Figure S1, Related to Figure 1

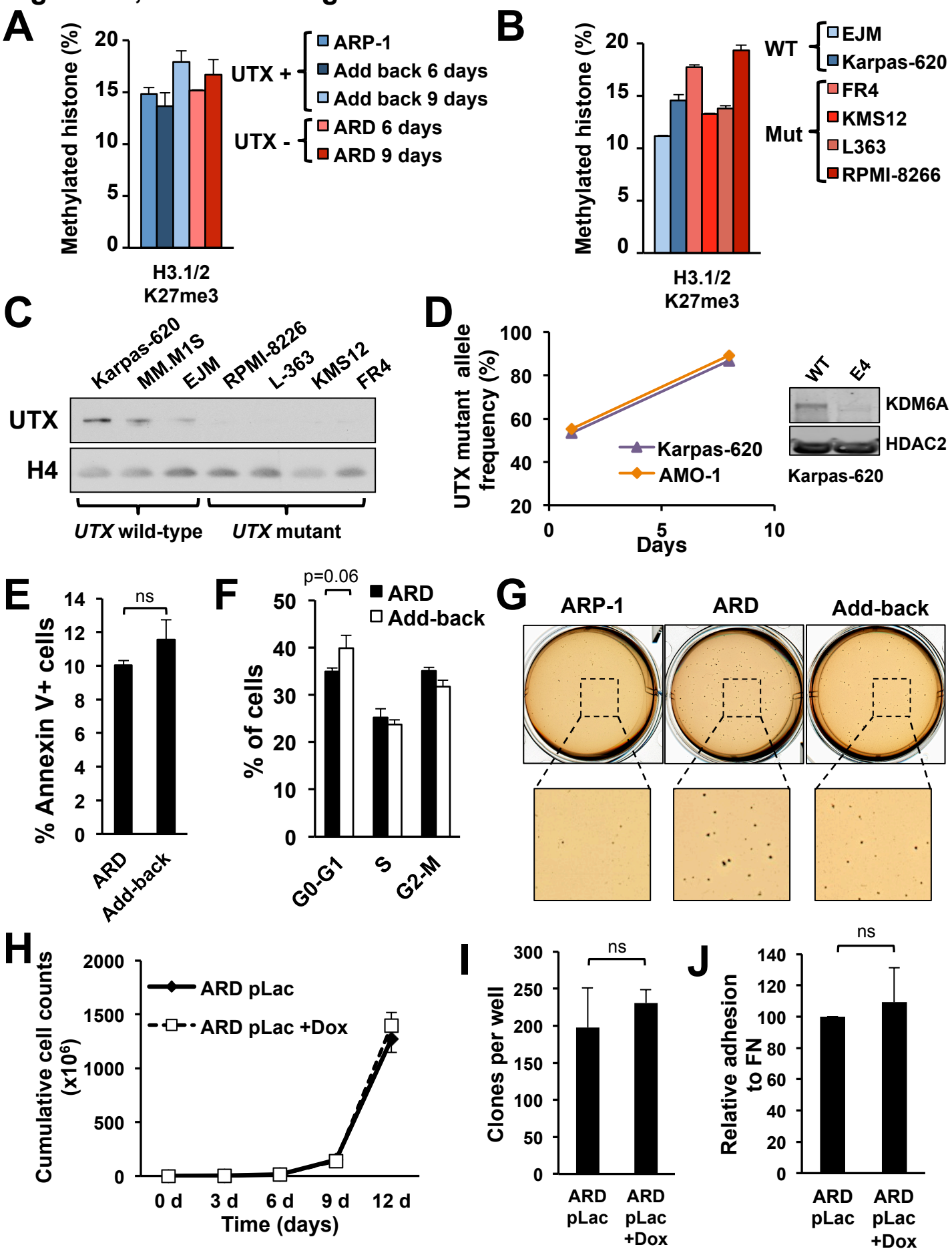


Figure S1. Loss of UTX does not alter global levels of H3K27me3 but promotes the proliferation and clonogenicity of MM cells, related to Figure 1.

(A) Histones were extracted from ARP-1 and the add-back system in ARD cells grown with or without doxycycline for 6 and 9 days. Histones were analyzed by mass spectrometry. The percentage of each methylation state from histone H3.1 and H3.2 variants is represented in technical triplicates +/- SD.

(B) Histones were extracted from a panel of *UTX* wild-type (blue) or mutant (red) cell lines. Histones were analyzed by mass spectrometry. The percentage of each methylation state from histone H3.1 and H3.2 variants is represented in technical triplicates +/- SD.

(C) Nuclear extracts were obtained from a panel of MM cell lines and immunoblotted with the indicated antibodies.

(D) Left: CRISPR-mediated gene disruption was performed in AMO-1 and Karpas-620 cells targeting the *UTX* locus using gRNAs targeting exon 4. Allele frequency was measured at different time points as indicated. Right: Karpas-620 cells described above were immunoblotted with the indicated antibodies.

(E) ARD cells harboring the inducible UTX add-back system were treated with doxycycline for 9 days and Annexin V positive cells were detected by flow cytometry. Values from 3 biological replicates +/- SD are presented.

(F) ARD cells were treated as in C, and the cell cycle profile was analyzed by flow cytometry using propidium iodide. The percentage of cells in each phase from 3 independent biological experiments +/- SD is presented.

(G) ARP-1 and ARD cells harboring the inducible system and with or without doxycyline were cultured in soft agar. Representative wells and magnifications are shown.

(H) ARD cells transduced with a control plasmid (pLac) were grown in the absence (pLac) or presence (pLAc + Dox) of doxycycline. Cells were collected every three days, counted and the initial number of cells replated in fresh media with or without drug. The cumulative number of cells at each time point of three independent experiments +/- SD is represented.

