Supplemental Material

Supplemental Methods

Antibodies

The following antibodies used in western blots and/or ChIP-seq were all generated in the Shilatifard lab: anti-H3K4me1, anti-H3K4me2, anti-H3K4me3, anti-H3, anti-Lpt, anti-dUtx, anti-UTX, and anti-MLL4. The following antibodies were purchases commercially: anti-GFP (Santa Cruz, B-2), anti-H3K4me1 [for wing disc IF] (Abcam, 8895), anti-FLAG (Sigma, M2), anti-Rbbp5 (Bethyl Labs, A300-109A), anti-Tubulin (DSHB, E7).

Size Exclusion Chromatography

Size exclusion separation of 293T cell extracts were performed with a Superose 6 column (GE Healthcare) on a SMART HPLC system (Pharmacia) in S100 buffer (30mM Tris-HCl pH 7.0, 140mM KCl, 3mM MgCl2).

CRISPR/Cas9 MLL4-deletions

Human MLL4-CT deletion gRNAs were: GTGGTGTCGGCGGGTTACTC and GCATCCATTTCCGACAATTCC.

MLL4-NT deletion gRNAs were: GGAGCAGCTTTTGTACGAGC and GGGACATCTCCATCGTGATA.

Guide-RNAs for MLL4 promoter deletions to generate ΔMLL4 HCT116 cells were: GAGGGGACTGATATGCACCGG and GTGCATGGTCGGCAGGCGTAT.

Chromatin Immunoprecipitation

Cells were harvested and washed with ice-cold PBS for two times, and fixed with paraformaldehyde (1% final) for 10 min at RT. Then quench the paraformaldehyde with 2.5M (1/20) glycine and wash the cell pellets twice with PBS. Sonication: the cell pellet was resuspended with lysis buffer 1 (50 mM HEPES, pH=7.5, 140 mM NaCl, 1 mM EDTA, 10% Glycerol, 0.5% NP-40, 0.25% Triton X-100, 1 X protease inhibitors) and incubated on nutator at 4 °C for 10 min. Spin down at 500 g for 5 min and discard supernatant. The pellet was washed with lysis buffer 2 (10 mM Tris-HCl, pH=8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 X protease inhibitors) and resuspended with lysis buffer 3 (10 mM Tris-HCl, pH=8.0, 1 mM EDTA, 0.1% SDS, 1 X protease inhibitors). Adjust the final volume to be 10 times of the pellet with lysis buffer 3. Sonication with 1 ml Covaris tubes at 10% duty cycle, 175 peak intensity power, 200 cycles per burst for 60-1200s. Sonicated DNA size was checked on a 2% agarose gel. We next added 10% of 10X ChIP Dilution Buffer (10% Triton x-100, 1 M NaCl, 1% Na-Deoxycholate, 5% N-Lauroylsarcosine, 5 mM EGTA) to the lysate and spun at 20,000 g for 15 min at 4 °C to pellet debris. Supernatant was collected and the concentration measured by nanodrop. A small aliquot was saved as input. Antibody was added (~ 10 ug per antibody) to

each cell lysate and incubated at 4 °C on nutator overnight. The next morning, protein A/G agarose beads (Santa Cruz) were washed in RIPA buffer (50 mM HEPES, pH=7.5, 500 mM LiCl, 1 mM EDTA, 1.0% NP-40, 0.7% Na-Deoxycholate) before being incubated with chromatin/antibody solution at 4 °C on nutator for 4 hours. Samples were next spun down in 15 ml conical tubes at 200g for 5 min at 4 °C and supernatant was discarded. Beads were pelleted in 15 ml conical tubes at 200g for 5 min at 4 °C and washed in RIPA Buffer 4 times at 4 °C on nutator for 10 min each time. Beads were washed once with 1 ml of cold TE + 50 mM NaCl and spun at 1000 g for 3 min at 4 °C and remove any residual buffer. Beads were resuspended in 200µl Elution Buffer (50 mM Tris-HCl, pH=8.0, 10 mM EDTA, 1.0% SDS) and incubated in 65 °C water bath for 30 min, vortexing gently twice in between. Beads were spun down at 1000 g for 3 min at RT and supernatant was transferred to a new tube. To reverse crosslinks, a 50µl aliquot was diluted in 150 µl (3 volumes) Elution Buffer and incubated at 65 °C for 6-15 h. We added 4 µl of 20 mg/ml Protease K and incubated at 55 °C for 2 h to degrade protein. DNA was purified using Qiagen PCR Purification columns.

