

Supplementary Information to the publication

MLL-fusion-driven leukemia requires SETD2 to safeguard genomic integrity

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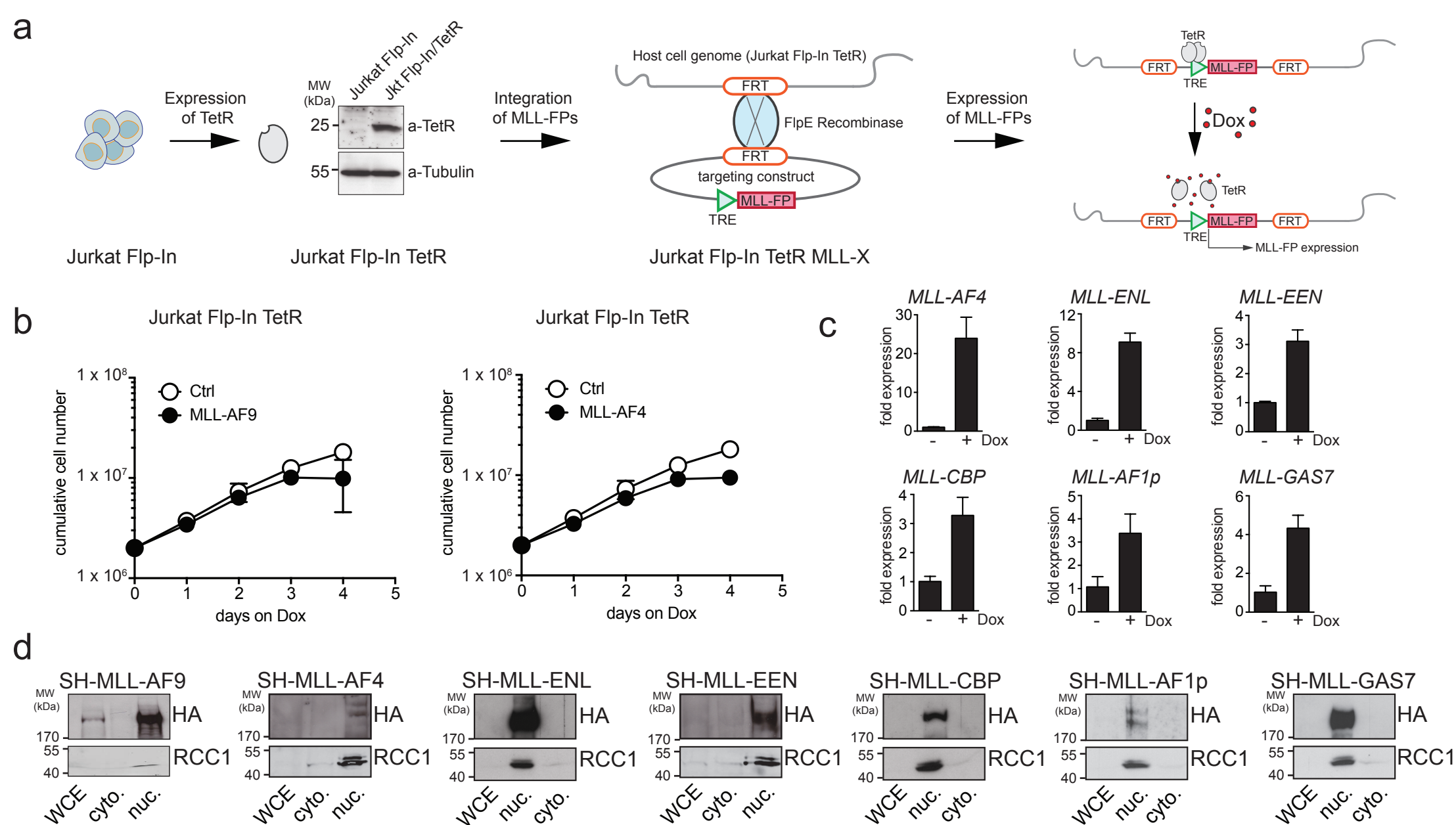
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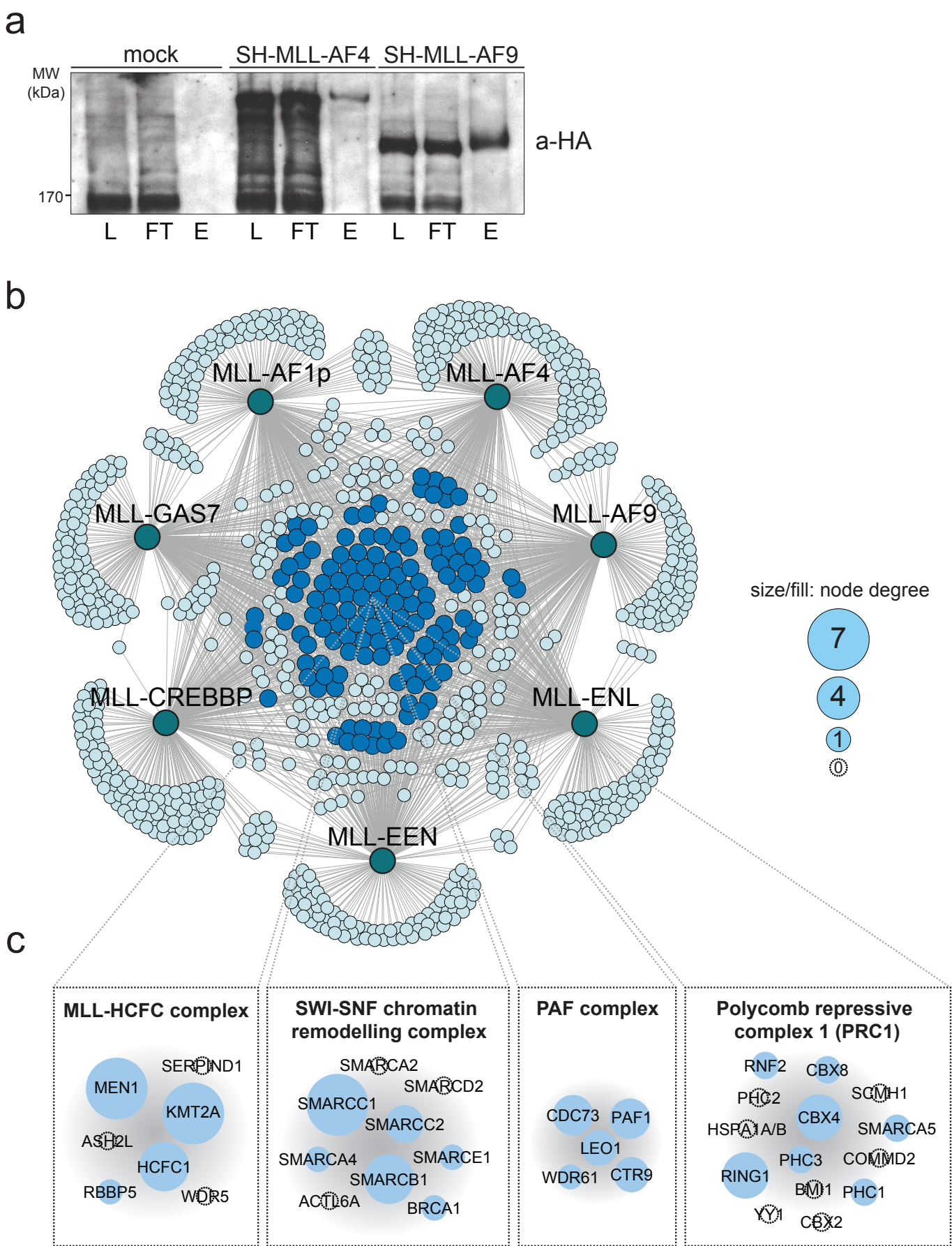
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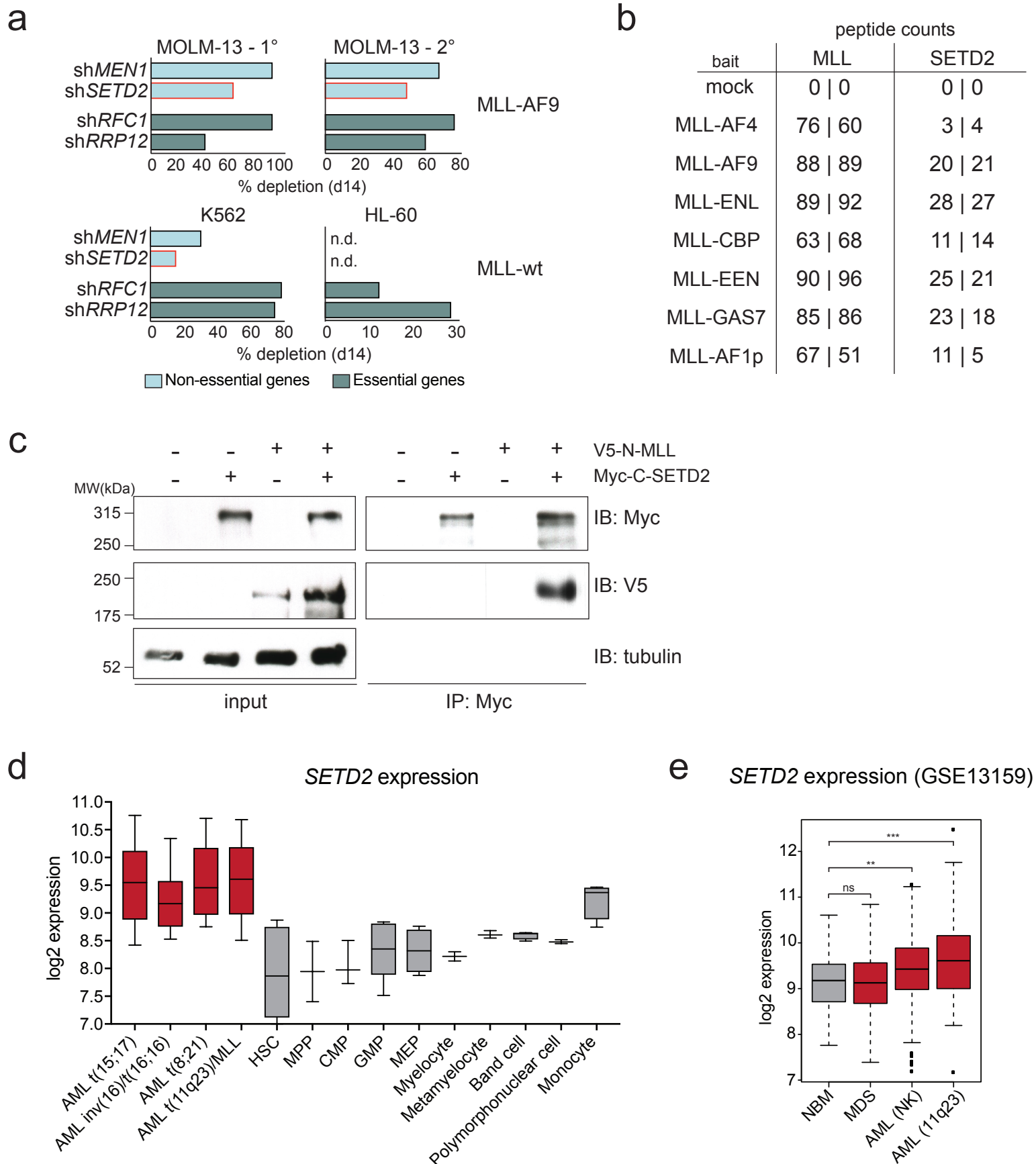
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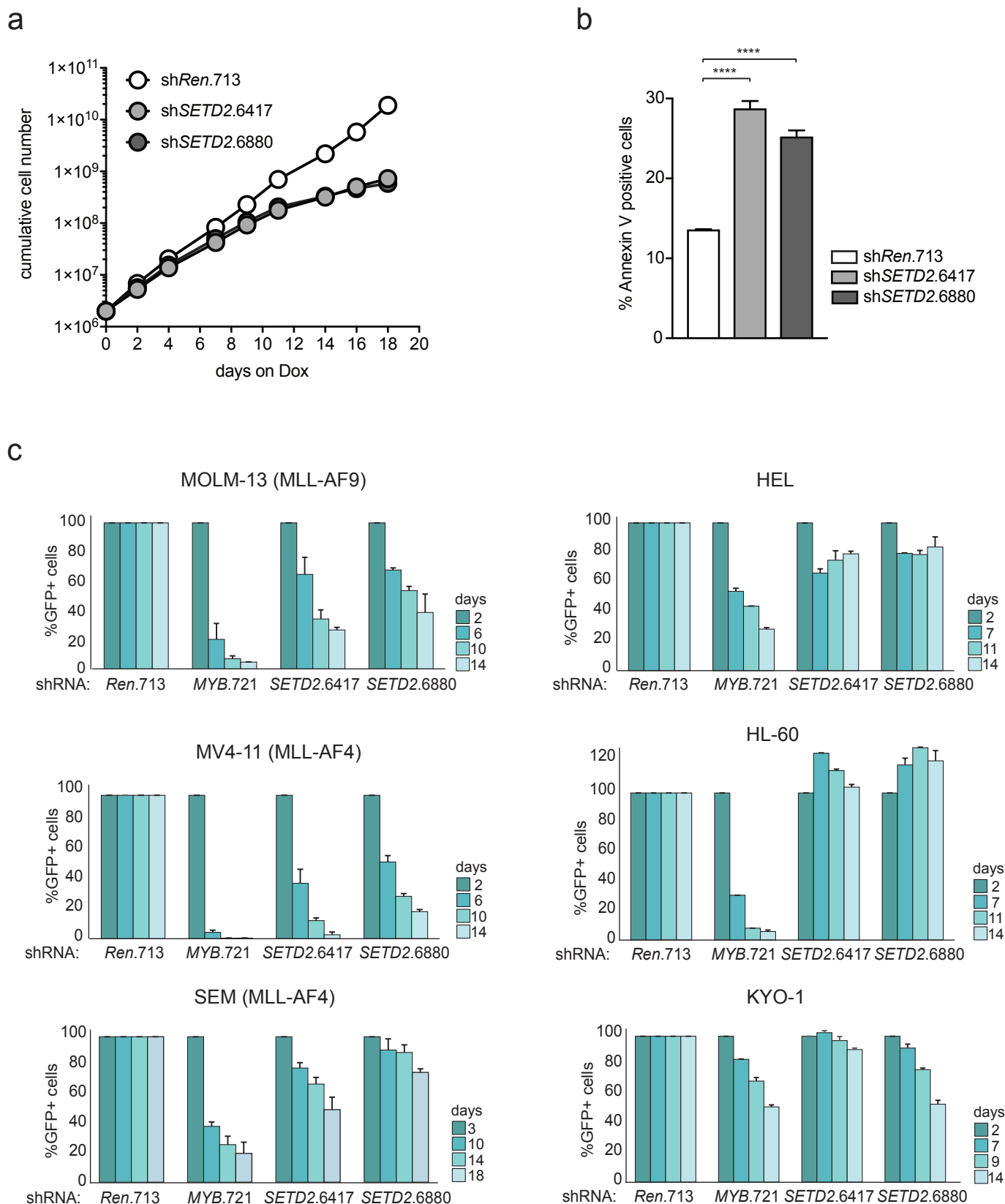
Supplementary Figure 1. Generation and characterization of isogenic cell lines for expression of affinity-tagged variants of MLL-fusion proteins. (a) Schematic illustration of the workflow for establishing the cell line models for affinity purification of MLL fusion proteins. The Flp-In system involves introduction of a Flp Recombination Target (FRT) site into the genome of the Jurkat cell line. An expression vector encoding for MLL-fusion protein is integrated into the genome via Flp recombinase-mediated DNA recombination at the FRT site. **(b)** Growth curves of Jurkat Flp-In cell lines expressing indicated MLL-fusion proteins (mean±s.d. n=3). **(c)** Cells expressing Strep-HA (SH)-tagged MLL fusion proteins were treated with Dox for 24h and transgene expression was monitored by qPCR (mean±s.d. n=3). **(d)** Extracts from Jurkat cells expressing indicated MLL-fusion proteins were fractionated and analyzed for expression of hemagglutinin (HA). RCC-1 was used as loading control (WCE: whole cell extract; nuc: nuclear fraction; cyto: cytosolic fraction).