(I) Cells treated as in F were cultured in soft agar. The mean colony number per well of three biological triplicates +/- SD is presented.

(J) Calcein-AM labeled ARD cells harboring the control plasmid were cultured over FN and the adhesion determined by fluorescence intensity. Values are presented as percentage of those obtained for ARD cells. The average of three independent experiments +/- SD is presented.

Figure S2, Related to Figure 1

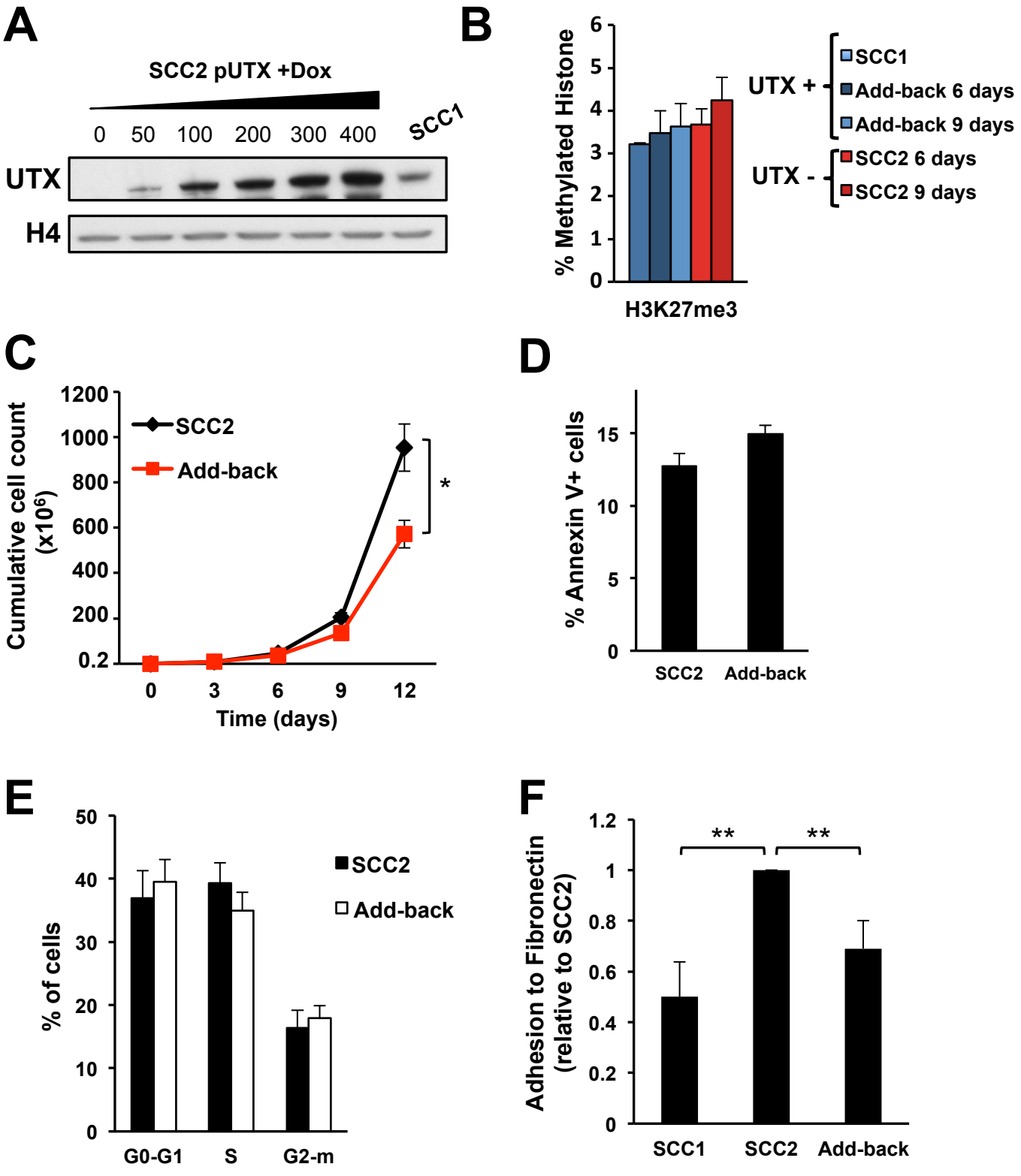


Figure S2. **Loss of UTX does not alter global levels of H3K27me3 but promotes the proliferation and adhesion of MM SCC1 cells, related to Figure 1.**

(A) SCC2 cells were infected with lentiviruses harboring a tetracycline-inducible UTX. Cells were treated with the indicated amounts of doxycycline (ng/ml) for 3 days and nuclear extracts were obtained and immunoblotted with the indicated antibodies.

(B) Histones were extracted from SCC1 and the add-back system in SCC2 cells grown with or without doxycycline for 6 and 9 days. Histones were analyzed by mass spectrometry. The percentage of each methylation state from histone H3.1 and H3.2 variants is represented in technical triplicates +/- SD.

(C) The add-back system in SCC2 cells was grown in the absence (SCC2) or presence (add-back) of doxycycline to re-express UTX. Cells were collected every three days, counted and the initial number of cells replated in fresh media with or without drug. The cumulative number of cells at each time point of three independent experiments +/- SD is represented.

(D) SCC2 cells harboring the inducible UTX add-back system were treated with doxycycline for 9 days and Annexin V + cells were detected by flow cytometry. Values from 3 biological replicates +/- SD are presented.

(E) SCC2 cells were treated as in D, and the cell cycle profile was analyzed by flow cytometry using propidium iodide. The percentage of cells in each phase from 3 independent biological experiments +/- SD is presented.

(F) Calcein-AM labeled SCC1, SCC2 cells and the add-back system were cultured over FN and the adhesion determined by fluorescence intensity. Values are presented as percentage of those obtained for SCC2 cells. The average of three independent experiments +/- SD is presented. Mann-Whitney U test: * $p<0.05$; ** $p<0.01$.