Supplemental Table 1.

Unfiltered mass-spectrometry data is available for all three individual replicates. The following measurements and information are given for each peptide identified: Accession number, Peptide Count, NSAF (normalized spectral abundance factor), emPAI (exponentially modified protein abundance index), Spectral Count, Percent Sequence Coverage, and Description of protein identified.

Supplemental Figure Legends

Supplemental Figure 1.

A. Cartoon depiction of the *trr[1]* complementation assay. Virgin females carrying *trr[1]*/FM7 are crossed to males carrying the trr-rescue, mini-white transgene on the third chromosome. Presence of red-colored, non-bar eyes in the male F1 progeny indicates the transgene was able to rescue *trr[1]*-associated lethality.
B. *trr[1];;trr-Del4* flies reared at 29ºC display ectopic L3/L4 cross-vein phenotypes identical to catalytic-dead *trr-C/A* flies, as previously reported (Rickels et al. 2017).

Supplemental Figure 2.

A. Doxycycline-inducible 293Trex cells expressing the GFP-MLL4-UTX-interacting domain (567aa) were fractionated by size-exclusion chromatography. UTX co-elutes with endogenous MLL4 complex in cells without dox; however, induced expression of the MLL4-GFP fragment competes for UTX binding, as evidenced by co-elution in later fractions.
B. Western blots comparing MLL4 and UTX protein levels in WT versus MLL4-KO HCT116 cells, in which UTX levels are diminished in the absence of MLL3 or MLL4.
C. RNA-sequencing track example shows UTX (*KDM6A*) mRNA levels are unchanged in the absence of MLL4.

Supplemental Figure 3.

A. Alignments of the same peptide sequence shown in Fig. 3A also including five *Drosophila* species and multiple invertebrate species for improved alignment. Note the interrupting sequences present in *Drosophila*, which are predicted to form extended coils by HHPred secondary sequence prediction software (https://toolkit.tuebingen.mpg.de/#/tools/hhpred).

Supplemental Figure 4.

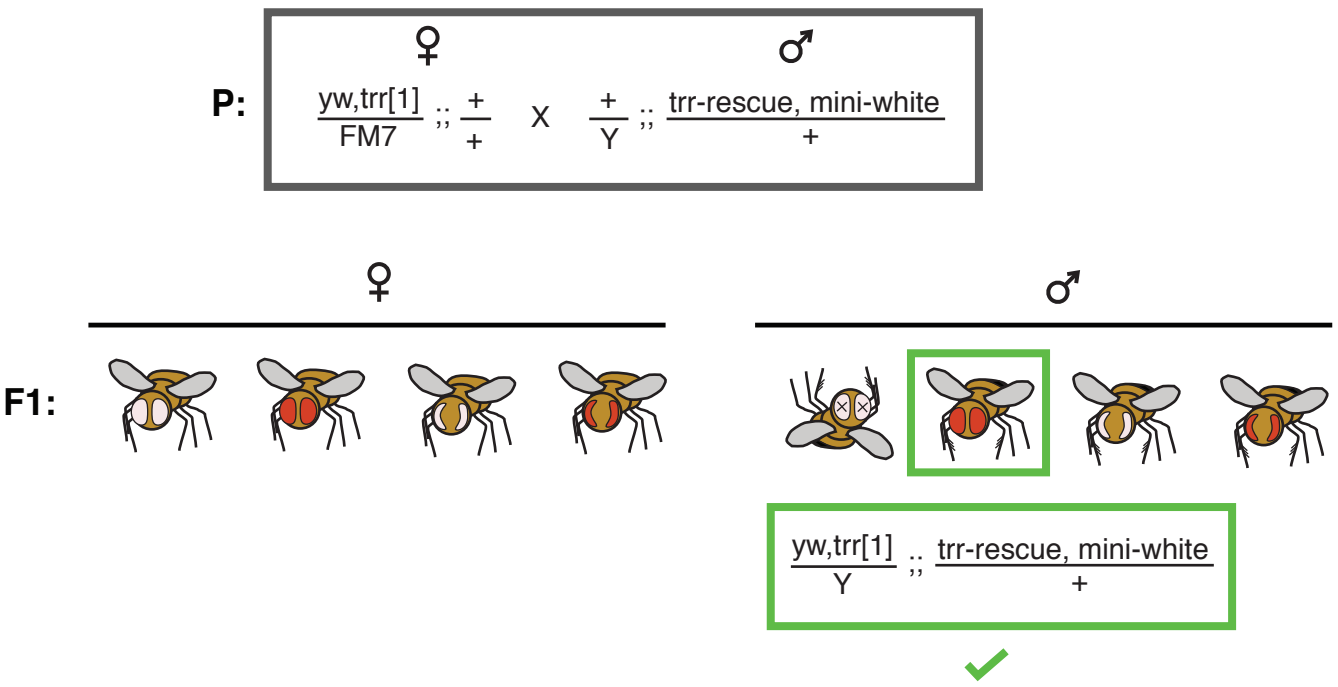
A. Western blots comparing nuclear UTX levels in WT, ΔMLL4, MLL4-ΔNT, and MLL4-ΔCT HCT116 cells.
B. Western blots show the MLL4-HMG-USD expression construct does not require an exogenous NLS to fully restore nuclear UTX levels in ΔMLL4 cells. Also, an MLL3-HMG-USD restores UTX levels similar to the MLL4-HMG-USD.
C. Immunoprecipitation experiments reveal UTX most likely interacts with MLL3/4 through its N-terminal TPR domains. Flag-tagged constructs were created to express overlapping NT, mid, and CT fragments of UTX for transfection and immunoprecipitation. The NT-fragment pulls down endogenous MLL4 in 293T cells.
D. UTX-NT co-immunoprecipitates with the MLL4-HMG-USD-GFP when co-transfected in MLL4-KO HCT116 cells.
E. Western blots showing strong nuclear UTX stabilization after ~72 hours of doxycycline-induced HMG-USD transgene expression in MLL4-KO HCT116 cells.

