Supplemental Experimental Procedures

Generation of *Setd2*$^{flox/flox}$ mice

The heterozygous floxed *Setd2* mice (*Setd2*$^{+/flox}$) were generated by Taconics. Briefly, the targeting vector of the *Setd2* locus was linearized and electroporated into Art B6/3.6 ES cells (genetic background: C57BL/6 NTac). The vector incorporates loxP sites flanking exon 6 containing the SET domain, a domain responsible for histone methytransferase activity. After positive and negative selection with puromycin and thymidine kinase, clones were screened by PCR analysis, and validated by Southern Blot analysis. The targeted C57BL/6 NTac ES cells were microinjected into BALB/c blastocysts and implanted into pseudopregnant NMRI females. Male chimeras, selected by coat color contribution of the ES cells to the BALB/c host, were bred to C57BL/6 females and germline transmission was confirmed by PCR genotyping. All mice were generated and maintained in an AAALAC accredited facility and approved by the Texas A&M University Health Science Center IACUC committee.

Cell culture and generation of *SETD2*-null human cells

SETD2-null 786-0 (human renal cell carcinoma (RCC)) cells and human kidney (HKC) cells were generated using TAL effector nucleases targeted to exon 3 of *SETD2*. Cells were transfected with a pair of transcription activator-like effector nuclease (TALENs) constructs using Amaxar® Cell Line Nucleofector® Kit V (Lonza), and single cell sorted to isolate individual clones. Individual clones were screened for loss of the H3K36me3 mark and sequenced at the *SETD2* target site to confirm the presence of an inactivating mutation. Allelic sequencing confirmed *SETD2* inactivation through frameshift mutations at the TALEN target site in all *SETD2* alleles. The truncated *SETD2* (tSETD2) construct was sequence-optimized for expression in human cells, tagged with a FLAG sequence on the C-terminus, and synthesized by Life Technologies (Carlsbad, CA). tSETD2 was sub-cloned into the pINDUCER20 vector (Meerbrey et al., 2011). R1625C and R2510H mutations were generated by PCR-mediated mutagenesis. Inserts and mutations were verified by sequencing. Stable cell lines expressing tSETD2 or R1625C and R2510H mutants of tSETD2 carrying GFP- and HA-tags were obtained by transducing 786-0 *SETD2*-inactivated cells with lentiviral particles. The transduced cells were single cell sorted for GFP expression using FACSAria II Flow Cytometer (BD Bioscience). GFP expressing colonies were further tested for recombinant gene expression by immunoblot analysis (HA-tag and H3K36me3) and immunofluorescence (H3K36me3). Cells were cultured in DMEM (Dulbecco's Modified Eagle Media) media supplemented with 10% FBS (fetal bovine serum).

Coimmunoprecipitation assays and antibodies

Endogenous SETD2 was immunoprecipitated from cell extracts using anti-SETD2 antibody (Abcam, or Sigma-Aldrich) and protein A/G agarose beads (Santa Cruz Biotechnology) using 1X cell lysis buffer (20mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, and protease inhibitor cocktail (Roche)). The immunoprecipitated complex was washed using phosphate buffered saline (PBS) (3.2mM Na2HPO4, 0.5mM KH2PO4, 1.3mM KCl, 135mM NaCl, pH7.4) containing 1% Triton X-100 (PBST). To identify direct interaction between SETD2 and α-tubulin, immunopurified SETD2 from MEF extracts bound to the protein A/G agarose beads was incubated with 1μg of recombinant α-tubulin (TUBA1A) protein (Origene) for 1 hour, and washed at least three times using PBST buffer. To ascertain whether K40 of α-tubulin is required for interaction with SETD2, overexpressed SETD2 was co-immunoprecipitated using whole cell extracts from HEK293T cells expressing human SETD2 and EGFP-α-tubulin or EGFP-K40R-α-tubulin. Following immunoprecipitation, the samples were subjected to SDS-PAGE gel electrophoresis under denaturing conditions, and subsequently immunoblotted using antibodies against SETD2 (Abcam or Sigma-Aldrich), GFP (Abcam), Histone H3 (Abcam) or α-tubulin (Santa Cruz Biotechnology, DM1A). TrueBlot anti-rabbit or mouse IgG-HRP (Rockland) were used as secondary antibodies.

