richard A. Young, and Rudolf Jaenisch

Embryogenesis requires the timely and coordinated activation of developmental regulators. It has been suggested that the recently discovered class of histone demethylases (UTX and JMJD3) that specifically target the repressive H3K27me3 modification play an important role in the activation of “bivalent” genes in response to specific developmental cues. To determine the requirements for UTX in pluripotency and development, we have generated Utx-null ES cells and mutant mice. The loss of UTX had a profound effect during embryogenesis. Utx-null embryos had reduced somite counts, neural tube closure defects and heart malformation that presented between E9.5 and E13.5. Unexpectedly, homozygous mutant female embryos were more severely affected than hemizygous mutant male embryos. In fact, we observed the survival of a subset of UTX-deficient males that were smaller in size and had reduced lifespan. Interestingly, these animals were fertile with normal spermatogenesis. Consistent with a mid-gestation lethality, UTX-null male and female ES cells gave rise to all three germ layers in teratoma assays, though sex-specific differences could be observed in the activation of developmental regulators in embryoid body assays. Lastly, ChIP-seq analysis revealed an increase in H3K27me3 in Utx-null male ES cells. In summary, our data demonstrate sex-specific requirements for this X-linked gene while suggesting a role for UTY during development.

KDM6A | polycomb

During differentiation and embryogenesis, developmental genes are temporally and spatially regulated by multiple cellular mechanisms. Specific histone modifications such as methylation of lysine residues are associated with either the active or silent state of gene transcription. For example, histone 3 lysine 4 trimethylation (H3K4me3) is commonly found at transcriptional start sites and is generally a mark for transcriptional initiation, whereas histone 3 lysine trimethylation (H3K27me3) is associated with repressed gene expression (1–3). The presence of both H3K4me3 and H3K27me3 at the same gene is termed the “bivalent state” (4). In ES cells, developmental regulators are in a “bivalent state” that creates a poised condition for activation during differentiation (5). To understand the functional role of histone modifications, it is important to understand how bivalent domains are maintained in ES cells and resolved during differentiation.

Although the machinery that deposits the trimethyl modification on histone H3K27, polycomb repressive complex 2 (PRC2), has been studied extensively, the class of enzymes that remove methyl marks from this lysine was only recently identified (6–9). UTX, UTY, and JMJD3 form a family of JmJC-domain containing proteins that specifically demethylate H3K27me3/me2. UTX is encoded on the X chromosome but escapes X inactivation in females and is ubiquitously expressed (10). In males, its homolog on the Y chromosome, UTY, shares similar patterns of expression with some sex-specific differences (11). Both proteins contain tetratricopeptide repeats in their amino terminus that mediate protein–protein interactions, whereas the JmJC domain is located in the C terminus. However, UTY is not active when tested in vitro, although the amino acids critical for demethylase function are conserved (6, 7, 12).

To further understand the events regulating H3K27me3 in ES cells and during development, we generated Utx-null ESCs and mutant mice. We demonstrate a significant difference in the phenotype of male and female UTX-deficient embryos. Although a small number of Utx-null males survived postnatally, female mutant embryos died at midgestation. The surviving mutant males were smaller than control mice but were fertile with normal spermatogenesis. We derived both male and female Utx-null ES cells from the inner cell mass of blastocysts and show that UTX is dispensable for ES cell self-renewal and germ layer specification consistent with the in vivo phenotype. However, when mutant ES cells were differentiated, differences in gene activation were detected. Furthermore, in the absence of UTX, H3K27me3 levels were increased and H3K4me3 levels were decreased at developmental regulators. Our results demonstrate a requirement for UTX and suggest an important role for its Y-homolog, UTY, in male embryogenesis.

Results

UTX is Critical for Embryonic Development. To study the function of UTX in ES cells and embryogenesis, male UtxKO ES cells were generated by gene targeting of V6.5 ES cells using a “knockout first” construct designed by EUCOMM (Fig. S1A). Correctly targeted cells (UtxKO) displayed hypomorphic expression of UTX, which was completely ablated after Cre addition (UtxKO). Conversely, when the cells were treated with Flipase, UTX expression was restored to wild-type levels (UtxWt) (Fig. S1B). All three ES cell lines were injected into blastocysts to generate chimeras. Both the UtxKO and UtxWt ES-cell-injected blastocysts gave rise to chimeras that transmitted the mutant allele.