Figure S3, Related to Figure 2

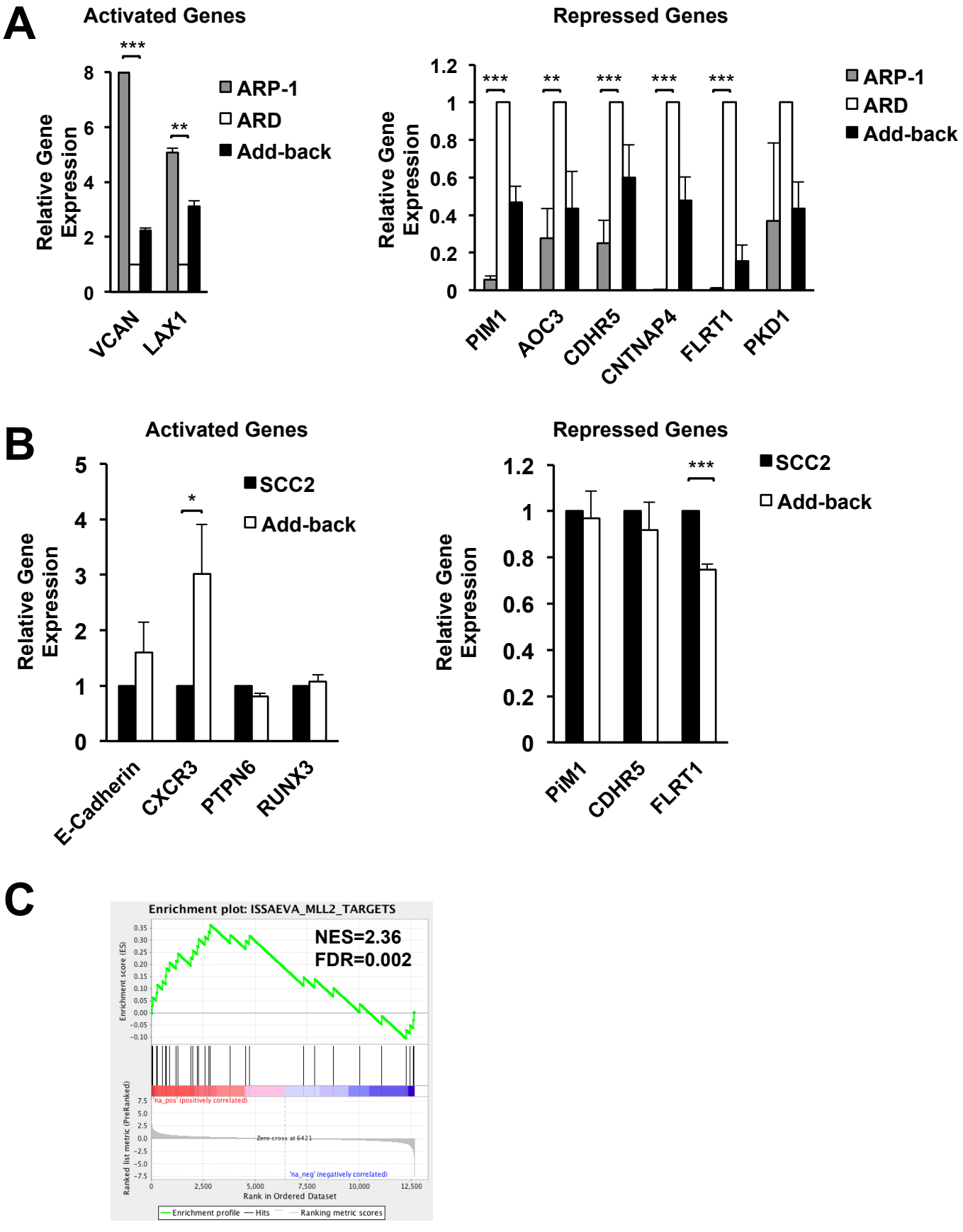


Figure S3. Identification of UTX target genes in MM cells, related to Figure 2.

(A) Validation of genes differentially expressed between ARP-1 and ARD and between ARD and UTX add-back by real time PCR. Gene expression normalized to GAPDH, from three independent experiments (+/-SD), is presented relative to that observed in ARD cells.

(B) Validation of genes responsive to UTX levels in SCC2 add-back system. Gene expression normalized to GAPDH, from three independent experiments (+/-SD), is presented relative to that observed in SCC2 cells.

(C) GSEA plot of datasets identified comparing ARD and add-back RNA-seq signatures. Mann-Whitney U test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Figure S4, Related to Figure 3

