Drosophila UTX Coordinates with p53 to Regulate ku80 Expression in Response to DNA Damage

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Abstract

UTX is known as a general factor that activates gene transcription during development. Here, we demonstrate an additional essential role of UTX in the DNA damage response, in which it upregulates the expression of ku80 in Drosophila, both in cultured cells and in third instar larvae. We further showed that UTX mediates the expression of ku80 by the demethylation of H3K27me3 at the ku80 promoter upon exposure to ionizing radiation (IR) in a p53-dependent manner. UTX interacts physically with p53, and both UTX and p53 are recruited to the ku80 promoter following IR exposure in an interdependent manner. In contrast, the loss of utx has little impact on the expression of ku70, mre11, hid and reaper, suggesting the specific regulation of ku80 expression by UTX. Thus, our findings further elucidate the molecular function of UTX.

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Introduction

Maintaining genomic stability is crucial for ensuring the accurate cellular functioning of organisms ranging from bacteria to humans [1,2]. During the course of evolution, cells have evolved multiple mechanisms, collectively known as the DNA damage response (DDR), that facilitate the cellular response to DNA damage [3,4,5]. These mechanisms include cell-cycle arrest, DNA repair and apoptosis [6,7]. In addition, cells responding to DNA damage display a specific gene expression profile that facilitates DNA repair [8]. For example, CSA and HR23A are upregulated by the transcription factor USF-1 in response to UV damage [9]. In the normal diploid human lung fibroblast line MRC-5, exposure to ionizing radiation results in the upregulation of Ku70 via a p53/ATM-dependent mechanism [10]. DNA damage induces CRT1 transcription, which is downstream of DUN1 in the DNA damage pathway in yeast. In turn, CRT1 becomes hyperphosphorylated and dissociates from DNA, resulting in the transcriptional induction of three of the four RVR genes [11]. Over the last few years, a wealth of new information has been uncovered about the DDR, including the identification of many novel proteins involved in this process [12], but whether these proteins are regulated at the gene transcription level in response to DNA damage remains poorly understood.

The ubiquitously transcribed TPR gene on the X chromosome, or UTX, was first described as a gene that escapes from X chromosome inactivation [13,14]. It is now clear that the UTX gene encodes a JmjC-domain-containing protein with histone lysine demethylase activity specific for the tri-methylated lysine 27 residues of histone H3 (H3K27me3) [15,16,17,18,19,20], and it is officially referred to as KDM6A in the human genome. Several recent studies have found that UTX is a major constituent of the COMPASS complex, which includes myeloid/lymphoid or mixed-lineage leukemia (MLL), a SET-domain containing protein homologous to Drosophila Trithorax [21,22,23,24,25], and regulates transcription by coordinating the methylation of histone H3K4 and the demethylation of H3K27 [26]. In addition, based on the recently established link between a super elongation complex and MLL, UTX might play a role as a general factor that is involved in the activation of gene transcription [25,27]. Interestingly, sporadic mutations and the abnormal expression of UTX have been linked to many types of human cancers, suggesting that UTX plays a role in tumorigenesis. However, the functional role of UTX in tumorigenesis remains elusive. Because the DDR is generally accepted as a crucial safeguard against cancer, we hypothesize that UTX is involved in the DDR and plays an important role in maintaining genome integrity.

In this study, we demonstrated that UTX plays an essential role in the DDR in Drosophila. UTX is specifically required for the p53-dependent expression of ku80 through mediating the demethylation of H3K27me3 upon exposure to ionizing radiation (IR). However, UTX is not required for the expression of other DNA repair genes, such as ku70 and mre11, or the apoptotic genes hid and reaper (ypr). UTX is physically associated with p53, and IR
exposure induces the recruitment of both UTX and p53 to the ku80 promoter in an interdependent manner. These data favor a model in which UTX is a specific co-player in a p53-dependent cell survival response to DNA damage. Both UTX and p53 are functionally conserved from flies to humans. Therefore, our data demonstrate the role of UTX in the maintenance of genomic stability and might shed light on how UTX influences tumorigenesis.