To generate UTX-null animals, conditional mutant males with the germ-line active Nestin-cre allele (see experimental procedures) were crossed with UtxKO/Wt females to generate a 1:1:1:1 expected ratio of UtxKO and UtxKO/Wt homozygous and UtxKO/KO females. Table 1 shows that UtxKO males were underrepresented in the progeny of this cross indicating that the loss of Utx is partially lethal for males. Significantly, no live UtxKO/KO females were identified, indicating fully penetrant lethality.

To determine the time of lethality, embryos at different stages of gestation were isolated, characterized, and genotyped. Consistent with loss of embryos during gestation, we observed a high frequency of resorptions at embryonic day (E) 12.5 and E13.5 (Table 1). Importantly, only abnormal or partially resorbed Utx-null female embryos were detected at E11.5, and no live female...
embryos were detected after E12.5 (Fig. 1A). This finding indicates that UTX deficiency in female, in contrast to male embryos leads to fully penetrant embryonic lethality. Upon closer examination of the abnormal E10.5 embryos we detected heart malformations (although some of these embryos had heart muscle contractions) and neural tube closure defects. When embryos were isolated at E9.5, mutant female embryos displayed a delay in development as indicated by a reduced number of

<table>
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<tr>
<th>Age</th>
<th>Utx&lt;sup&gt;Wt&lt;/sup&gt; (%)</th>
<th>Utx&lt;sup&gt;KO&lt;/sup&gt; (%)</th>
<th>Subtotal (%)</th>
<th>Utx&lt;sup&gt;KO/Wt&lt;/sup&gt; (%)</th>
<th>Utx&lt;sup&gt;KO/KO&lt;/sup&gt; (%)</th>
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<td>E8.5</td>
<td>3 (60)</td>
<td>2 (40)</td>
<td>5</td>
<td>4 (57)</td>
<td>3 (43)</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>E9.5</td>
<td>4 (50)</td>
<td>4 (50)</td>
<td>8</td>
<td>6 (50)</td>
<td>6 (50)</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>E10.5</td>
<td>11 (42)</td>
<td>15 (58)</td>
<td>26</td>
<td>10 (50)</td>
<td>10 (50)</td>
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<tr>
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<td>61</td>
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This table provides a summary of observed progeny at different developmental time points obtained from matings between Utx<sup>Flx</sup> males with a germ-line Cre deleter (Ncre) and Utx<sup>KO/Wt</sup> females. Expected ratios and observed ratios are provided at the bottom with the latter calculated from the progeny genotyped at 3 wk of age. The timing of gestation was determined based on the morning of the appearance of a plug as being E0.5.

Fig. 1. UTX is required for development in a sex-specific manner. (A) Utx is required for development of female embryos. Representative embryos isolated at the respective time from a natural mating between a mutant male and heterozygote female (Utx<sup>KO/Wt</sup>) are shown. All images are taken at the same magnification (8×) except for the E9.5 embryos, which were taken at a higher magnification (20×). (B) A representative litter from E9.5 that demonstrates the variability in phenotypes of Utx-null female embryos. (C) UTX-deficient males are smaller than wild-type males throughout their lifespan. Error bars represent the SD of the weight taken at that age. A representative image of a Utx-null male and wild-type littermate is shown. (D) UTX-deficient males die prematurely compared with wild-type littermates. The percent survival of wild-type (n = 42) and Utx<sup>KO</sup> males (n = 22) followed for 200 d after birth.
somites and a failure to turn. Fig. 1B demonstrates the spectrum of defects in Utx-deficient sibling females obtained from a single litter. In contrast to Utx homozygous mutant female embryos, hemizygous mutant male embryos displayed a milder phenotype. At E9.5, UtxKO males appeared relatively normal, but abnormalities were detected in some mutant embryos by E11.5. Importantly, however, normal appearing UtxKO male embryos were observed at E13.5 and later stages of gestation, though at a lower than expected frequency, indicating some loss of mutant male embryos before E13.5. (Fig. L4 and Table 1). This result indicates that loss of both Utx copies in females results in a more severe phenotype than deletion of Utx in males.