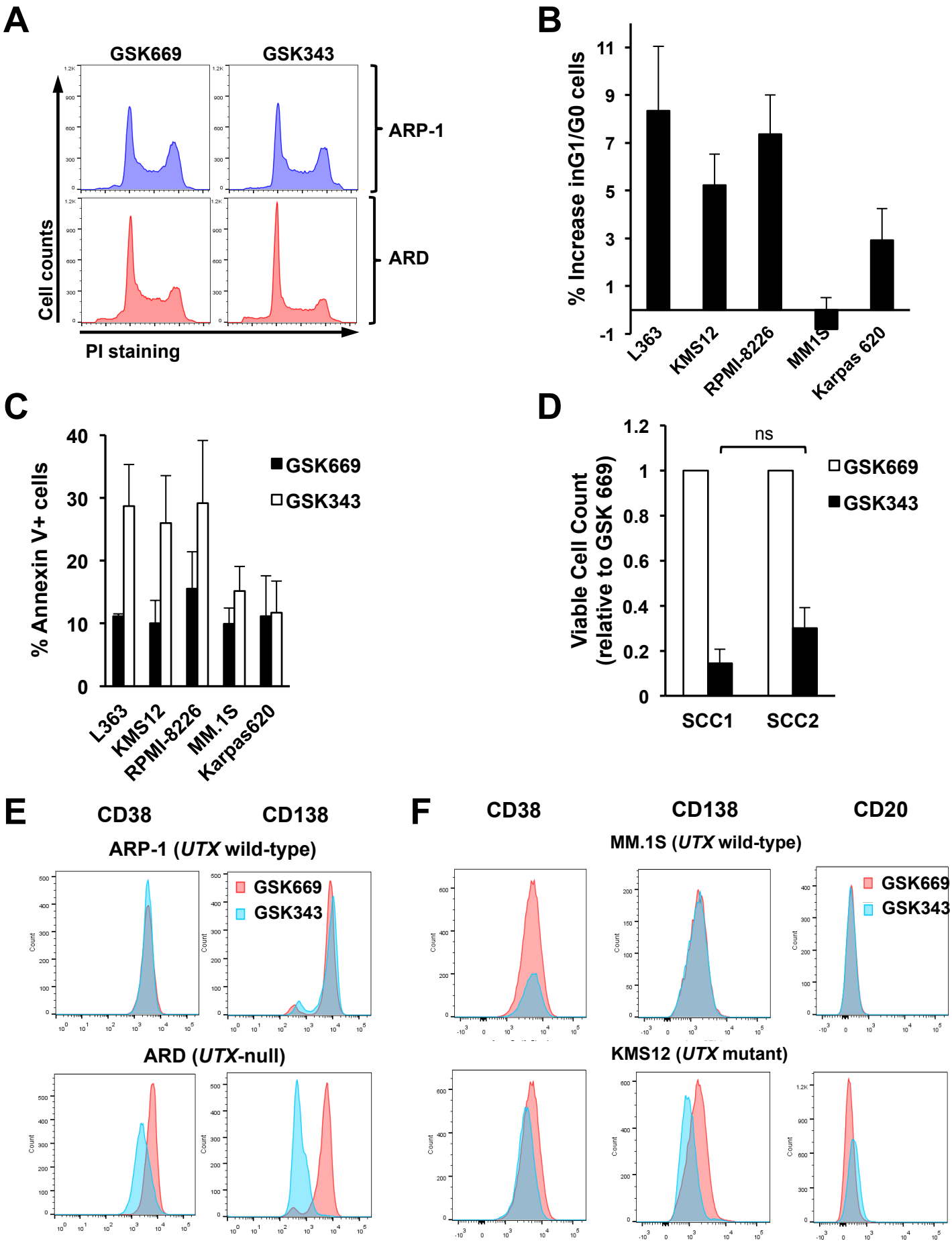


Figure S4. **Loss of UTX confers sensitivity to the EZH2i GSK343, related to Figure 3.**

(A) ARP-1 and ARD cells were treated with GSK343 or GSK669 for 4 days and the cell cycle profile was analyzed by flow cytometry using propidium iodide. Representative profiles are shown.

(B) A panel of MM was treated with GSK343 or GSK669 for 4 days and the cell cycle profile was analyzed by flow cytometry using propidium iodide. The percentage of cells in each phase from 3 independent biological experiments +/- SD is presented.

(C) The panel of MM cells was treated with GSK343 or GSK669 7 days and Annexin V positive cells were detected by flow cytometry. Values from 3 biological replicates +/- SD are presented as relative to values obtained for GSK669.

(D) SCC1 and SCC2 cells were treated with GSK343 or GSK669 for 7 days. Viable cell counts of cells treated with GSK343 are presented as relative to cell counts of cells treated with GSK669. The average from 3 biological independent experiments +/- SD is shown.

(E) ARP-1 and ARD cells were treated as in C and the expression of surface markers was analyzed by flow cytometry.

(F) *UTX*-mutant and wild-type MM cells were treated as in C and the expression of surface markers was analyzed by flow cytometry.