GST and peptide pull down assays

GST-SETD2 fusion protein expressing constructs were generated in a pGEX-4T-1 (GE Healthcare) vector by cloning fragments of human *SETD2* cDNA (GenScript) into the *BamHI* and *XhoI* sites via PCR using In-Fusion® HD Cloning Kit (Clontech). The following constructs were generated: G1 (1 – 299 amino acids (a. a.) of human SETD2), G2 (284 -624 a. a.), G3 (616 – 1066 a. a.), G4 (1060 – 1497 a. a.), G5 (1464 – 2023 a. a.),

G6 (2018 – 2374 a. a.), G7 (2018 – 2441 a. a.), G8 (2438 – 2564 a. a.), G9 (1464 – 2374 a. a.), G10 (1464 – 2441 a. a.), G11 (1464 – 2564 a. a.), G12 (1418 – 1714 a. a.). GST-SETD2 constructs were transformed to BL21 *E. coli* and induced using 0.5mM IPTG. Cells were collected by centrifugation and digested with 0.5mg/ml lysozyme (Thermo Scientific) and 250 Units Benzonase (Millipore) on ice for 30 mins in PBST buffer. The cells were subsequently sonicated for 30 seconds to collect the soluble fractions. Insoluble fractions were collected using detergents added to 2% Sarkosyl, 40mM CHAPS, 4% Triton X-100 buffer. The GST-SETD2 fusion proteins were purified using glutathione-agarose beads (Pierce), and expression of the constructs analyzed on SDS-PAGE gels stained with Coomassie blue. In parallel, the purified GST-SETD2 fusion proteins were incubated with 1μg of recombinant α-tubulin (TUBA1A) protein (Origene). Following washing using PBST, the protein complexes were run on SDS-PAGE gels. Immunoblot analyses were performed using a mouse anti-α-tubulin antibody (Santa Cruz Biotechnology). For the peptide pull-down assay a biotin-labeled K40 peptide of α-tubulin was commercially synthesized containing +/- 10 amino acids around the K40 residue of α-tubulin (IQPDGQMPSDKTIGGGDDSFT-biotin, ThermoFisher Scientific). Five micrograms of the K40 peptide was incubated with 5μg of purified GST-SET domain (G12) protein or GST control protein in PBST with streptavidin-agarose beads overnight at 4℃. The incubated peptides were washed at least four times using PBST, and immunoblotted using anti-GST antibody (Santa Cruz Biotechnology).

***In vitro* methylation assays and mass spectrometry analysis**

Commercially available microtubule proteins (Cytoskeleton) were polymerized according to the manufacturer's protocol. Briefly, microtubules (1mg/ml) were polymerized in warm polymerization buffer (15mM PIPES (pH 7.0), 1mM $MgCl_2$, 20μM paclitaxel) at RT for 15 mins with occasional mixing. Depolymerized microtubules were prepared by dissolving bovine microtubules (Cytoskeleton) in PBS. *In vitro* methylation of the microtubules (0.5μg) was performed in the presence of 1μg of recombinant SETD2 (1392-2564 a. a) (Active Motif) incubated in PBS buffer with protease inhibitor cocktail (Sigma-Aldrich) using S-[methyl-^{3}H]-Adenosyl-L-Methionine (SAM) as a methyl donor (RT, overnight). Histone proteins were purified from HEK293T cells by acid extraction according to a histone extraction protocol (http://www.abcam.com/protocols/histone-extraction-protocol-for-western-blot) and 2μg of histone proteins were used in the above assay as a positive control. *In vitro* methylation of recombinant TUBA1B-myc (Origene) protein (0.5μg) was performed as described above in PBS with or without 0.5μg of histone deacetylase 6 (HDAC6) (Active Motif). *In vitro* methylation assays using alternate sources of recombinant α-tubulin (GST-TUBA4A from Abnova) or the K40 peptide as substrates, were not successful (data not shown). Samples were run on 12% SDS-PAGE gels and transferred to PVDF membranes. β-mercaptoethanol (1:1,000 dilution) was added to both the running buffer and transfer buffers to dissociate polymerized tubulin proteins. To enhance the β-ray activity and signal, EN3HANCE spray (PerkinElmer) was sprayed on the membrane. The film was exposed for one week prior to visualization. For mass spectrometry analysis, α-tubulin (TUBA1B-myc) protein purified from HEK293T cells (Origene) was eluted from a SDS-PAGE gel and digested with *Asp-N* (Promega). The resulting peptides were purified using C18 spin columns and were analyzed using a LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. Methylation was analyzed using the Proteome Discoverer 1.3 with MASCOT search engine.