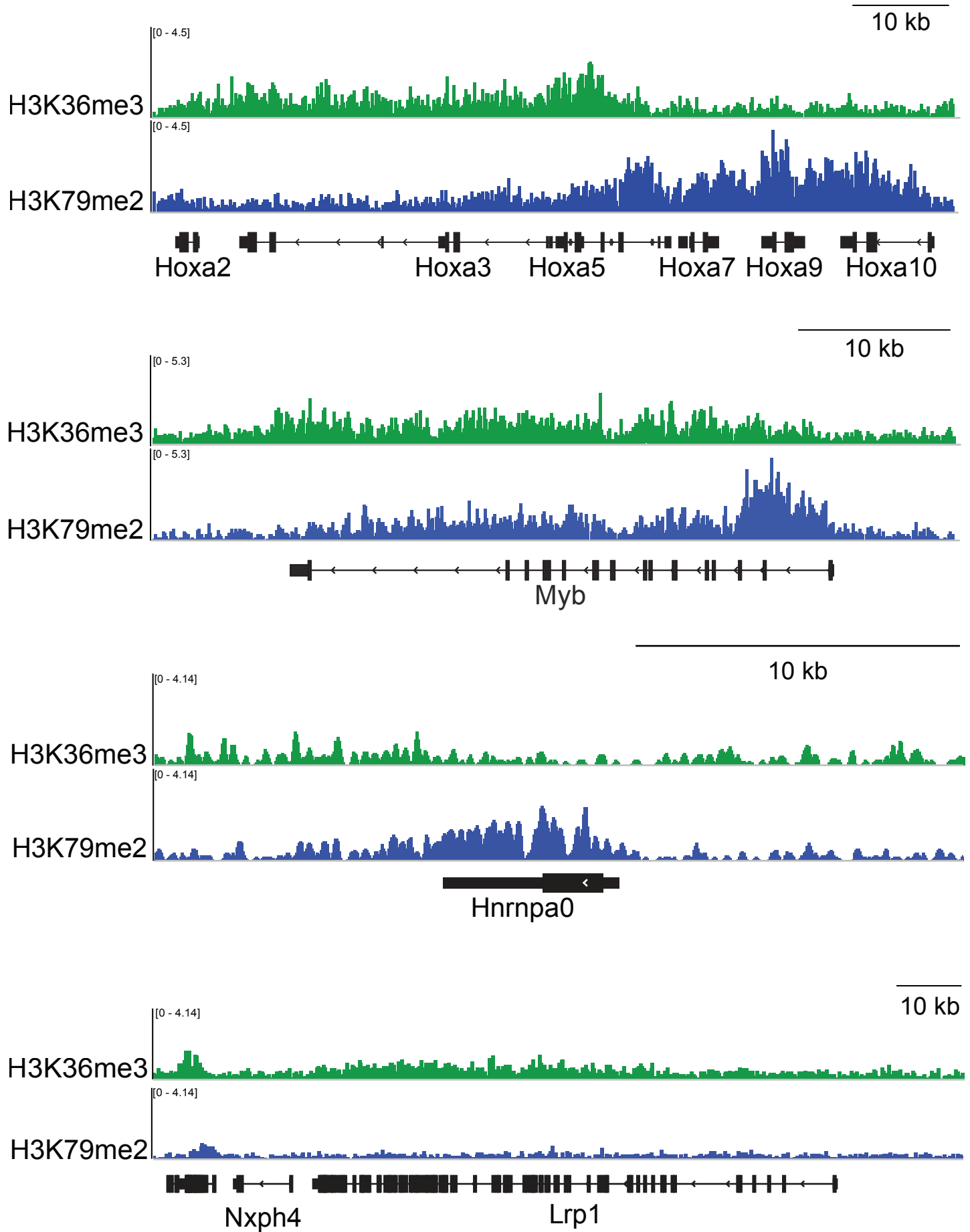
Supplementary Figure 2. Identification of protein complexes among the MLL-fusion protein-interactome. (a) Western blot (WB) analysis of lysate (L), flow-through (FT) and eluate (E) from StrepTactin-purifications of indicated affinity-tagged MLL-fusion proteins. **(b)** Network representation of the AP-MS-derived protein-protein interaction network of seven MLL-fusion proteins (dark green nodes), showing the top 300 interactors per bait ranked by p-value (light blue nodes). Nodes labelled in dark blue represent 128 proteins that interact with ≥ 5 of the 7 MLL-fusion proteins. **(c)** Representation of enriched protein complexes previously reported to be associated with MLL. The size and the color of the nodes correspond to the number of interaction partners.