Materials and Methods

Drosophila Genetics

All Drosophila lines were cultured in standard medium at 25°C. The P-element insertion mutant of utx, with a genotype of y1 w67c23; P{GSV6}GS10564/SM1, was obtained from the Drosophila Genetic Resource Center at the Kyoto Institute of Technology (http://kyotofly.kit.jp/cgi-bin/stocks). The P-element was mobilized using P[delta2–3] as the source of P-element transposase according to standard protocols. A total of 176 independent white revertant lines were analyzed via PCR using genomic primers. One imprecise excision line, designated utxD95 (containing a 1,691 bp deletion from ggttatttgtatgtatgtat to taaccaatcagtgggcaat), was recovered. The utxD stock was kindly provided by Andreas Bergmann [28].

Kc Cell Culture, RNAi knockdown and Transfection

Kc167 (Kc) cells were ordered from DRSC (Drosophila RNAi Screening Center) and were routinely cultured in Schneider’s Drosophila medium (Life Technologies) containing 5% FBS (Life Technologies) at 25°C. RNAi-mediated gene knockdown experiments were performed essentially as described previously [29]. Double-stranded RNAs (dsRNAs) targeting control, utx and p53 sequences were synthesized as described elsewhere [26]. The following primer pairs were designed and used for the synthesis of the dsRNAs: utx forward, 5’-gaattaatacgactcactatattgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg
FBS was then supplemented at a 5% concentration. Total RNA was isolated two days later with the RNeasy mini kit (QIAGEN), and 2 μg of RNA was reverse transcribed using Superscript III reverse transcriptase (Life Technologies). We generated UTX mutant plasmid using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent technologies). The following primer pair is designed and used for the synthesis of utx mutant plasmid: utx-j mutant forward, 5′-cctggcgcgcaagcgaacaacaacttctgctcaatcaaca-3′
and utx-j mutant reverse, 5’-gttcgctgccgcagggctacttccgge-
caccttgc-3’. For rescue experiment, we first treated the cells with
dsRNA, and 2 days later re-plate the cells for transfection. Ten
million cells were plated in 25 cm² flask with medium containing
5% FBS and incubate at 25°C for 24 hours. Then each million
cells transfected with 2 µg plasmid and X-tremeGENE Transfec-
tion Reagent (Roche). Incubated for 24 hours then following
experiment.

Ionizing Radiation (IR) and Survival Assay
For the IR and qRT-PCR experiments using Kc cells, γ-ray
irradiation was applied to the cells at 2 or 4 days after RNAi
treatment, and the cells were then harvested for total RNA

extraction using the RNeasy mini kit (QIAGEN). To assess cell
survival, RNAi-treated Kc cells were irradiated with 4 or 8 Gy of
IR, seeded at a density of 1 × 10⁶ cells/ml into 6-well plates and
counted at 2 and 4 days after irradiation. For hatching rate
quantification, embryos were collected at 0–4 hours after embryo
laying and irradiated with 10 Gy of IR, and the hatched larvae
were counted after 3 days. For utxΔ95/Δ95 flies, utxΔ95/CyoGFP
flies were crossed with utx1/CyoGFP flies, and the non-fluorescent
embryos were collected. For the third instar larva experiments, the
total irradiation dosage was 40 Gy. Total RNA was isolated at 2
hours after irradiation using the RNeasy mini kit (QIAGEN).

Drosophila UTX Mediates Ku80 Expression after IR

Figure 3. p53 and UTX are recruited in an interdependent manner to the ku80 promoter region. (A) ku80 expression following IR
exposure in Kc cells subjected to RNAi treatment, as indicated. (B, C) ChIP analysis of the physical occupancy of p53 and UTX at the
ku80 promoter region. Note that knockdown of utx eliminates the increase in p53 binding, and knockdown of p53 reduces the binding of UTX to the ku80 promoter. (D) ChIP assay for H3K27me3 at the ku80 promoter in Kc cells treated with control or utx RNAi after IR. (E) Coimmunoprecipitation was performed
using anti-p53 and anti-UTX antibodies and whole cell extracts of Kc cells. The immunoprecipitates were subjected to Western blot analysis with the
indicated antibodies. (F, G) Western blot analysis to confirm the knockdown efficiency of p53 RNAi. β-Tubulin (β-Tub) levels were used as a loading
control.
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Real-time Quantitative RT-PCR Analysis