Supplemental Figure 5.

A. A venn diagram showing of the 2112 ΔMLL4 down-regulated genes (red), 148 (green) are transcriptionally "rescued" after 8 days of HMG-USD expression.
B. ChIP-seq for H3K27me3 in ΔMLL4-T/O cells after 0, 2, 4 days of dox induction and presented as metapeak plots. Left is centered on total HMG-USD peaks (day 4) and right is centered on total H3K27me3 peaks (day0).

Supplemental Figure 1

A



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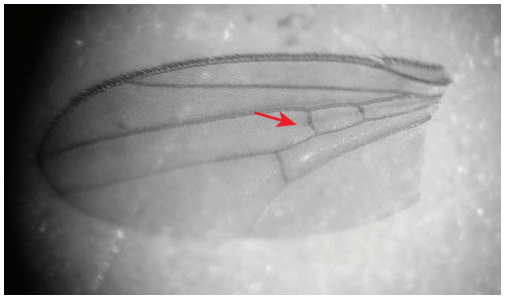
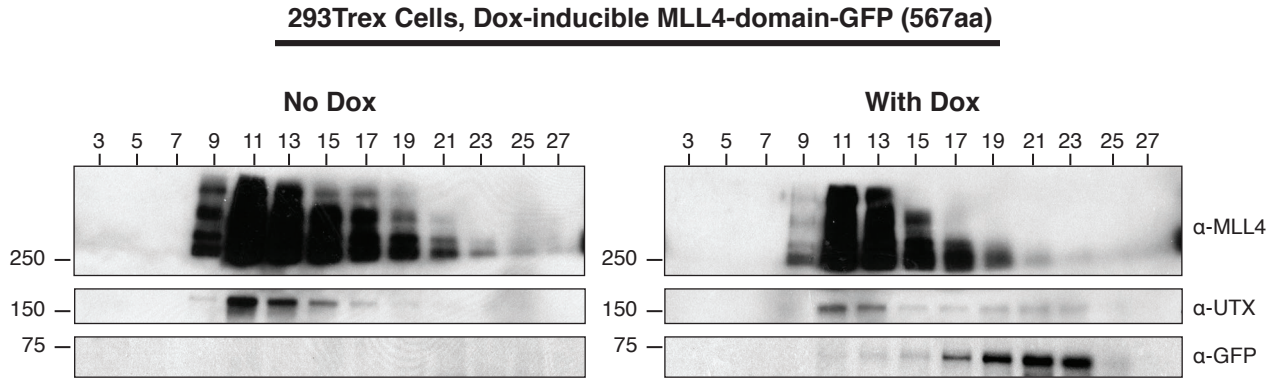