Figure S5, Related to Figure 3

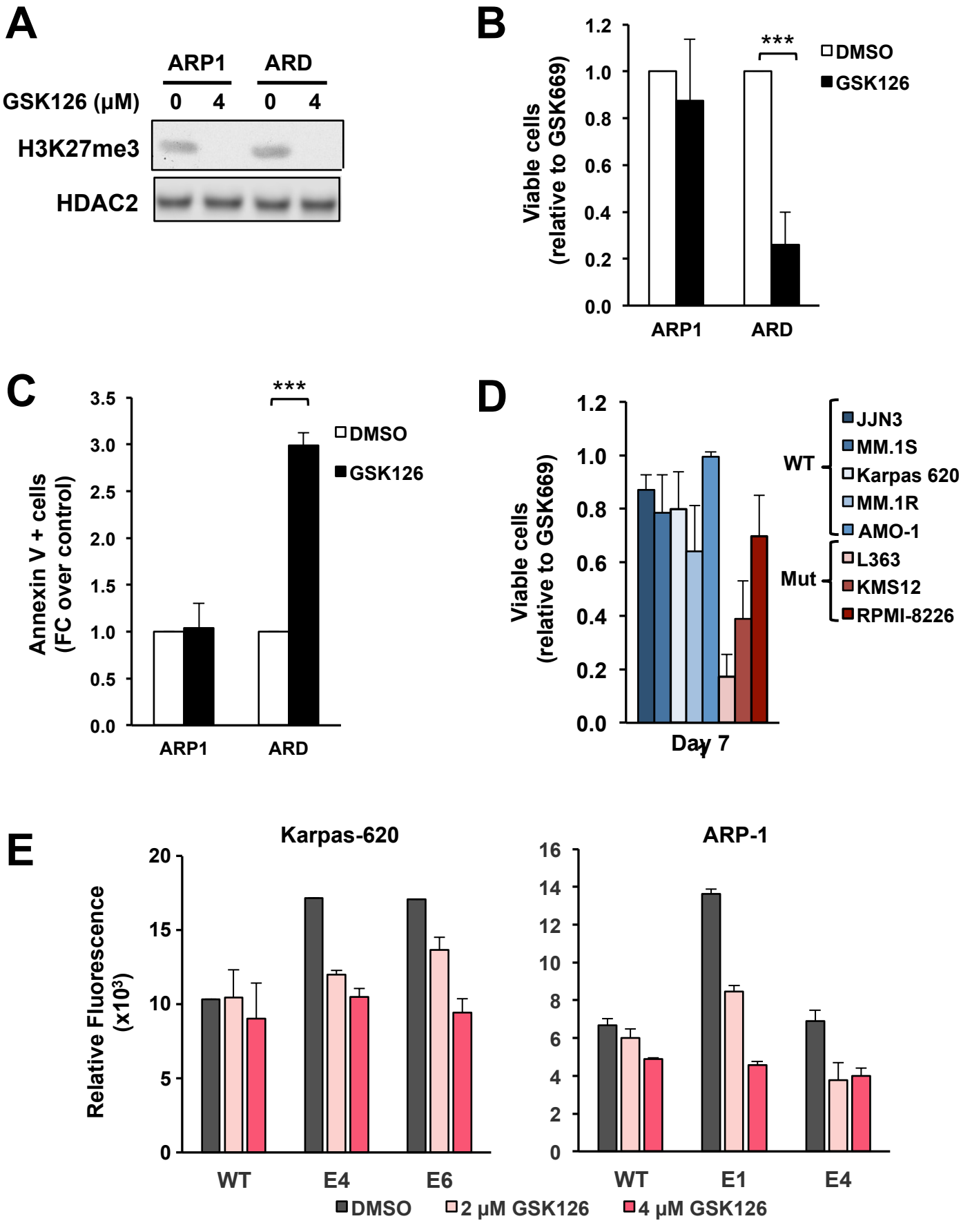


Figure S5. **Loss of UTX confers sensitivity to the EZH2i GSK126, related to Figure 3.**

(A) Nuclear extracts from ARP-1 and ARD cells treated with GSK126 4 μM or DMSO (0) as control for 7 days were immunoblotted with the indicated antibodies.

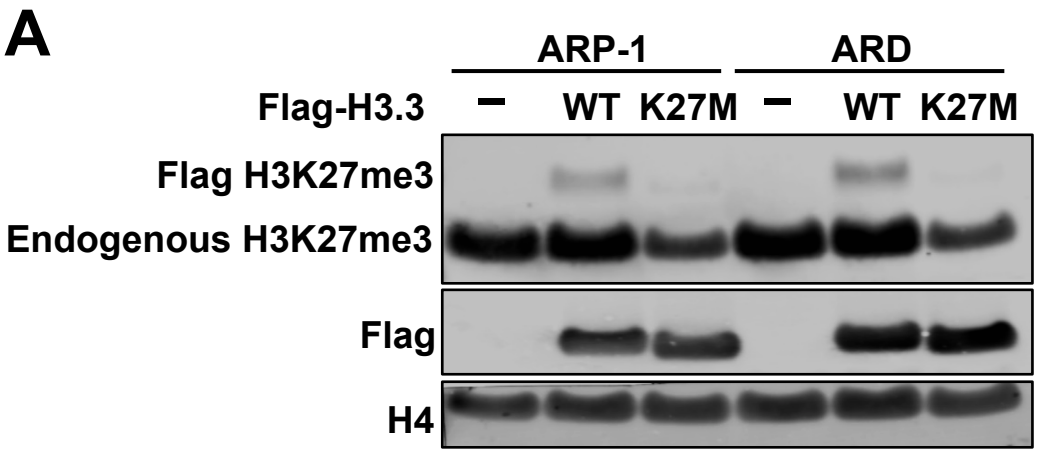
(B) ARP-1 and ARD cells were treated with GSK126 or DMSO for 7 days. Viable cell counts of cells treated with GSK126 are presented as relative to cell counts of cells treated with DMSO. The average from 3 biological independent experiments +/- SD is shown.

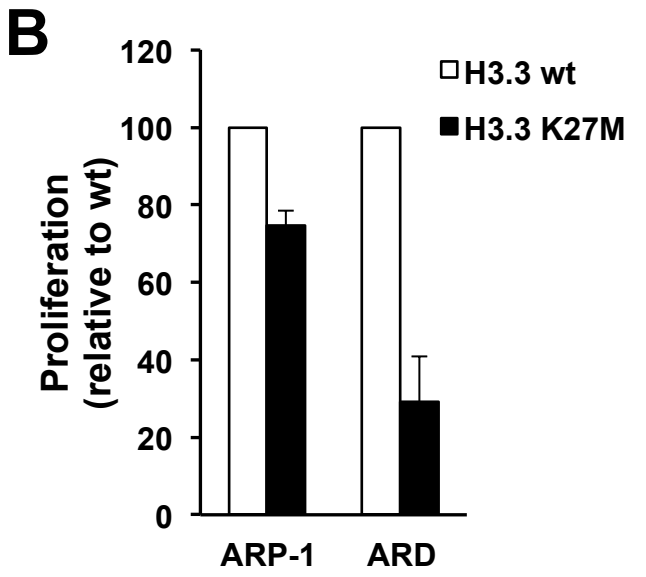
(C) ARP-1 and ARD cells were treated with GSK126 or DMSO for 7 days and Annexin V positive cells were detected by flow cytometry. Values from 3 biological replicates +/- SD are presented as relative to values obtained for DMSO.