Real-time quantitative RT-PCR (qRT-PCR) assays were performed using the Applied Biosystems 7300 Real-Time PCR System (Life Technologies) and FastStart Universal SYBR Green Master Mix (Roche Applied Science). The following primer pairs were designed and employed for qRT-PCR:

- **ku80**: forward, 5’-aagtccgcaaaatgtgtggc-3’; reverse, 5’-atttcatcggtgtcgcaacc-3’;
- **ku70**: forward, 5’-cccatggtcgatgactttgac-3’; reverse, 5’-gaaaattgaacgcacaaaacagg-3’;
- **mre11**: forward, 5’-ccaaaacggaggctgtcaat-3’; reverse, 5’-cgatccactaactctccagc-3’;
- **hid**: forward, 5’-ccaccgaccaagtgctatact-3’; reverse, 5’-ccatggatggaagggagtttc-3’;
- **rpr**: forward, 5’-ccagttgttaattccgaacg-3’; reverse, 5’-tcgcctgatcgggtatgtaga-3’;
- **pnr**: forward, 5’-gcaaggaggagcatgatctca-3’; reverse, 5’-ttggtgccgctcttcatatcc-3’;
- **b-tubulin**: levels were used as an internal control as described [29].

Figure 4. UTX is required for the expression of ku80 following IR exposure in Drosophila. (A) Schematic illustration of the gene structure of wild type utx and a utx mutant allele (utx<sup>495</sup>) generated via the imprecise excision of a P-element insertion. (B, C) Genomic PCR (B) and Western blot (C) analyses to verify the utx<sup>495</sup> genotype. (B) An approximately 250-bp band is detected in adult flies with a utx<sup>495</sup>/CyO genotype, but absent from the w<sup>1118</sup> genotype. For details, please see the Materials and Methods section. (C) An approximately 130-kDa band indicated by an arrow in w<sup>1118</sup> flies was not detected in the utx<sup>495</sup>/utx<sup>2</sup> third instar larvae. A non-specific band is indicated by an asterisk, and β-Tubulin (β-Tub) was used as a loading control. (D) qRT-PCR analysis of mRNA expression for the indicated genes before and after IR exposure in the w<sup>1118</sup> and utx<sup>495</sup>/utx<sup>2</sup> third instar larvae. The relative expression levels are normalized to β-tubulin levels. Note that ku80 is the only gene that requires utx for its expression.

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Immunoprecipitation and Chromatin

Immunoprecipitation (ChiP)

The immunoprecipitation experiments were performed with the PIERCE direct IP kit according to the manufacturer’s protocol. The ChiP assays were conducted using the Upstate ChIP assay kit, also following the manufacturer’s protocol. Briefly, approximately 3×10⁷ cells were collected, fixed and sonicated with a Bioruptor sonicator (Diagenode) to generate DNA fragments of approximately 500 bp in length. Next, immunoprecipitation was performed with either an antibody (3 µg) or normal rabbit IgG (3 µg), and the subsequent steps were performed as previously described [26]. The following primer pairs were used for qPCR: ku80 forward, 5′-gcaagcggttgtagaatatat-3′; reverse, 5′-gcggcttagccagcacaaagtg-3′; mre11 forward, 5′-tgctggcttcctttttgtcct-3′; reverse, 5′-tggtgagcgaaagagcaaga-3′; nri1 forward, 5′-tttttcagatggtgcaatcg-3′; reverse, 5′-ttttttcgagatgcgttcgc-3′; hid forward, 5′-agcaaaacaaagcagcgaaga-3′; reverse, 5′-tggtgagcgaaagagcaaga-3′; rpr forward, 5′-cggtctatgtgatggcgaaat-3′; reverse, 5′-ccacctttcgatgacagcc-3′; utx forward, 5′-accacctttcgatgacagcc-3′; reverse, 5′-accacctttcgatgacagcc-3′; utx forward, 5′-accacctttcgatgacagcc-3′; reverse, 5′-accacctttcgatgacagcc-3′; utx forward, 5′-accacctttcgatgacagcc-3′; reverse, 5′-accacctttcgatgacagcc-3′; utx forward, 5′-accacctttcgatgacagcc-3′; reverse, 5′-accacctttcgatgacagcc-3′; utx forward, 5′-accacctttcgatgacagcc-3′; reverse, 5′-accacctttcgatgacagcc-3′; utx forward, 5′-accacctttcgatgacagcc-3′; reverse, 5′-accacctttcgatgacagcc-3′.