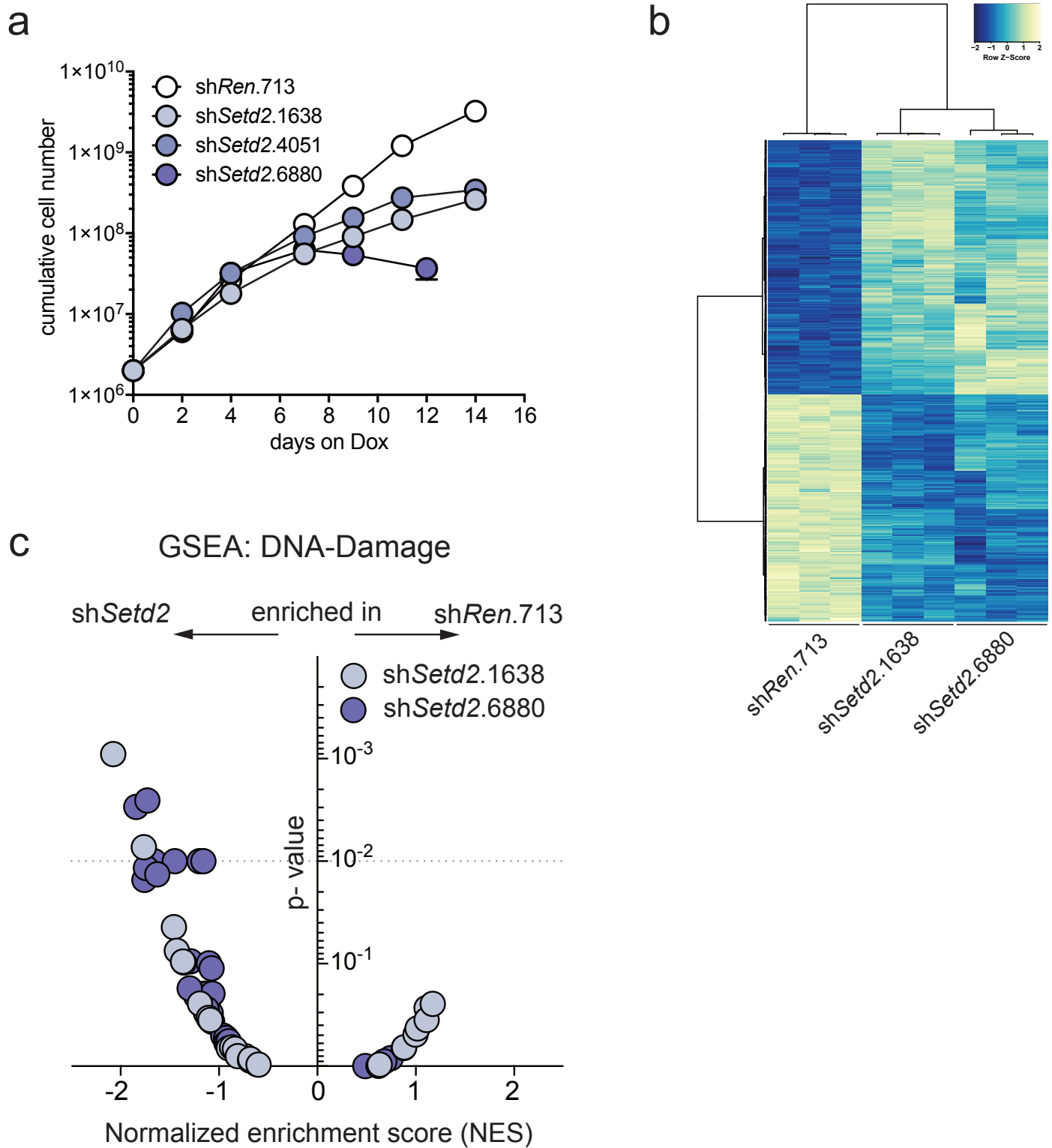
Supplementary Figure 3. shRNA screening of conserved MLL-interactors identifies SETD2 as a critical effector of MLL-fusion proteins. (a) Examples of depletion phenotypes of four selected genes in MLL-rearranged vs. MLL-wild type leukemia cell lines. Gene essentiality was assigned based on published datasets. (b) Peptide counts for MLL and SETD2 from AP-MS experiments of MLL-fusion proteins. (c) HEK293 rTA3 cells were transiently transfected with indicated constructs. Following immunoprecipitation using anti-Myc beads, eluates (IP) and whole cell extracts (input) were analyzed by immunoblotting with the indicated antibodies. (d) Expression levels of SETD2 in leukemia and normal hematopoiesis (bloodspot.binf.ku.dk). (e) Expression levels of SETD2 in different AML subtypes (NBM: Normal Bone marrow; MDS: Myelodysplastic Syndrome; AML (NK): Normal Karyotype; AML (11q23): AML harboring rearrangements of the MLL gene). ns, not significant, ** $p < 0.01$, *** $p < 0.001$ (t-test).