Supplemental Figure 2

A



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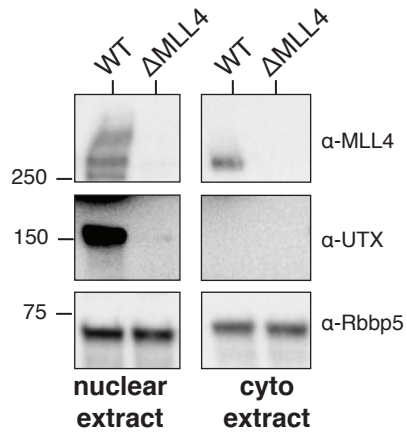

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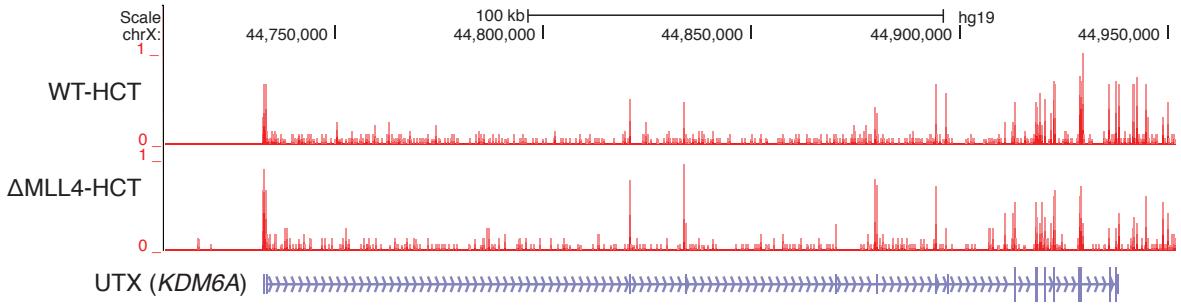