(D) A panel of *UTX* mutant and wild-type MM cell lines were treated as in C. Viable cell counts of cells treated with GSK126 are presented as relative to cell counts of cells treated with DMSO. The average from 3 biological independent experiments +/- SD is shown.

(E) *UTX*-wild-type MM cell lines were CRISPR/Cas9-mediated gene edited in the *UTX* locus using gRNAs targeting two different exons. Cells were treated with GSK126 at the indicated concentrations for 6 days and the relative proliferation was measured using Alamar Blue. Bars represent technical replicates +SD, and biological replicates are shown in Fig 3G.

Figure S6, Related to Figure 3





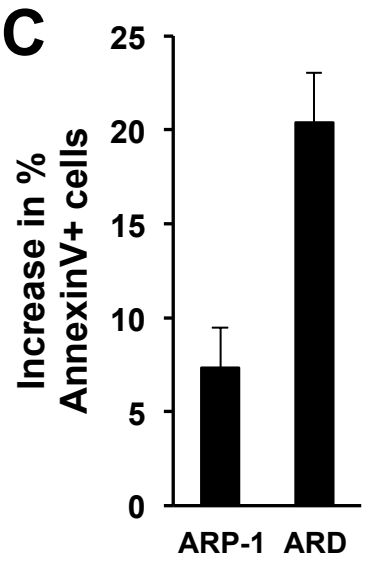


Figure S6. **Loss of UTX confers sensitivity to the loss of K27 on histone H3.3, related to Figure 3.**

(A) Nuclear extracts from parental ARP-1 and ARD cells and transduced with a plasmid encoding the histone H3.3 wild-type (WT) or H3.3 harboring the K27M mutation (K27M). Extracts were obtained from cells after selection and immunoblotted with the indicated antibodies.

(B) ARP-1 and ARD cells were transduced as in A and cell viability was measured 7 days post-selection. Results are presented as relative to those obtained in cells transduced with WT H3.3. The average from 3 biological independent experiments +/- SD is shown.

(C) Cells were transduced as in A and Annexin V positive cells were detected by flow cytometry at 7 days post-selection. The increase in the percentage of Annexin V positive cells in K27M-transduced cells compared to cells transduced with WT H3.3 is presented. The average from 3 biological independent experiments +/- SD is shown.

Figure S7, Related to Figures 4,5,6

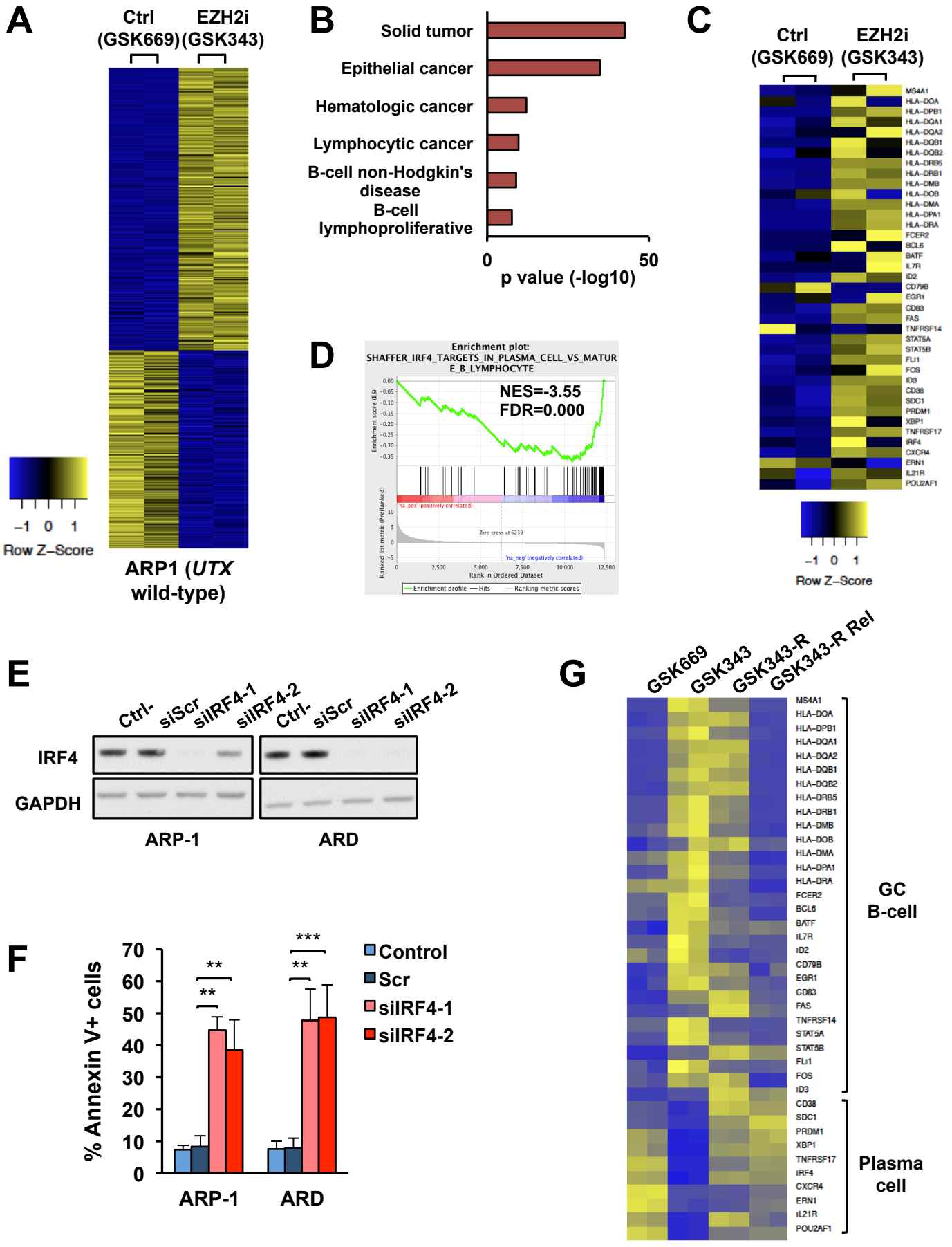


Figure S7. EZH2i leads to profound transcriptional changes of *UTX* mutant and wild-type cells with a de-differentiation process only present in null cells; whereas EZH2i-resistant cells avoid critical gene expression changes promoted by EZH2i, related fo Figures 4, 5 and 6.