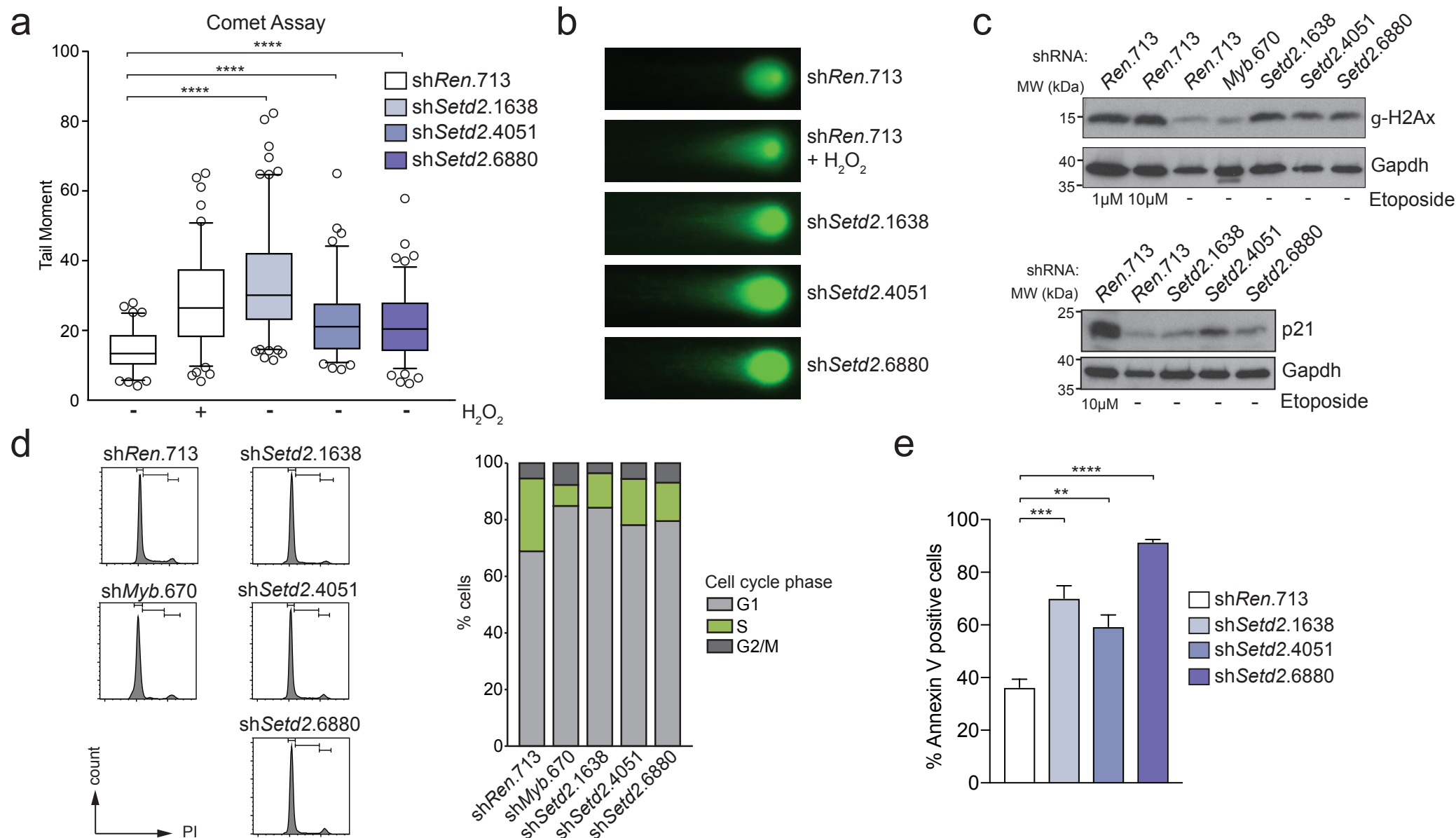
Supplementary Figure 4. shRNA-mediated knockdown of SETD2 impairs proliferation of human MLL-rearranged cell lines. (a) Growth curves of MV4-11 cells expressing indicated shRNAs (mean $\pm$ s.d. n=3). **(b)** Quantification of flow cytometric analysis of apoptosis as measured by Annexin V-staining in MV4-11 cells. Cells were treated with Dox for 11 days and stained according to manufacturer's protocol. **** p<0.0001 (t-test) (mean $\pm$ s.d. n=3). **(c)** Results of FACS-based competitive proliferation assay shown as the percentage of GFP-positive cells expressing individual SETD2-targeting shRNAs in the presence of Dox over 14-18 days in indicated human cell lines (mean $\pm$ s.d. n=2).



Supplementary Figure 5. MLL-target genes show high levels of H3K36me3 and H3K79me2. H3K36me3 (green) and H3K79me2 profiles (blue) of selected MLL-AF9 target genes Hoxa-cluster genes and Myb vs. non-MLL target genes (Hnrnpa0 and Lrp1).

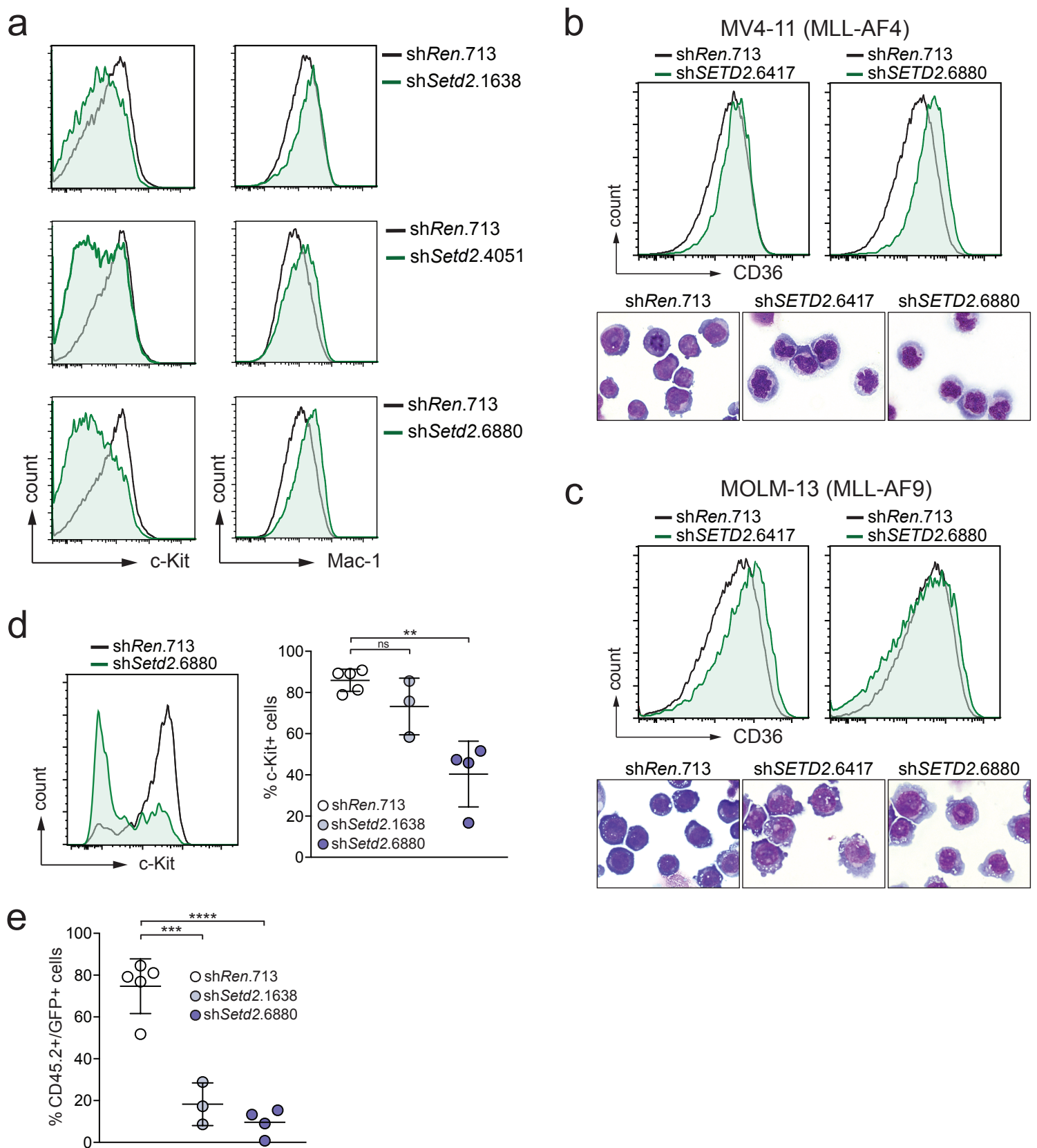


Supplementary Figure 6. Loss of SETD2 induces DNA damage-associated transcriptional changes in MLL-AF9 expressing cells. (a) Growth curve of mouse *MLL-AF9/NrasG12D* AML cells expressing indicated shRNAs. **(b)** Heatmap representation of differentially expressed genes in *MLL-AF9/NrasG12D* AML cells upon *Setd2* knockdown (mean±s.d. n=3). **(c)** Diagram showing results from GSEA of gene sets associated with DNA damage (from MSigDB) in *MLL-AF9/NrasG12D* AML cells upon shRNA-mediated knockdown of *Setd2*.