Antibodies

Rabbit polyclonal anti-UTX antiserum was generated against a bacterially expressed GST-tagged UTX fragment (1–113aa). In the Western blot assays, the blots were first incubated with the proper concentrations of primary antibodies, followed by incubation with the indicated HRP-anti-rabbit IgG or HRP-anti-mouse IgG secondary antibodies (Sigma) and visualized using an ECL kit (Thermo Scientific). The antibodies used in the Western blot analysis were as follows: anti-UTX serum (1:1,000), anti-β-Tubulin (1:5,000, Sigma Cat. No. F1804) and anti-p53 (1:1,000, DSHB Cat. No. 25F4); anti-UTX antiserum and anti-H3K27me3 (UPSTATE Cat. No. 07–449) were used for ChIP.

Results

The utx Gene is Essential for Cell Survival After IR Exposure

The UTX protein has been extensively studied regarding its function as a demethylase and its H3K27 demethylase-indepen-
dent activity during development [15,18,28]. Previous studies indicate that UTX is also associated with human cancers [30,31]. However, how UTX functions as a tumor suppressor is unclear. For this reason, we hypothesized that UTX is involved in the maintenance of genomic stability in response to DNA damage, as loss of UTX function results in genome instability and tumori-
genesis. Under basal conditions, the RNAi-mediated depletion of utx did not affect the growth of Kc cells at 2 days after soaked with double-strend RNAs and caused only a slight slower growth at 4 days (Fig. 1A). We challenged 4 days RNAi-treated cells with IR at doses of 4 and 8 Gy and examined relative cell viability after 2 days. We found that both doses of IR induced a significant reduction in the viability of the cells treated with utx RNAi compared to controls RNAi cells. The reduction of cell survival is indeed due to the loss of utx, since over-expression of wild type UTX in RNAi cell could rescue the viability (Fig. 1B). UTX protein levels were significantly reduced after the RNAi-mediated knockdown of utx but remained steady after IR, suggesting that IR does not regulate the expression of UTX (Fig. 1C, 1D). These data indicate that UTX is required for cell survival/growth upon genotoxic insult in Kc cells.

UTX Upregulates ku80 Through Promoter Demethylation in Response to IR Exposure

In a previous microarray analysis that conducted in our laboratory, we identified a list of genes that are upregulated following IR treatment in Drosophila Kc167 (Kc) cells (Table S1). This list included genes known for their roles in DSB repair, such as ku70, ku80 and mre11, similar to what has been previously reported in fly embryos [32]. To further determine the role of UTX in the DDR, we investigated whether UTX is involved in the regulation of these genes in response to IR exposure. Interestingly, we found that the RNAi-mediated knockdown of UTX expression significantly inhibited the upregulation of ku80, but not that of ku70 and mre11, following IR treatment (Fig. 2A). Over-expression of wild type UTX in utx RNAi cell restored the upregulation of ku80 expression (Fig. 2B). These data suggest that UTX regulates ku80 expression in a gene-specific manner in DDR. This notion is further supported by the fact that both utx and ku80 RNAi-treated cells showed similar cell sensitivity to IR.

Table 1. The hatching rate of Drosophila embryo.

| Genotype | γ-ray irradiation | Normalized hatching rate |
|----------|------------------|--------------------------|
|          | 0 Gy | 5 Gy | 10 Gy | 5 Gy | 10 Gy |
| w1118    | 499/520 (96.0%) | 98/185 (53.0%) | 28/166 (16.9%) | 55.3% | 17.6% |
| utxΔ113/utxΔ113 | 188/270 (69.6%) | 42/127 (33.1%) | 7/170 (4.0%) | 47.7% * | 5.7% * |

*p<0.05 and *p<0.01, as analyzed by Fisher’s Exact Test for normalized hatch rate upon IR treatment of 0–4 hrs w1118 and utxΔ113/utxΔ113 embryo.