Although at a significantly reduced frequency than expected, postnatal UTX-deficient males were viable (Table 1). These males were smaller than their littermates at birth and throughout adulthood (Fig. 1C), which could already be detected in utero (Fig. L4). In addition, these animals had reduced survival compared with wild-type littermates (Fig. 1D). Although UtxKO ES cells did not contribute to the germ line in chimeric animals, UTX-deficient males displayed normal spermatogenesis and were functionally fertile when mated with wild-type females or UtxKO/Wt females (Fig. S2 and Table S1). Although it is possible that UTX plays a role in germ cell development as indicated by the UtxKO ES-derived chimeras (none had germ-line transmission of the mutant allele), our data suggest that UTX is not required for male fertility. Together, it is clear that there exists a sex-specific requirement for UTX during development.

**Utx Is Not Required for ES Cell Maintenance but Is Necessary for Proper Activation of Developmental Regulators.** To better understand the sex-specific loss of UTX mutant embryos, UtxKO male, UtxKO/Wt female, and heterozygous UtxKO/Flx female ES cells were isolated from the inner cell mass of explanted blastocysts. The three lines were derived from embryos of the same female, and the absence of UTX in the two male cell lines was confirmed by Western blot.

To determine whether UTX plays a role in maintaining the activity of key pluripotency genes, UTX-null male and female ES cells were grown under self-renewal conditions and stained for key pluripotency markers (Fig. S1C). The expression pattern of Oct3/4, Nanog, and SSEA-1 was unaffected by the loss of UTX. Similarly, we found that the deletion of UTX in ES cells did not affect the cell cycle and proliferation (Fig. S1D) of UTX-null ES cells. Thus, UTX does not play a role in ES cell self-renewal.

To investigate whether the loss of UTX has a critical role in differentiation, UtxKO ES cells were tested in a teratoma formation assay. Two independent UtxKO male ES cells (generated by in vitro targeting of existing V6.5 ES cells) and the two ES lines (UtxKO, UtxKO/Flx) derived from explanted blastocysts were compared with UtxKO/Flx UtxKO/Wt, and UtxKO/Flx control ES cells for their ability to generate all three germ layers. For all of the in vitro assays performed, there was no observed difference in phenotype between UtxKO and UtxKO/Wt (V6.5) male ES cells. The teratomas derived from UTX-deficient ES cells displayed tissues from all three germ layers, consistent with UTX-null embryos surviving to midgestation (Fig. 2A). Of note, however, was the presence of a large number of giant trophoblast cells in the teratomas derived from the UtxKO/Flx female ES cells; these trophoblast cells were not present in the other teratomas analyzed.

We used the embryoid body assay to further investigate the differentiation capacity of UTX-deficient ES cells. UtxKO, UtxKO/Flx, and UtxKO/Flx/Wt ES cells were plated as hanging drops in media without LIF to form embryoid bodies, replated 2 d later on nonadherent plates and allowed to differentiate for 10 d. Interestingly, the in vivo sexual dimorphism appeared to be phenocopied: H&E sections revealed a diminished differentiation potential for UtxKO/Wt female ES cells compared with the male UtxKO ES line. The heterozygous UtxKO/Flx/Wt ES cells displayed typical differentiation potential based on the presence of complex structures in each of the EBs (Fig. 2B).

Analysis of gene expression by quantitative PCR (qPCR) revealed differences in phenotype between UtxKO and UtxKO/Wt ES cells. qRT-PCR was performed on RNA harvested from ES cells and male and Table 1). This result indicates that loss of both Utx copies in females results in a more severe phenotype than deletion of Utx in males.