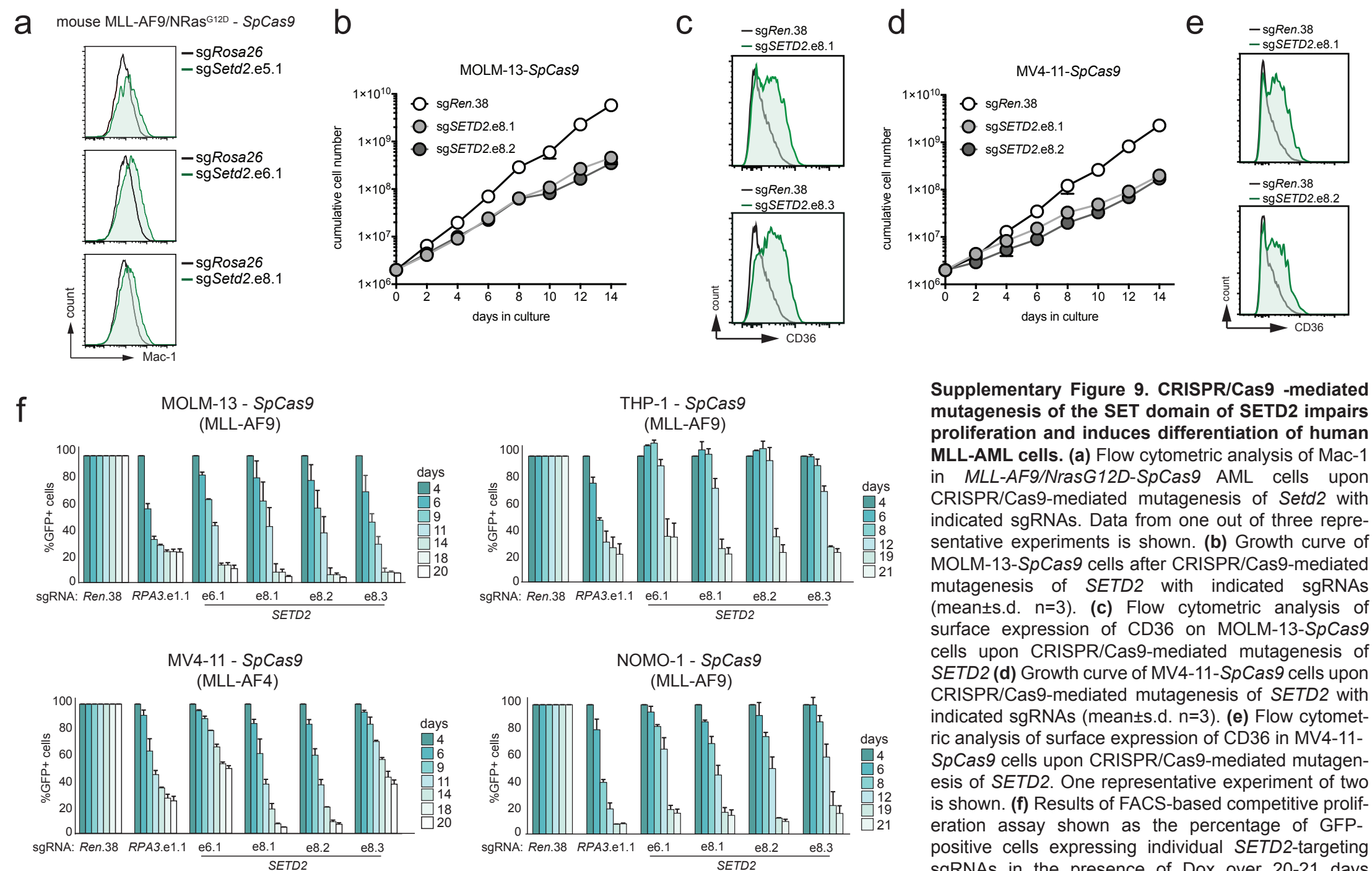


Supplementary Figure 7. Loss of Setd2 leads to accumulation of DNA damage, p21 expression, cell cycle arrest and apoptosis in MLL-fusion-expressing cells.

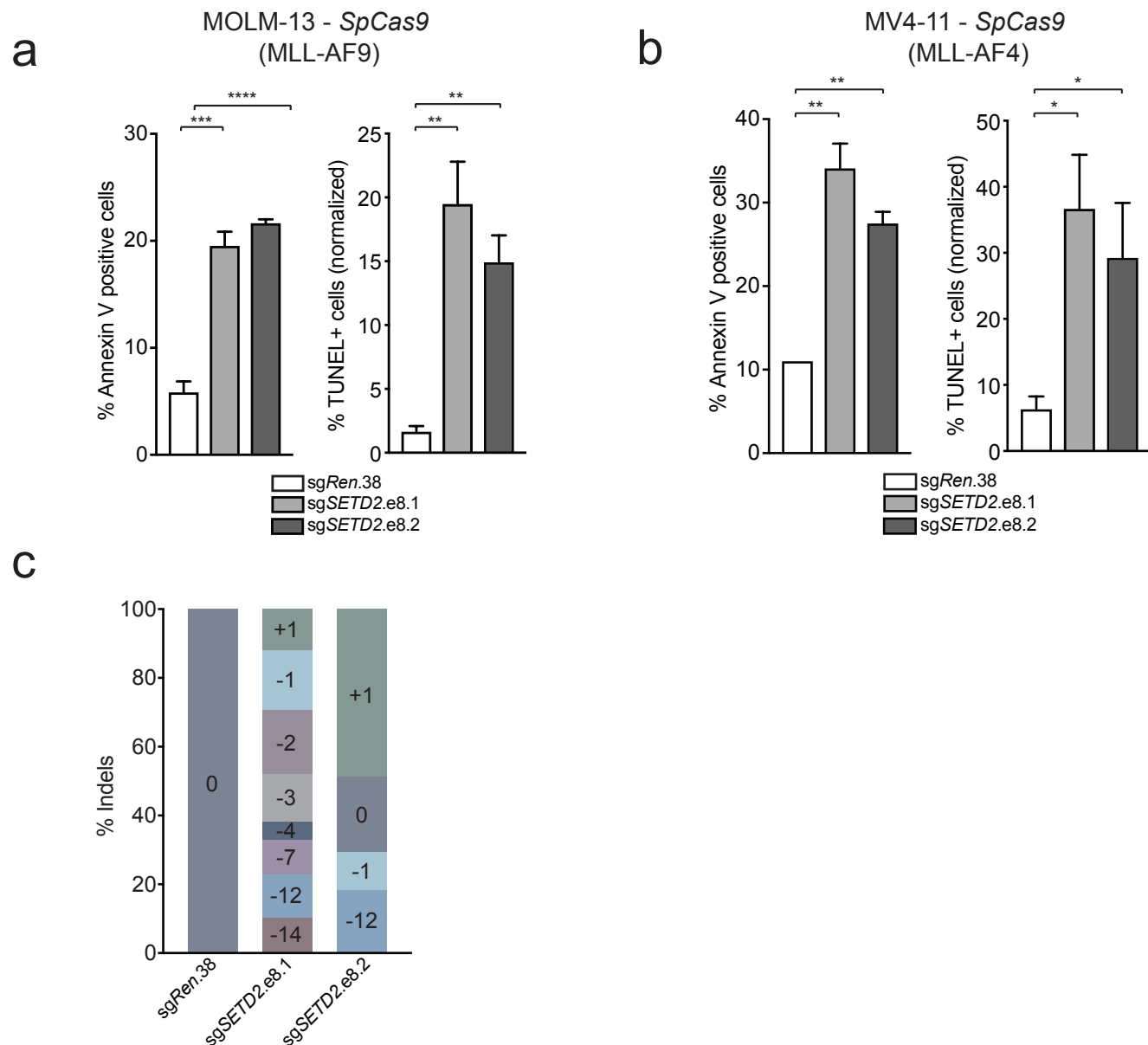
(a) Quantification of tail moments in an alkaline comet assay performed after shRNA-mediated knockdown of *Setd2* in *MLL-AF9/NrasG12D* AML cells. Quantification of >100 cells is shown (mean±s.d.). Cells treated with 150 μM H₂O₂ were used as a positive control. **(b)** Representative micrographs of *MLL-AF9/NrasG12D* AML cells showing DNA breaks upon shRNA-mediated knockdown of *Setd2*. **(c)** Western blot analysis of γ-H2AX- (top) and p21 levels (bottom) in *MLL-AF9/NrasG12D* cells expressing indicated shRNAs after 3 days and 5 days of Dox treatment, respectively. Cells treated with etoposide were used a positive control. **(d)** Cell cycle analysis of *MLL-AF9/NrasG12D* AML cells expressing indicated shRNAs after Dox treatment (8 days). Histograms (left) and quantification (right) are shown. **(e)** Quantification of flow cytometric analysis of apoptosis as measured by Annexin V staining of *MLL-AF9/NrasG12D* cells expressing indicated shRNAs. Cells were treated with Dox for 9 days and processed according to the manufacturer's protocol. ** p<0.01, *** p<0.001, **** p<0.0001 (t-test) (mean±s.d. n=3).