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Table 2. The hatching rate of Drosophila embryo.

| Genotype | UV irradiation | Normalized hatching rate |
|----------|---------------|--------------------------|
|          | 0 J/m² | 10 J/m² | 100 J/m² | 10 J/m² | 100 J/m² |
| w1118    | 499/520 (96.0%) | 124/220 (56.4%) | 93/205 (45.4%) | 58.8% | 47.3% |
| utxΔ113/utxΔ113 | 188/270 (69.6%) | 55/145 (37.9%) | 69/179 (38.5%) | 54.5% * | 55.3% * |

*p>0.05, as analyzed by Fisher’s Exact Test for normalized hatch rate after 48 hrs upon UV treatment of 0–4 hrs w1118 and utxΔ113/utxΔ113 embryo.

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Next, we explored whether UTX regulates directly or indirectly the expression of ku80 upon IR treatment. Chromatin immunoprecipitation (ChIP) assays revealed that UTX is recruited to the promoter region of ku80 upon IR treatment (Fig. 2C).

Although UTX has predominantly been shown to regulate transcription by demethylating H3K27, it has also been found that UTX regulates the mesoderm differentiation of embryonic stem cells, independent of its H3K27 demethylase activity in mouse [33]. Therefore, we investigated whether the observed UTX-mediated ku80 expression was dependent on the demethylase activity of UTX.

Using ChIP assays, we found that IR treatment dramatically reduced the levels of H3K27me3 in ku80, but not in utx depleted cells. Over-expression WT UTX could reduce the levels of H3K27me3 in utx RNAi cells after IR, but not JMJC domain mutant UTX which disrupt UTX enzyme activity (Fig. 2D) [28]. Furthermore, we found that over-expression JMJC domain mutant UTX could not rescue cell survival and ku80 expression compare with WT UTX in utx RNAi cell after IR (Fig. 1B, Fig. 2B). These data indicate that UTX functions as a histone demethylase in its regulation of ku80 upon IR exposure. To further assess whether DNA damage genes are activated in association with the altered levels of H3K27me3, we examined the promoter regions of these genes before and after IR treatment. We found that IR treatment induced a dramatic reduction of H3K27me3 levels in ku80, but not in ku70 or mre11 (Fig. 2E, 2F). As a control gene, we also examined a known Polycomb target gene, panner (panr), and we found that IR caused no apparent changes in either the expression or the recruitment of UTX to panr [26]. These data indicate that during DNA damage, UTX specifically and directly regulates the expression of ku80 by demethylating histone H3K27me3 at the ku80 promoter.
UTX Coordinates with p53 to Directly Facilitate the Expression of ku80 Following IR Treatment

Previous studies have indicated that ku80, as well as ku70, is among the p53 target gene list in fly embryos [32] and larvae [34]. In Kc cells, we found that the expression of ku80 also requires p53, as the RNAi-mediated knockdown of p53 significantly reduced the expression of ku80 following IR treatment (Fig. 3A). In addition, using ChIP analysis, we showed that IR caused marked p53 enrichment in the ku80 promoter (Fig. 3B). Next, we asked whether UTX and p53 coordinate their activities to regulate the expression of ku80. The RNAi-mediated knockdown of UTX expression significantly inhibited the recruitment of p53 to the ku80 promoter, suggesting that UTX is required for the regulation of ku80 expression (Fig. 3C). Intriguingly, we found both the UTX recruitment and the reduction of H3K27me3 levels in the ku80 gene were also prevented by the knock-down of p53 (Fig. 3C, 3D). These data indicate that both p53 and UTX directly regulate ku80 expression within the same pathway, thus requiring coordinated action between p53 and UTX. Further supporting this notion, we found that p53 co-immunoprecipitated with UTX, and UTX was similarly able to co-immunoprecipitate with p53, indicating a physical interaction between the two proteins (Fig. 3E). However, we did not observe a direct interaction in GST pull-down assays, suggesting that the interaction between p53 and UTX is indirect. The UTX protein level is not affected by the change of p53 level as confirmed by the Western blot (Fig. 3F). Similarly, UTX does not regulate p53 expression (Fig. 3G). These data exclude the possibility that UTX and p53 interact in DDR by the mutual regulation of expression. Together, these data support a molecular model in which p53 and UTX form a complex to regulate ku80 expression and mediate the DDR following exposure to IR.