(A) Heatmap of RNA-seq analysis in ARP-1 cells treated with the EZH2i GSK343 4 μM or a inactive control compound (GSK669) for 7 days (n=2).

(B) IPA of the top "Diseases and Disorders"" of the differentially expressed genes between ARD treated with GK343 and control cells.

(C) Heatmap of GC B-cell and plasma cell markers in ARP-1 cells treated with GSK669 or GSK343. Z-score is presented.

(D) GSEA plots of datasets identified comparing ARD treated with GSK669 or GSK343.

(E) ARD and ARP-1 cells were transfected with the indicated siRNAs and total proteins were extracted after 3 days and immunoblotted with the indicated antibodies.

(F) Cells were transduced as in E and Annexin V positive cells were detected by flow cytometry. Values from 3 biological replicates +/- SD are presented.

(G) Heatmap of RNA-seq analysis of ARD cells treated with the EZH2i GSK343 4 μM or a inactive control compound (GSK669) for 7 days, ARD-R cells and ARD-R after release from the drug for 7 days (n=2). GC B-cell and plasma cell markers are depicted. Z-score is presented.

Supplemental experimental procedures

Generation of GSK343-resistant cells

ARD cells were grown in the presence of GSK343 0.5 μM, increasing the concentration of drug by 0.5 μM every 7-10 days. Cells growing in GSK343 4 μM for three weeks were considered resistant. STR profiling confirmed that the cells matched the parental genotype.

Gain of Function Models

ARD and KMS34 SCC2 cells were transduced with the lentiviral vector pInducer20 harboring a Tet operator-controlled UTX cDNA (pUTX) or a control plasmid (pLac). Lentiviruses were generated by transfection of 293T cells with these plasmids, in addition to the packaging vectors psPAX2 and pMD2.G (Addgene) (Salmon and Trono, 2007), using Fugene 6 (Roche Applied Science, Indianapolis, IN) at a 1:3 DNA (μg) to Fugene 6 (μl) ratio. Lentiviruses were added to MM cell suspensions in the presence of 6 μg/ml polybrene (Millipore) and transduced for 48 h followed by selection in G418 (Corning). For the induction of UTX, ARD and SCC2 cells were grown in presence of 25 or 5 ng/mL of doxycycline, respectively. For H3.3 experiments, ARD and ARP-1 cells were transduced with pCDH-EF1-MCS-Puro vectors harboring Flag-HA-epitope tagged H3.3 WT or K27M mutant (gift from David Allis). Virus generation and transductions were performed as above and infected cells were selected with puromycin. For BCL6 and IRF1 overexpression analyses, cells were transduced with plasmids harboring such transcripts and tagged with GFP (Origene, RC219007L2, RC203500L2). Virus generation and

transductions were performed as above and the percentage of GFP positive cells was detected daily by flow cytometry (BD LSR II).

Loss of Function Models

For *IRF4* knockdown, cells were transfected with 150 nM siRNA using Neon (1000V, 50 ms, 1 pulse, Invitrogen). siRNAs were obtained from Dharmacon (MQ-019668-01). A scrambled siRNA (D-001206-14, Dharmacon, Lafayette, CO) was used as a control.

Immunoblotting

Nuclear proteins were extracted using the Nuclear Complex Co-IP Kit (Active Motif, Carlsbad, CA), following the manufacturer's instructions. For extraction of total proteins, cells were disrupted in 1% NP-40 lysis buffer (140 mM NaCl, 10 mM Tris-HCl pH 8, 1% NP-40) supplemented with proteinase inhibitors (Roche). Proteins were electrophoretically separated, blotted, and detected using enhanced chemiluminescence. Primary antibodies were: UTX (Genetex GTX121246), H3K27me3 (Millipore, 07-449), pan-H4 (Abcam, Cambridge, MA; Ab7311), RUNX3 (Santa Cruz, 101533), BIK (Cell signaling, 4592), IRF4 (Active motif, 61511), c-MYC (Abcam, ab32072), BATF (Cell Signaling, 8638), IRF1 (Cell signaling, 8478), BCL6 (Active motif, 61193) and GAPDH (Millipore, MAB374). Secondary antibodies were horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse IgG (GE Healthcare Life Sciences, Piscataway, NJ).

cDNA Preparation and Real Time PCR

Total RNA was extracted using the RNeasy Plus kit (Qiagen, Valencia, CA). Reverse transcription was performed using 2 μg of total RNA and the iScript Advanced cDNA Synthesis Kit (BioRad, Hercules, CA). Real time PCR determinations were performed using the Lightcycler 480 SYBR Green I Master reagent (Roche) or the TaqMan Universal PCR Master Mix (Lifetechnologies) on the Lichtcycler 480 II (Roche). Primers used are listed in Table S1.

Cell Proliferation and Soft Agar Assays

For the proliferation assays, 100,000 cells per well were seeded in 2 ml of media in 6-well plates, and doxycycline was added to half of the wells containing cells with UTX or the control plasmid. Every 3 days, live cells were counted by the trypan blue exclusion method, and the initial number of cells was replated in fresh media with or without doxycycline. For treatment of *UTX*-edited cells with EZH2 inhibitors, cells were seeded at $5x10^4$ cell/ml and treated with GSK126 or DMSO. At day 3, cells were split 1:4 and drug treatment refreshed. At day 6, 100 μl of cell suspension was mixed with 10 μl Alamar blue (Invitrogen), incubated at 37°C for 2-3 hours and fluorescence was detected on FluostarOptima plate reader (590/584). Clonogenicity of MM cells was determined by plating 1,000 cells in 6-well plates, with a bottom layer of 0.6% agar and a top layer of 0.3% agar containing the cells. One ml of media with or without doxycycline was added on top of the agar and renewed every 3 days. After a 10-day incubation, colonies were visualized using MTT reagent.