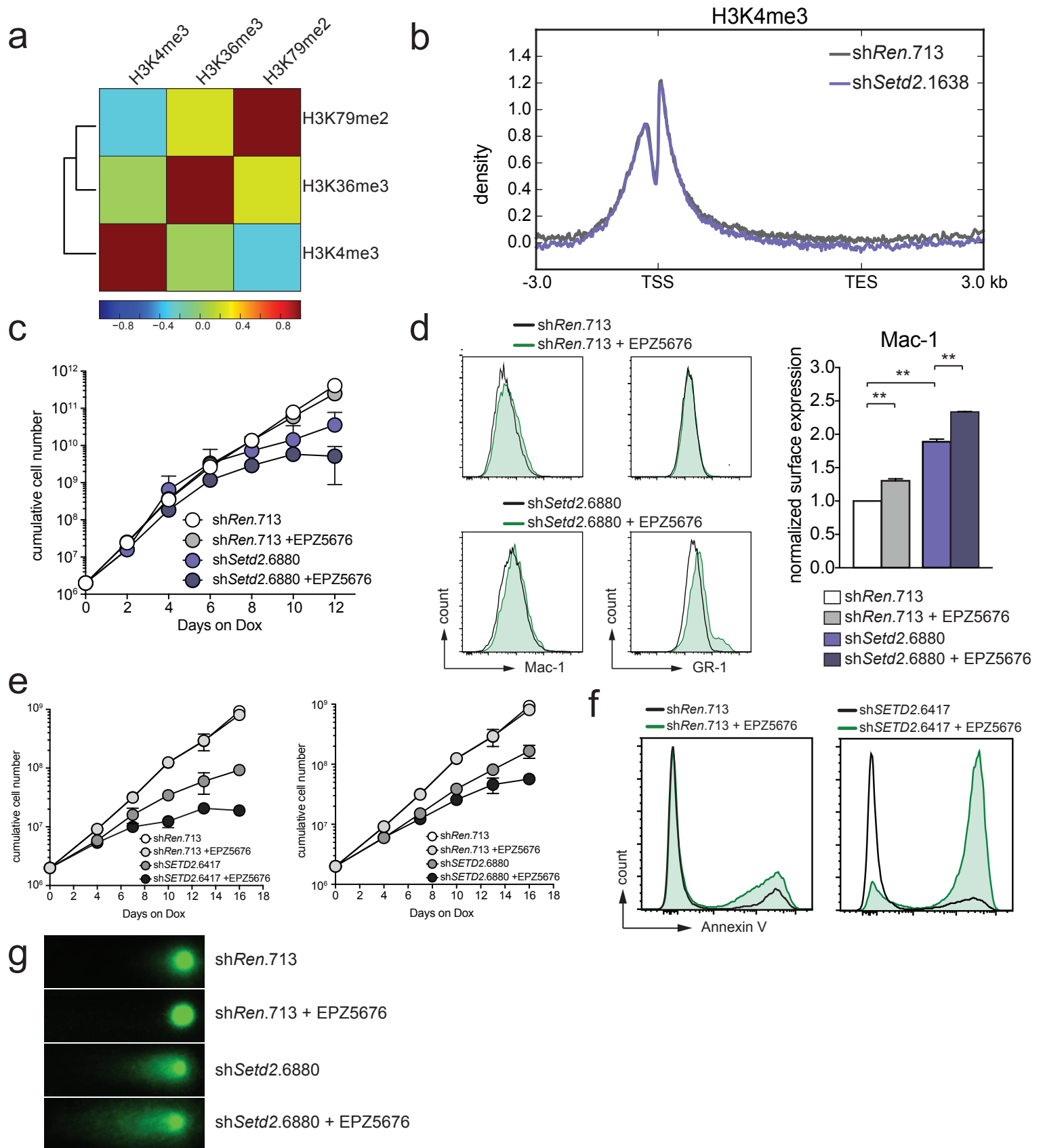
Supplementary Figure 8. shRNA-mediated SETD2 downregulation leads to myeloid differentiation of leukemia cells *in vitro* and *in vivo*. (a) Flow cytometric analysis of c-Kit and Mac-1 in *MLL-AF9/NrasG12D* AML cells upon *Setd2* knockdown. Data from one out of three representative experiments are shown. (b) Flow cytometric analysis of CD36 in MV4-11 cells upon *SETD2* knockdown (top). Data from one out of three representative experiments are shown. Micrographs of cytopsin preparations of MV4-11 cells after expression of indicated shRNAs (bottom). (c) Flow cytometric analysis of CD36 on MOLM-13 cells upon *SETD2* knockdown (top). Data from one out of three representative experiments are shown. Micrographs of cytopsin preparations of MV4-11 cells after expression of indicated shRNAs (bottom). (d) Flow cytometric analysis of c-Kit on *MLL-AF9/NrasG12D* AML cells upon *Setd2* knockdown *in vivo* (left). Quantification of flow cytometric analysis of c-Kit on *MLL-AF9/NrasG12D* AML cells upon *Setd2* knockdown with indicated shRNAs *in vivo* (right) (mean±s.d. n≥3) (e) Quantification of flow cytometric analysis of CD45.2/GFP on *MLL-AF9/NrasG12D* AML cells upon *Setd2* knockdown with indicated shRNAs *in vivo*. ns, not significant, ** p<0.01, *** p<0.001, **** p<0.0001 (t-test) (mean±s.d. n≥3).



Supplementary Figure 9. CRISPR/Cas9 -mediated mutagenesis of the SET domain of SETD2 impairs proliferation and induces differentiation of human MLL-AML cells. (a) Flow cytometric analysis of Mac-1 in *MLL-AF9/NrasG12D-SpCas9* AML cells upon CRISPR/Cas9-mediated mutagenesis of *Setd2* with indicated sgRNAs. Data from one out of three representative experiments is shown. (b) Growth curve of MOLM-13-*SpCas9* cells after CRISPR/Cas9-mediated mutagenesis of *SETD2* with indicated sgRNAs (mean±s.d. n=3). (c) Flow cytometric analysis of surface expression of CD36 on MOLM-13-*SpCas9* cells upon CRISPR/Cas9-mediated mutagenesis of *SETD2* (d) Growth curve of MV4-11-*SpCas9* cells upon CRISPR/Cas9-mediated mutagenesis of *SETD2* with indicated sgRNAs (mean±s.d. n=3). (e) Flow cytometric analysis of surface expression of CD36 in MV4-11-*SpCas9* cells upon CRISPR/Cas9-mediated mutagenesis of *SETD2*. One representative experiment of two is shown. (f) Results of FACS-based competitive proliferation assay shown as the percentage of GFP-positive cells expressing individual *SETD2*-targeting sgRNAs in the presence of Dox over 20-21 days (mean±s.d. n=2).