UTX Regulates ku80 Expression in Drosophila

Using Drosophila as an in vivo model system, we next investigated whether the expression of ku80 is also regulated by UTX in Drosophila. To address this question, we generated a utx mutant allele, utx^{495}, through the imprecise excision of a P-element inserted into the utx locus (Fig. 4A). Genomic PCR analysis and sequencing data confirmed the existence of a deletion of five exons (1,691 base pairs) in utx^{495}, as indicated by FlyBase gene annotation [http://flybase.org/ (Figs. 4A & 4B). We found that animals homozygous for utx^{495} only rarely survive to adults but utx^{495}/utx^{iso} trans-heterozygotes can develop into adults and show no detectable morphological defects. Those results consistent with recently published article [35]. A reported EMS allele, utx^{iso}, bearing a nonsense mutation in the JmjC domain, has also been used [28]. Both utx alleles are null, as verified by the missing UTx band in a Western blot analysis of trans-heterozygous (utx^{495}/utx^{iso}) third instar larvae performed using an anti-UTX antiseraum raised against the N-terminal 103 aa portion of the protein (Fig. 4C). As shown in Figure 4D, 8 Gy of IR dramatically upregulated the levels ku80, ku70 and moe1 in w^{1118} (wild type control) third instar larvae, similar to what was observed in Kc cells (Fig. 2A), indicating the expression of these genes upon DNA damage in Drosophila. However, IR treatment elicited a significantly reduced induction of ku80 expression in utx^{495}/utx^{iso} third instar larvae compared to wild type larvae, whereas the expression of other genes remained relatively constant, suggesting an gene-specific requirement of UTX for ku80 expression during the DDR. We therefore conclude that UTX is essential for the expression of ku80 both in cell and Drosophila.

Furthermore, to determine whether UTX is involved in DNA repair in Drosophila, we quantified the hatching rate of transheterozygous utx null (utx^{495}/utx^{iso}) and w^{1118} embryos treated with IR. The utx null embryos exhibited a markedly lower hatching rate of 69.6% compared to the wild type embryos, which displayed a hatching rate of 96.0% (Table 1). Treatment with 10 Gy of IR severely reduced the hatching rate for both genotypes. However, we conclude that the effect of IR was more significant for utx null embryos, as demonstrated by the statistically significant reduction of the normalized hatching rate (Table 1). These data suggest that utx mutant embryos are more sensitive to IR stress than wild type embryos. In addition, we found that the hatching rate of Drosophila embryo does not significantly change between wild type and mutant after UV irradiation (Table 2). Therefore, UTX might play an essential role in DNA repair through regulating ku80 expression both in cell and Drosophila.

UTX is Not Responsible for the Upregulation of Apoptotic Genes in Response to DNA Damage

Previous studies have shown that p53 upregulates the apoptotic genes reaper and hid following treatment with IR [32]. Given that together with p53, UTX coordinates regulates the expression of ku80 upon IR exposure, we sought to determine whether UTX also participates in the regulation of apoptotic gene expression in response to DNA damage. To investigate this notion, we evaluated the changes in reaper and hid expression levels following IR treatment. Figures 5A and 5B show that independent of UTX, the expression levels of these apoptotic genes were upregulated to the same extent following IR exposure both in cell and Drosophila. These results were consistent with the findings of previous works showing that overexpression of UTX in primary human fibroblasts induces cell cycle arrest, but not apoptosis [36]. These data suggest that p53, but not UTX, is required for DNA damage-induced apoptosis. Interestingly, we found that the levels of H3K27me3 at the reaper and hid promoters were also reduced following IR treatment, similar to what was observed for the ku80 promoter (Fig. 5C). These data suggest that other demethylases might be responsible for the upregulation of apoptotic genes in response to DNA damage.