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that developmental regulators were not properly activated (Fig. 1B). Markers of all layers, endoderm (Gata4 and Gata6), ectoderm (Otx2), and mesoderm (T) were analyzed and found to lack proper activation. In contrast, pluripotency genes such as Nanog and Oct3/4 were appropriately silenced during differentiation. Although differences in gene expression were not observed for all developmental regulators tested, our data suggest that UTX is important for the correct and coordinated activation of several and that the loss of two copies of Utx in female ES cells has an increased effect over the loss of only one allele in male ES cells.

To exclude the possibility that UTX deficiency affects imprinting, the expression of all known imprinted genes was analyzed in UTX-null (UtxKO/KO) and heterozygous (UtxKO/Flx) female ES cells grown in self-renewal or differentiation conditions (+RA). No significant difference in the normalized expression was observed (Fig. S1E), indicating that abnormal imprinting is not a major cause for the difference in male and female mutant embryos. We also looked at whether the expression of UTY changes in the absence of UTX-deficient male embryos at E10.5. We performed quantitative PCR analysis on RNA harvested from the heads of wild-type and UTX-null male embryos and observed a slight decrease in UTY transcripts (Fig. S3A). Reduced embryo size or delayed development may explain the reduced level of UTY in the UTX-deficient male embryos.

**Loss of UTX in Male ES Cells Results in a Genome-Wide Increase of H3K27me3.** During ES cell differentiation, developmental regulators are activated, which coincides with the removal of H3K27me3 at their promoters. UTX is implicated in this process because it occupies the promoters of developmental genes during differentiation and the transient knockdown of UTX leads to an increase of H3K27me3 at these targets (6, 9). Consistent with this concept, UTX seems to be excluded from specific targets in ES cells under self-renewal conditions but is recruited to developmental regulators upon differentiation (8, 13). In H3K27me3 can functionally compete with PRC2 promoters of Hox genes in fibroblasts to regulate the steady state levels of H3K27me3 at these genes (9, 14). The possibility that UTX and PRC2 compete at promoters to regulate H3K27me3 levels in fibroblasts suggests that UTX may also function to modulate H3K27me3 at bivalent genes in ES cells.

To determine whether UTX has an unappreciated function in ES cells, we used chromatin immunoprecipitation followed by massively parallel DNA sequencing (ChIP-seq) and probed the deposition of H3K4me3 and H3K27me3 in male wild-type and male UtxKO ES cells grown under self-renewal or differentiation conditions. Visual inspection of density profiles for H3K27me3 at representative genes revealed that H3K27me3 levels increased at transcription start sites (Fig. 2D). These genes also showed a slight decrease in H3K4me3. In contrast, the loss of UTX did not result in the presence of H3K27me3 at pluripotency genes, such as Pou5f1, indicating that the increase is specific to bivalent genes (Fig. S3A). Genome-wide, the average levels of H3K27me3 were significantly increased for all H3K27me3 target genes in the UtxKO ES cells compared with UtxFlx control cells, whereas the average levels of H3K27me3 at active genes were maintained (Fig. S3B). These data indicate that UTX regulates the levels of H3K27me3 in ES cells and this may affect their potential for differentiation.

**UTX-Deficient ES Cells Are Responsive to Retinoic Acid (RA)-Induced Differentiation.** To determine whether Utx-deficient ES cells are less responsive to differentiation as a result of the increased levels of H3K27me3 at developmental regulators, ChIP-seq analysis was performed on ES cells differentiated with RA. Previous reports have shown that UTX is required for gene activation following RA treatment by removing H3K27me3 (6, 9). UtxKO and UtxFlx ES cells were treated with RA for 48 h, crosslinked, and collected for ChIP-seq analysis. Consistent with previous reports showing that Hox genes are a UTX target during differentiation, the level of H3K27me3 in UtxKO cells treated with RA was higher than that in wild-type controls (Fig. 3A). However, close inspection of the gene tracks revealed that the levels of H3K27me3 in differentiated UtxKO ES cells were lower than in undifferentiated UtxKO ES cells, indicating active demethylation of H3K27me3 in the absence of UTX. Moreover, the levels of H3K4me3 increased at the promoters throughout the cluster, indicating that the genes were responsive to RA in the absence of UTX.