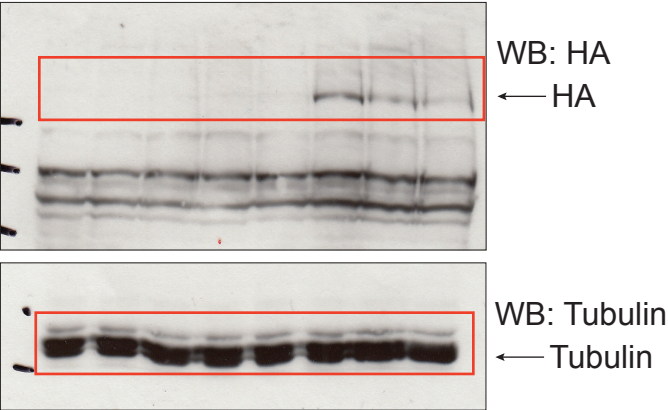
Supplementary Figure 10. Mutagenesis of the SETD2 SET domain leads to apoptosis of MLL-fusion expressing cells. (a) Flow cytometric apoptosis detection as measured by Annexin V- (left) and TUNEL staining (right) in MOLM-13-*SpCas9* cells upon CRISPR/Cas9-mediated mutagenesis of *SETD2* with indicated sgRNAs (mean±s.d. n=3). **(b)** Flow cytometric apoptosis detection as measured by Annexin V- (left) and TUNEL staining (right) in MV4-11-*SpCas9* cells upon CRISPR/Cas9-mediated mutagenesis of *SETD2* with indicated sgRNAs (mean±s.d. n=3). **(c)** Analysis of indel formation by *SETD2*-targeting sgRNAs in MV4-11-*SpCas9* cells using Sanger sequencing and TIDE analysis. * $p>0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ (t-test).



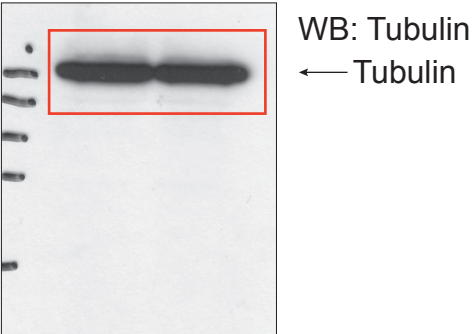
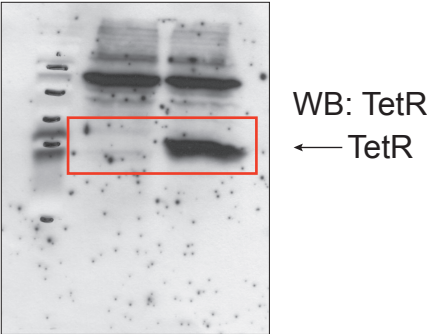
Supplementary Figure 11. SETD2 loss disrupts the H3K36me3-H3K79me2 signature on MLL-target genes and sensitizes AML cells to DOT1L inhibition. (a) Heatmap representation of the Pearson correlation coefficients between changes in the respective histone marks over gene bodies from ChIP-Rx experiments after shRNA-mediated knockdown of *Setd2* in *MLL-AF9/NrasG12D* cells. (b) Metagene plots of ChIP-Rx data for H3K4me3 after *Setd2* knockdown. (c) Growth curves of *MLL-AF9/NrasG12D* AML cells treated with EPZ5676 (500 nM) upon shRNA-mediated mutagenesis of *Setd2* with indicated shRNAs (mean $\pm$ s.d. n=3). (d) Flow cytometric analysis of Mac-1 and Gr-1 expression in *MLL-AF9/NrasG12D* cells treated with EPZ5676 (500nM) upon shRNA-mediated knockdown of *Setd2* (left). Quantification of surface expression of Mac-1 on *MLL-AF9/NrasG12D* AML cells treated with EPZ5676 (500nM) after shRNA-mediated knockdown of *Setd2* (mean $\pm$ s.d. n=2) (right). (e) Growth curves of MV4-11 cells upon shRNA-mediated knockdown of *SETD2* with indicated shRNAs treated with EPZ5676 (50nM) (mean $\pm$ s.d. n=3). (f) Flow cytometric analysis of apoptosis induction as measured by Annexin V-staining in MV4-11 cells treated with EPZ5676 (50nM) after shRNA-mediated knockdown of *SETD2*. (g) Representative micrographs of *MLL-AF9/NrasG12D* AML cells showing DNA breaks after treated with EPZ5676 (500nM) upon shRNA-mediated knockdown of *Setd2*. ** p < 0.01 (t-test).