Cell Cycle and Apoptosis Analysis

For cell cycle determinations, cells were fixed with 70% ethanol for 1 hour, incubated with RNAase A 0.2 mg/mL (Sigma) for 1 hour at 37 °C and stained with propidium iodide (PI) 10 μg/mL (Sigma). Apoptosis was measured using the Annexin V-Cy5 Apoptosis Detection Kit (Biovision, Mountain View, CA). Flow cytometric analysis of these cells was performed using a Becton Dickenson LSR II.

Adhesion assays

ARD, ARP-1 and ARD cells containing the UTX inducible plasmid and treated with doxycycline for 6 days, were fluorescently labeled by incubation with calcein-AM (Sigma) 1 μg/ml for 30 minutes in serum-free media. Cells were washed with PBS and resuspended in adhesion media (RPMI, 0.5% BSA, 10mM HEPES buffer). One hundred μl containing 50,000 cells were added to 96-well plates containing a confluent monolayer of Hs-5 cells or coated with fibronectin (EMD Millipore), and incubated for 30 minutes at 37 C. Unbound cells were washed out with adhesion media, 100 μl of PBS were added per well and fluorescence was measured using a plate reader (Fluorostar Ultima, BMG labtech).

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation was performed using a ChIP kit (Millipore), with the modifications previously described (Martinez-Garcia et al., 2011). The antibodies used were: H3K27me3 (07-449), H3K27ac (Active motif, #39133), H3K4me3 (Millipore, #17-614), and Rabbit IgG (Millipore, #PP64). qPCR was performed as above. Enrichment was calculated as percentage of total input DNA precipitated.

Analysis of markers by flow cytometry

Cells were stained with 5 ug/ml of antibodies directed against CD38, CD138 and CD20 (eBiosciences, 11-1389-42, 48-0388-41, 17-0209-41), incubated with on ice for 30 min, washed, and analyzed on a LSR II.

Histone Preparation and Mass Spectrometry Analysis

Acid extracted histones from isolated nuclei were digested with trypsin and chemically derivatized using propionic anhydride (Garcia et al., 2007). Histone peptides were analyzed by a nanoLC-QqQ mass spectrometer (Dionex UltiMate 3000 and ThermoFisher Scientific TSQ Quantum) using the selected reaction monitoring method. Data were analyzed using Skyline software (MacCoss Lab, University of Washington) (MacLean et al.).

CRISPR-Cas9 editing and allele frequency quantification

One µl of annealed crRNA:tracrRNA ribonucleotides (at 10 µM in 10mM Tris pH 8.0) was incubated with 1 µl of Cas9 protein (10µM in OPTIMEM) at room temperature for 5 min (crRNA, tracrRNA and Cas9 were purchased from IDT, Coralville, Iowa). The complex was mixed with 1µl of 10µM of unspecific oligonucleotide (IDT) and mix with 5x105 cells (in 10µl buffer R). Immediately, electroporation was performed with NEON apparatus using the following settings: 1 pulse 20ms 1600 Volts. Cells were plated in advanced RPMI supplemented with Pen/strep and 5% FBS and passaged every 2-3 days. One day and 8 days post-electroporation, 1x105 cells were collected and DNA was

extracted using QuickExtract. The region of interest was amplified by PCR (Dreamtaq, Thermo Fisher Scientific). Amplicons were purified on column (DNA clean, Zymo), and analyzed by next generation sequencing at the Center for Computational and Integrative Biology at Massachusetts General Hospital, Boston.

The crRNA recognition sites are the following: *UTX* exon 4: CAGCATTATCTGCATACCAG; UTX exon 6: AGCTTTTGTCGAGCCAAGGA. Primers for generation of amplicons were: *UTX* exon 4 forward; 5'-TGT GGT GGG AAT CTT GTT ACC-3', reverse; 5'-GCA CAA ACA TAA ATA CTC TCA ACC C-3'. *UTX* exon 6 forward; 5'-GTT TCA ATG TAC TAC CAA GCA AGA A-3', reverse; 5'-ACC CAA CAA CCT ACC TTT AAA CT-3'.

Mouse models

Animal experiments were approved in compliance with the Northwestern University Animal Care and Use Committee (ASP# 2013-3123). Six-week-old female C57BL6 Nu/Nu mice (Jackson Laboratory) were acclimated for at least for 48 h before cell injection. A total of 5×10^6 cells transduced with a plasmid harboring the luciferase gene (pFU-2LT) were resuspended in 100 μL cold PBS, mixed with 100 μL of CultreX PathClear BME (3432-005-02, Trevigen), and injected subcutaneously in the dorsal region next to both thighs. Once tumors were detectable, half of the mice (n=7) were given doxycycline 2 mg/mL in water containing 0.04 g/mL of sucrose. The water was changed every other day to ensure delivery of a stable dose of doxycycline. Once a week, Luciferin (150 mg/kg) (Gold Biotechnology) was injected intraperitoneally and images were taken 10–15 min later using IVISR Spectrum (Caliper Life Sciences, Inc.).

Bioluminiscence was quantified using Living Images software. For inhibition of EZH2, tumors were formed as above and mice were treated with daily IP injections of GSK126 50 mg/kg (Activebiochem), or vehicle (20% captisol, pH 4.5). Tumors growth was measured weekly as above.

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