We performed a metagene analysis of the average levels of H3K27me3 of two different gene sets to investigate whether increased methylation was specific to the Hoxb cluster. The first group contained genes that maintained a bivalent state following RA treatment, and the second cluster comprised genes that resolved the bivalent domain by losing the H3K27me3 modification at their transcription start site. Consistent with our previous analyses, the average levels of H3K27me3 in UtxKO ES cells grown under self-renewal conditions were higher for both gene sets (Fig. 3B). Additionally, the levels of H3K4me3 for both gene sets showed a slight but significant decrease in UtxKO ES cells compared with UtxFlx cells, which is in agreement with the chromatin state observed at individual gene tracks (Figs. 2D and 3A). The metagene analysis of “resolved” bivalent genes for UtxKO ES cells treated with RA confirmed that Utx-deficient ES cells were responsive to RA as evidenced by both the reduction in the average H3K27me3 levels and increase in H3K4me3 levels for this group of genes (Fig. 3C). In contrast, the levels of H3K27me3 remained unchanged for genes that did not resolve following RA treatment, the second cluster comprised genes that re-solved the bivalent domain by losing the H3K27me3 modification at their transcription start sites (Fig. 3A). Additionally, the levels of H3K4me3 for both gene sets showed a slight but significant decrease in UtxKO ES cells compared with UtxFlx cells, which is in agreement with the chromatin state observed at individual gene tracks (Figs. 2D and 3A). The metagene analysis of “resolved” bivalent genes for UtxKO ES cells treated with RA confirmed that Utx-deficient ES cells were responsive to RA as evidenced by both the reduction in the average H3K27me3 levels and increase in H3K4me3 levels for this group of genes (Fig. 3C). In contrast, the levels of H3K27me3 remained unchanged for genes that did not resolve following RA treatment, the second cluster comprised genes that re-solved the bivalent domain by losing the H3K27me3 modification at their transcription start sites (Fig. 3A). Additionally, the levels of H3K4me3 for both gene sets showed a slight but significant decrease in UtxKO ES cells compared with UtxFlx cells, which is in agreement with the chromatin state observed at individual gene tracks (Figs. 2D and 3A). The metagene analysis of “resolved” bivalent genes for UtxKO ES cells treated with RA confirmed that Utx-deficient ES cells were responsive to RA as evidenced by both the reduction in the average H3K27me3 levels and increase in H3K4me3 levels for this group of genes (Fig. 3C). In contrast, the levels of H3K27me3 remained unchanged for genes that did not resolve following RA treatment, the second cluster comprised genes that re-solved the bivalent domain by losing the H3K27me3 modification at their transcription start sites (Fig. 3A). Additionally, the levels of H3K4me3 for both gene sets showed a slight but significant decrease in UtxKO ES cells compared with UtxFlx cells, which is in agreement with the chromatin state observed at individual gene tracks (Figs. 2D and 3A).
UTX is not required for RA-induced differentiation. (A) Increased levels of H3K27me3 at the Hoxb cluster in the absence of UTX. H3K27me3 (red) and H3K4me3 (green) gene tracks for male ES cells with restored UTX expression (Utx+) and Utx-null (Utx−) male ES cells that were untreated or treated with RA. During differentiation, bivalent genes can be maintained (yellow shading) or resolved (blue shading). Maintained bivalent genes are defined as those that maintain significant levels of H3K4me3 and H3K27me3 during RA-induced differentiation. Resolved bivalent genes are defined as genes that lost H3K27me3, but retained H3K4me3. The y axis floor is set at 0.5 reads per million. Gene models are shown below the density profiles. (B) Bivalent genes in UTX-deficient ES cells have increased levels of H3K27me3 and a reduction in H3K4me3. Metagene representations of the average H3K4me3 (green lines) and H3K27me3 (red lines) levels around transcription start sites (±2 kb) for wild-type (solid lines) and Utx-deficient (dashed lines) ES cells. (C) Demethylation of H3K27me3 in UTX-deficient ES cells in response to RA. Metagene representations of the average H3K4me3 (green lines) and H3K27me3 (red lines) levels around transcription start sites (±2 kb) for wild-type (solid lines) and Utx-deficient (dashed lines) ES cells. H3K27me3 levels are reduced at resolved genes for both wild-type and UTX-deficient ES cells. (D) UTX-deficient male ES cells are transcriptionally responsive to RA. Violin plots summarizing the gene expression of wild-type (Utx+) and UTX-deficient (Utx−) ES cells grown in self-renewal conditions (no RA) or under differentiation conditions (+RA). Maintained and resolved bivalent genes were analyzed.
targeting of the Utx allele resulted in the elimination of Utx transcript and protein, quantitative RT-PCR and Western blot analysis was performed. ES cell lines that were targeted with the full targeting construct displayed hypomorphic expression of Utx with ∼50% of mRNA transcripts than the parental v6.5 ES cells (Fig. 51B). As expected, when these hypomorphic lines were exposed to Cre, the remaining transcripts were eliminated, resulting in the ablation of protein. When the hypomorphic lines were treated with Flipase, the normal levels of Utx mRNA were restored.