Immunocytochemistry and live cell imaging

After fixation as described in Experimental Procedures, the cells were washed using PEM/PEG buffer and blocked in blocking buffer (5% skim milk in PEM/PEG buffer) for 1 hour at RT. Cells were incubated in blocking buffer using primary antibodies overnight at 4^{o}C. Primary antibodies were diluted as follows; mouse anti-K40 acetylated α-tubulin (1:4,000, Santa Cruz Biotechnology), rabbit anti-H3K36me3 (1:4,000, Active motif), rabbit pan-trimethyl lysine (1:4,000, Cell Signaling Technology), mouse anti-γ-tubulin clone GTU-88 (1:4,000, Sigma), rabbit anti-detyrosinated tubulin (1:4,000, Chemicon), mouse anti-polyglutamylated tubulin clone GT335 (1:4,000, Enzo Life Sciences or Adipogen), anti-kinetochore (CREST, 1:6,000, Antibody Inc). After five rounds of washing for 10 mins each using PEM/PEG buffer, cells were incubated for 1 hour in blocking buffer with corresponding secondary antibodies (Alexa fluor (H+L) goat anti-mouse 546 and goat anti-rabbit 488 (Invitrogen)) at a dilution of 1:5,000. Following more than three washes, for 10 mins each using PEM/PEG buffer, cells were then post-fixed to stabilize the signal for 10 mins using 4% paraformaldehyde in PEM/PEG buffer. Cells were counterstained using DAPI for subsequent visualization of nuclei. Coverslips were mounted in SlowFade Gold (Molecular Probes) and imaged using deconvolution and confocal microscopy (DeltaVision Elite (GE), Ti Eclipse (Nikon) or InCell6000 (GE)). For live cell imaging, cells were plated on 6cm tissue culture dishes with glass bottoms (Thermo Scientific). The cells were subsequently visualized using a Nikon TiE confocal microscope using a 10x NA=0.3 lens. A Kohler alignment was performed before image acquisition in order to optimize the light path for phase contrast. A total of 64 images, with a ten percent overlap, were collected every 6 mins for a total of 12 hours. All image processing was done in Fiji/ImageJ. An additional stage drift correction was also imposed using image registration tools (StackReg) in Fiji (Thevenaz et al., 1998).

Mitotic cells were manually identified and the elapsed time of mitosis determined. As a criterion for entry into mitosis the cells had to be fully rounded; likewise, exit of mitosis was determined by reattachment of the cells and visualization of two distinct daughters. Mitosis times were measured from the time when cells were fully rounded to the time when cells were reattached as two flat daughter cells. Only cells in which a full mitotic event was captured were included in analysis. All plots were generated using Prism (Graphpad).

Intensity profile quantitation

Multiple exposure-matched fields were captured on an InCell6000 (GE) from coverslips using a 20x objective. A line was placed over midbodies that had been manually identified. From this line a profile for both the acetylated-α-tubulin and H3K36me3 was calculated using ImageJ. Midbodies that resided directly over nuclei were excluded from analysis to exclude the possibility of measuring nuclear H3K36me3 label. A total of 102 midbodies from three independent experiments were segmented using this method. Midbody length and intensity were subsequently range normalized to generate an average profile.

Nuclear content quantitation

Exposure times were set using positive and negative controls; all subsequent images were acquired using the previously defined settings. Multiple fields per coverslip were collected by rastering across a defined area using robotic stages. Images were then processed and quantified using algorithms developed in Pipeline Pilot (Accelrys) designed for single cell nuclear property mining. The workflow consists of reading images into the software followed by a user defined background subtraction optimized to each run. An intensity threshold mask is then applied to the DAPI channel for crude identification of cell objects. The crude nuclear mask is then eroded and subsequently used as seed regions for a watershed mask, resulting in the final nuclear mask. Morphometric and intensity measures are then collected for each masked region. Nuclear content is then extrapolated using this data.

Flow cytometry

Cell cycle analysis was performed by flow cytometry and the samples prepared as outlined in Current Methods of Cytometry (Darzynkiewicz and Juan, 2001) under the section DNA content analysis of fixed cells with propidium iodide (PI). Cells were fixed in 70% ethanol overnight. Fixed cells were treated with 10mg/ml RNaseA in PBS for 20 mins and added to 5mg/ml PI and 0.1% Triton X-100. After equilibration at 4℃ in the dark for at least 1 hour, cells were analyzed BD FACSAria Fusion (BD). Gates were appropriately set to remove debris and doublets based off of cellular complexity and size.