Discussion

To understand the mechanism underlying UTX function in tumorigenesis, we explored whether UTX is involved in DNA damage response in Drosophila. In this study, we found that UTX, play an essential role in DNA damage response by upregulation of ku80, which is uniquely required for p53 activated ku80 expression (Fig. 2–5). In addition, the gene activity of utx is correlated with loss of histone demethylation at H3K27 (Fig.2), suggesting that UTX could function as a histone demethylase and serve a gene-specific co-activator of p53 gene activation. We therefore provide an example that p53 target genes expression may be regulated at the level of histone modifications.

It is clear that p53 plays a pivotal role in the DNA damage response (DDR). One of the functions of p53 is to activate its target gene after DNA damage as transcription factor. For instance, p53 has been best characterized in regulating expression of cell cycle genes and apoptosis gene [37]. However, the precise regulation mechanism of p53 is still not clear. It is interesting that in Drosophila ku80 upregulation mediated by p53 requires UTX, but not other genes in related to DNA repair and apoptosis. However, we did observe reduced H3K27me3 levels in apoptotic genes (Fig.2), which raise the possibility that there could be additional histone demethylases participating in DDR pathways that coordinate with p53 regulating expression of hid and reaper after DNA damage, and remaining to be determined in further studies.
In contrast, we did not detect reduced H3K27me3 levels in the \textit{ka70} promoter region following IR treatment. Further analysis revealed that the H3K27me3 level in the \textit{ka70} promoter region was lower than at the \textit{ka80} promoter. The expression of \textit{ka70} is independent of UTX, possibly due to the extremely low levels of H3K27me3 in the \textit{ka70} promoter region, which might not require demethylation for the expression of \textit{ka70} to occur (Fig. 2F). Thus, our data demonstrate the complexity of the function of p53 in the activation of target genes in response to DNA damage, particularly in terms of histone modification and the action of different demethylases (Fig. 6).

UTX has been reported to participate in many biological processes, including cell fate determination and animal development [15,18,23,36,38], largely depending on the transcriptional regulation of the target genes of UTX. UTX appears to play an important role in orchestrating several histone marker, including acetylation at H3K27 and ubiquitination at H2A [19,39,40], and mediates derepression of polycomb (Pc) target genes, such as HOX genes, by affecting Pc recruitment. These roles are consistent with UTX being a histone demethylase specific for H3K27 [20]. However, sporadic mutations of UTX have been linked to many types of human cancers [30,41,42] and it remains to be elucidated whether this is also sufficiently explained by its enzymatic activity. Indeed, several studies have proposed a role of UTX independent of its demethylase activity in chromatin remodeling and embryonic development [33,43,44]. In this study, we found UTX is also involved in DDR by upregulation of \textit{ka80} in \textit{Drosophila} after IR. Although there are no available data demonstrating that \textit{ka80} mRNA levels are increased following DSBs in human cells, our data provide evidence that UTX functions to maintain genome stability and shed light on the mechanism underlying the function of UTX in human cancer. Recent studies suggest that loss of polycomb-mediated silencing might promote the upregulation of DNA repair genes [43] and facilitate the recovery of cells from genotoxic insults. UTX might therefore be required for various cell defense mechanisms under environmental stress, thereby contributing to tumor suppression.

### Supporting Information

- **Figure S1**: qRT-PCR analysis to confirm the knockdown efficiency of \textit{ku80} RNAs. (TIF)
- **Table S1**: Contains partial of microarray data which shows fold change more than five times of genes expression up-regulated following IR. (DOCX)
- **Text S1**: Only contains the legend for Figure S1 and is not intended for publication. (DOC)

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### Author Contributions

Conceived and designed the experiments: ZH MF. Performed the experiments: CZ ZH WM YQ. Analyzed the data: ZH WX FT. Contributed reagents/materials/analysis tools: CZ ZH WM DM.

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