Derivation of ES Cells. Following protocols described in ref. 39, ES cells were isolated from blastocyst embryos using ESC derivation media: KOSR (knockout serum replacement, Gibco, catalog no. 1028-028), LIF ESERG (1× 107 units of ESERG per ml; Chemicon, catalog no. ESG1106), Mek1 inhibitor (PD98059; Cell Signaling Technology, catalog no. 9900), nonessential amino acids, glutamine solution, and pen/strep solution. Blastocysts were obtained from a Utx+/− female crossed with a NestinCre/−/− male. This first step of trypsinization was considered passage 1.

Mouse Blastocyst Injections and Teratoma Formation. All animal procedures were performed according to National Institutes of Health guidelines and were approved by the Committee on Animal Care at the Massachusetts Institute of Technology. B6D2F2 blastocysts were injected with Utx−/−, Utx−/+ or Utx+/+ ES cells (v6.5 is parental line) and transferred to Swiss pseudo-pregnant females. Teratoma formation was performed by depositing 1 × 10³ cells under the flanks of recipient SCID or Rag2−/− mice. Tumors were isolated 3–6 wk later for histological analysis.

Differentiation of ES Cells. Embryoid body. ES cells were trypsinized, preplated, and resuspended in ES medium minus LIF at 10⁶ cells per ml. Twenty-microliter drops were placed onto the lid of a bacterial plate, inverted, and incubated for 2 d at 37 °C. The hanging drops were put into suspension culture in a bacterial plate and incubated for an additional 8 d. ES cell lines that were targeted with the full targeting construct displayed hypomorphic expression of Utx with ∼50% of mRNA transcripts than the parental v6.5 ES cells (Fig. 51B). As expected, when these hypomorphic lines were exposed to Cre, the remaining transcripts were eliminated, resulting in the ablation of protein. When the hypomorphic lines were treated with Flipase, the normal levels of Utx mRNA were restored.

Generation of Hemizygous Mutant Males. To generate Utx−/− animals, a well characterized NestinCre transgenic strain was used to generate Tlox alleles in the germ line. Utx−/− or Utx−/+ animals were crossed with the NestinCre strain to generate conditional Utx−/− male animals. The males were fertile, transmitted an Utx−/− allele to their progeny, and gave rise to Utx−/− Utx−/+ females when crossed with wild-type females.

ChIP-Seq Experiments. Utx−/− ES cells and Utx−/− ES cells were grown in 15-cm plates on gelatin in either ES cell media + LIF or ES cell media without LIF but with 1 μM of RA. After treatment with RA for 48 h, a fraction of cells that were not crosslinked was harvested for RNA isolation and gene expression analysis; the remaining cells were chemically crosslinked. Chromatin immunoprecipitation was performed using antibodies versus H3K27me3 (Abcam) and H3K4me3 (Millipore). Detailed procedures can be found in SI Experimental Procedures. Illumina/Seqlexa sequence preparation, sequencing, and quality control were provided by Illumina. A brief summary of the technique and the sequencing protocol modified as well as data analysis are described in SI Experimental Procedures. Data sets have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo).

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