Cell fractionation and histone extraction

Cytoplasmic fractions and histone proteins of parental 786-0 cells and *SETD2*-null 786-0 cell lines stably transfected with EGFP, tSETD2, R1625C or R2510H expression vectors were prepared by gentle pipetting several times and incubated on ice for 10 mins in a cell fractionation buffer (320 mM sucrose, 10mM Tris-HCl (pH8.0), 2mM magnesium acetate, 0.1 mM EDTA, 0.5% NP-40, fresh 1mM dithiothreitol, protease inhibitor cocktail). Cytoplasmic fractions were collected after centrifugation at 1,000 g for 5 min and were used for immunoprecipitation experiments using α-TubK40me3 antibody. Histone proteins were extracted by adding equal volume of 0.2 N HCl to the pelleted nuclear fraction and stored at 4℃, overnight. After centrifugation at 10,000 g for 10 min, the soluble fractions were neutralized by addition of 10N NaOH (1/50 volume) and were used for immunoblots probed with the H3K36me3 and H3 antibodies.

RNAseq Analysis

RNAs from 786-O parental cells, left-TALEN treated (*SETD2* wildtype control) cells, and two *SETD2*-null cell clones, as well as HKC parental cells, left-TALEN treated (*SETD2* wildtype control) cells, and two *SETD2*-null cell clones were isolated (TRIzol Reagent, ThermoFisher). Total RNAs were either poly-A selected (786-O) (Oligotex, Qiagen) or rRNA depleted (HKC) (Ribominus Core Module v2, Ambion), fragmented (RNA Fragmentation Reagents, Ambion), converted to cDNA (SuperScript II, ThermoFisher), and used to generate libraries (TruSeq, Illumina). Libraries were paired-end sequenced (786-O: Illumina HiSeq2000, HKC: Illumina HiSeq2500), filtered for adapters with an FDR cutoff of 0.001 (TagDust v1.12), and aligned to hg19 using TopHat (v2.0.13) with default alignment parameters, and the addition of --bowtie1, --no-mixed, and --no-coverage-search. Files were filtered for properly paired reads only, and duplicate paired-end fragments were removed after alignment to account for PCR artifact. Read counts were generated with HTSeq (v0.6.1p1) default, non-stranded settings, which were used to identify differential genes at a p-value < 0.05 (DESeq2 v1.6.3). MA plots were generated using DESeq2 ($p < 0.05$).

Statistics

In order to determine statistical significance of the number of cells with more than one nuclei between the control and *Setd2* KO cells (Figure 5D), a Tukeys multiple comparison test was performed on the mean from three independent biological replicates. The mean and standard deviation were graphed for each sample and *p*-values reported as indicated. To determine if the number of defects in mitosis were independent of *Setd2* status (Figure 6B), a contingency matrix was constructed using the *Setd2* status and mitotic event label as factors, and the count of events from a pool of independently treated wells as the response variable. To estimate if the populations were drawn from the same distribution a chi-square test was performed on the contingency table and *p*-values reported on the graph. To determine if the rescue of mitotic defects in 786-0 cells was significant a two-way ANOVA test was performed using the percent normalized values from two biological replicates, each of which contained three technical replicates. This test showed significant interactions between samples and the rate of mitotic abnormalities. To further infer which interactions were significant a Tukey's multiple comparison test was used and the adjusted *p*-values reported (Figure 7B). Densitometric analyses performed on immunoblots of α-TubK40me3 levels in response to *SETD2* depletion and subsequent rescue with *SETD2* constructs (Figure 7D) were normalized to α-tubulin in order to control for loading variability. This normalized value was further scaled to the parental $SETD2^{+/+}$ control to account for inter-assay variability. Statistical significance was determined using ANOVA and Tukeys multiple comparison tests. The mean and standard error from 5 independent biological replicates is plotted as shown with reported *p*-values comparing highlighted samples. In all cases a *p*-value of less than 0.05 is considered to be significant. Statistical analysis was performed using a combination of Prism (GraphPad), Excel (Microsoft), R or Pipeline Pilot (Accelrys).

Supplemental References

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