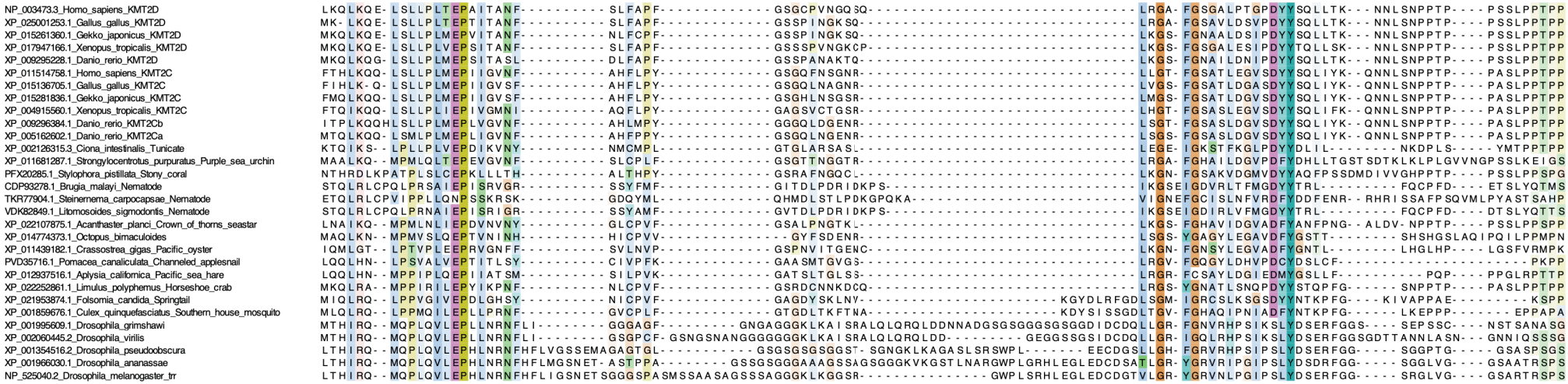
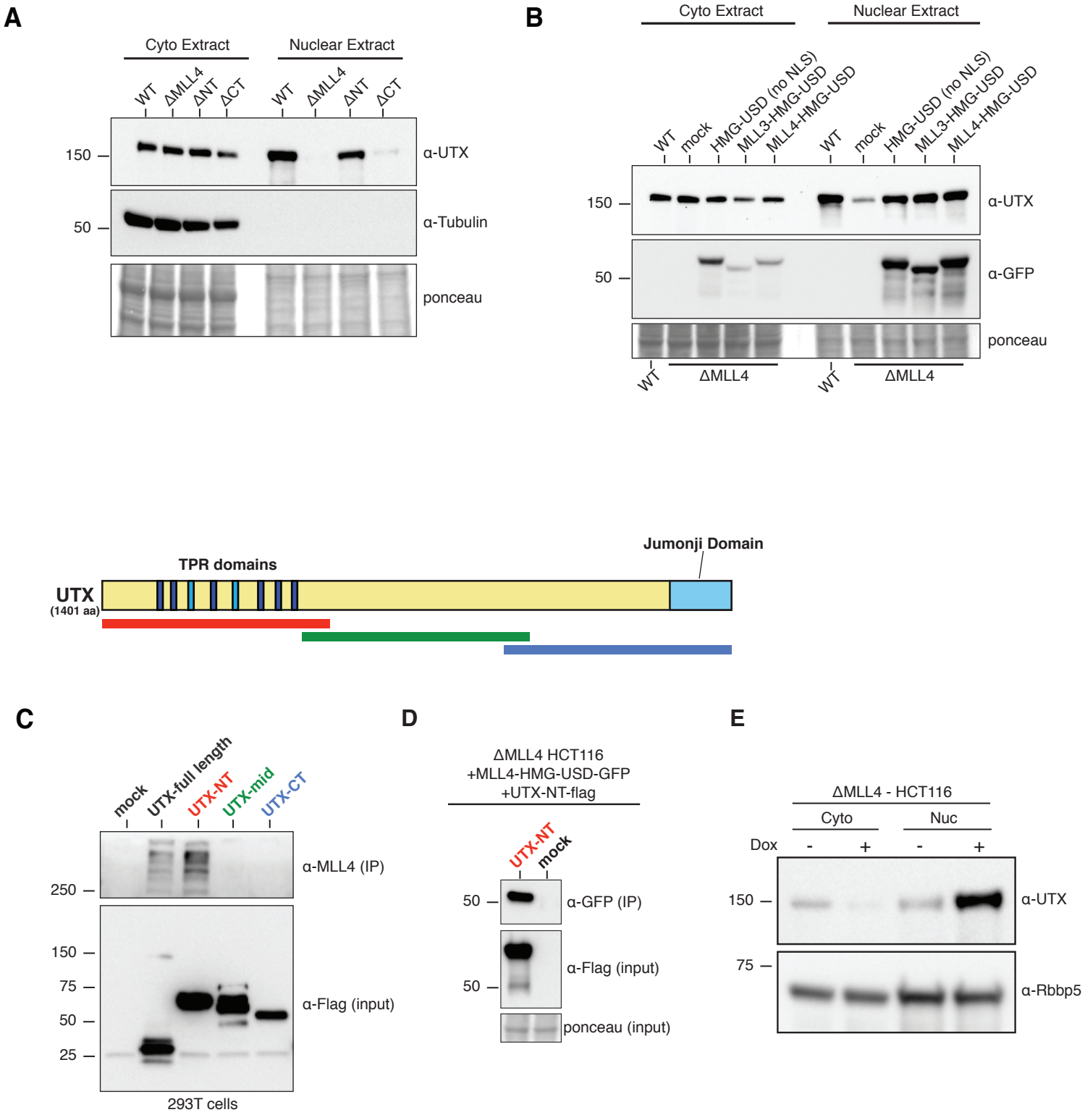


Supplemental Figure 3

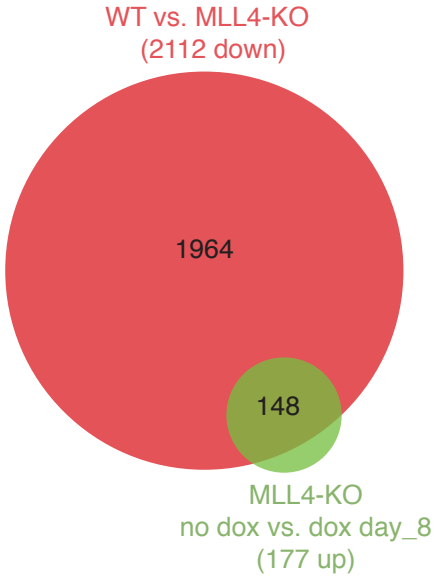


Supplemental Figure 4



Supplemental Figure 5

A



B