Supplementary Figure 12. Full, uncropped scans of Western blots

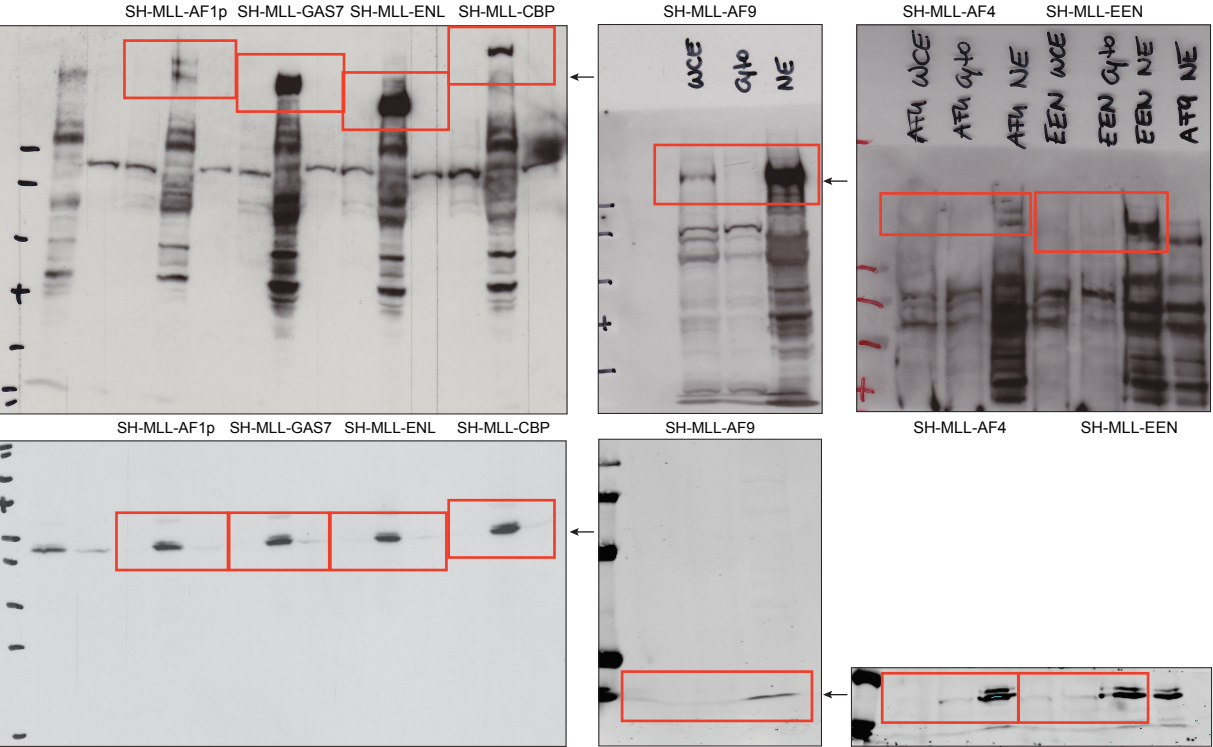
Figure 1a



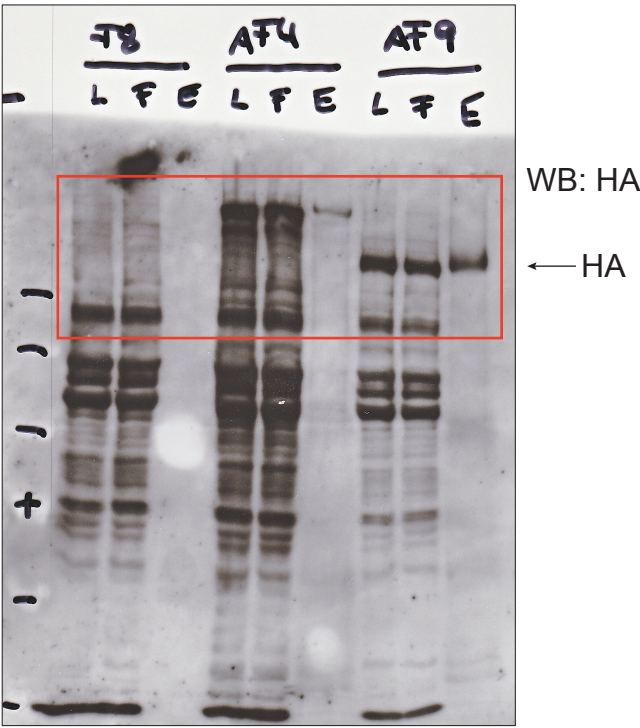
Supplementary Figure 1a



Supplementary Figure 1d



Supplementary Figure 2



Supplementary Figure 3c

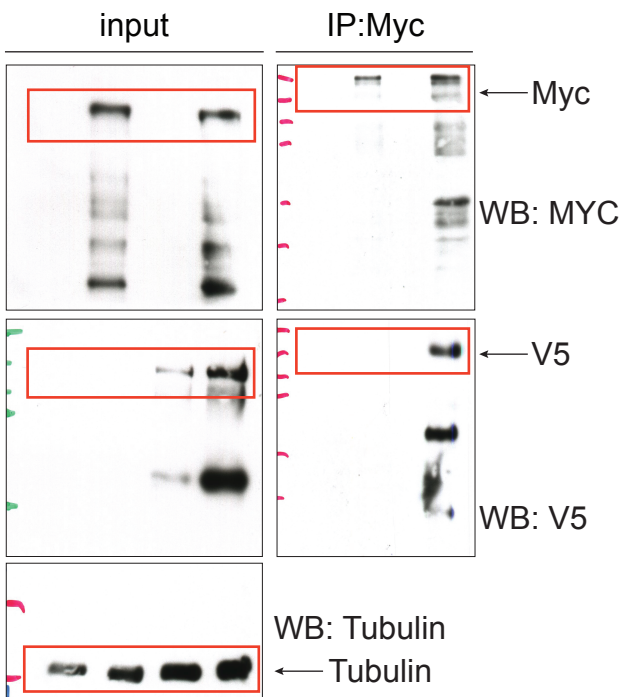


Figure 3c

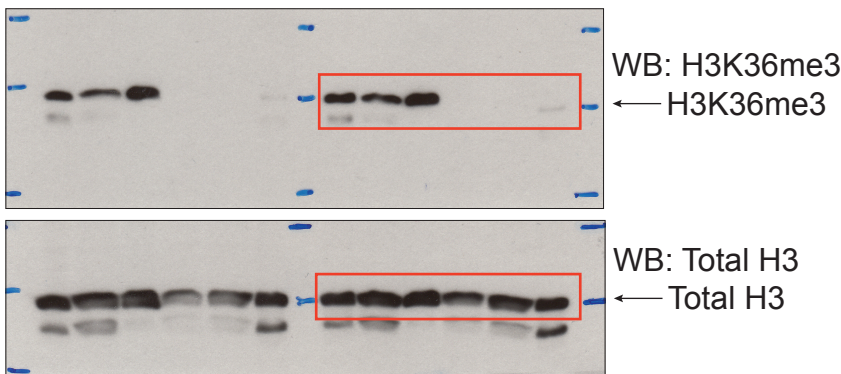
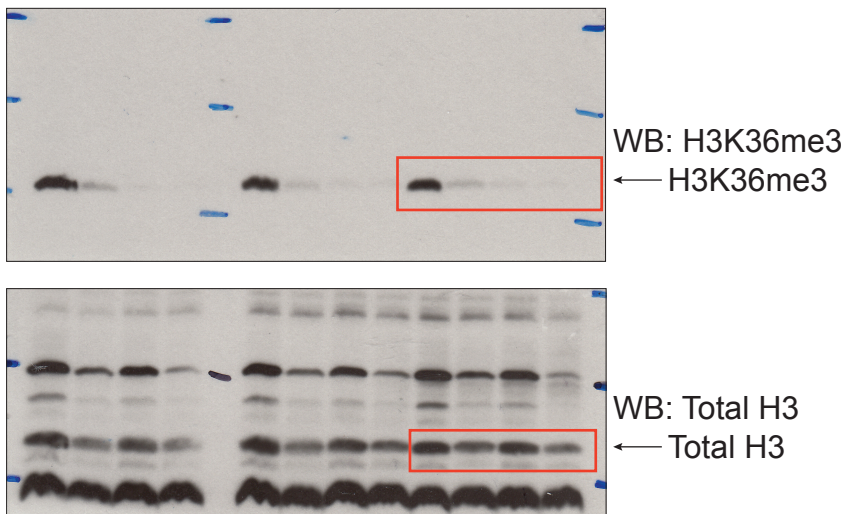
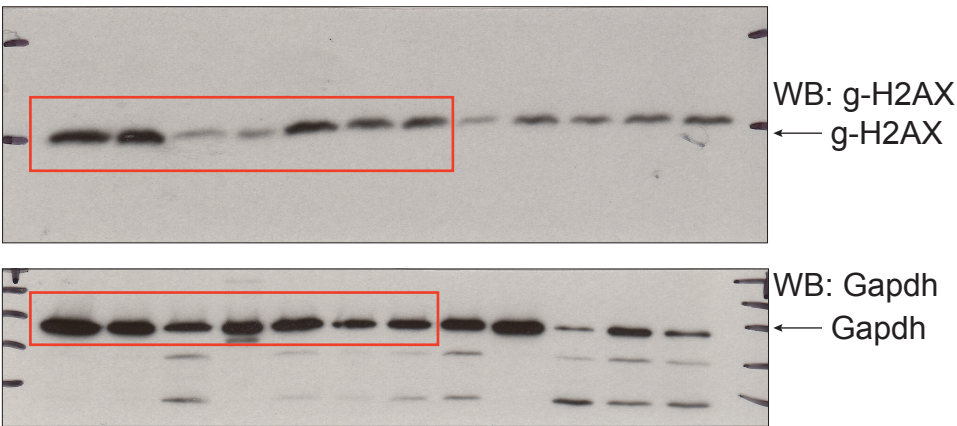
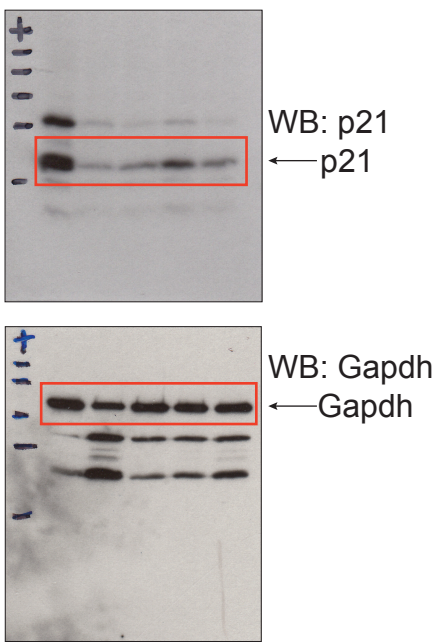


Figure 5c



Supplementary Figure 7c



Supplementary Table 1. Functional annotation of conserved MLL-fusion interactors

Community:1			
GO.id	Term	Pvalue	Adj.Pvalue
GO:0051084	'de novo' posttranslational protein folding	2.40E-09	1.80E-07
GO:0043044	ATP-dependent chromatin remodeling	1.00E-08	3.60E-07
GO:0045944	positive regulation of transcription from RNA pol II promoter	1.60E-05	0.00023
GO:0060216	definitive hemopoiesis	4.00E-04	0.00392
GO:0035162	embryonic hemopoiesis	0.00043	0.00392
GO:0044648	histone H3-K4 dimethylation	0.00124	0.00766
Community:2			
purple			
GO.id	Term	Pvalue	Adj.Pvalue
GO:0016925	protein sumoylation	6.80E-09	5.20E-07
GO:0019054	modulation by virus of host process	3.30E-08	1.30E-06
GO:0007077	mitotic nuclear envelope disassembly	3.10E-06	7.00E-05
GO:0043687	post-translational protein modification	4.20E-06	7.00E-05
GO:0010827	regulation of glucose transport	3.80E-05	0.00041
GO:0031047	gene silencing by RNA	7.20E-05	0.00061
Community:3			
green			
GO.id	Term	Pvalue	Adj.Pvalue
GO:0006977	DNA damage response, signal transduction by p53	3.70E-07	3.50E-05
GO:0000278	mitotic cell cycle	8.40E-06	0.00028
GO:0007411	axon guidance	8.90E-06	0.00028
GO:0031145	anaphase-promoting complex-dependent process	1.50E-05	0.00036
GO:0006521	regulation of cellular amino acid metabolic process	2.70E-05	0.00051
GO:0051437	positive regulation of ubiquitin-protein ligase activity involved in regulation of mitotic cell cycle transition	4.00E-05	0.00063
Community:4			
orange			
GO.id	Term	Pvalue	Adj.Pvalue
GO:0000184	nuclear-transcribed mRNA catabolic process	6.00E-09	3.20E-07
GO:0006413	translational initiation	3.00E-05	0.00041
GO:0006364	rRNA processing	3.40E-05	0.00041
GO:0006415	translational termination	6.20E-05	0.00053
GO:0000086	G2/M transition of mitotic cell cycle	7.70E-05	0.00053
GO:0006414	translational elongation	0.00012	0.00072
Community:5			
red			
GO.id	Term	Pvalue	Adj.Pvalue
GO:0000398	mRNA splicing, via spliceosome	7.70E-13	1.30E-11
GO:0008380	RNA splicing	1.70E-06	1.40E-05
GO:0006397	mRNA processing	4.20E-06	2.40E-05
GO:0006369	termination of RNA polymerase II transcription	1.30E-05	5.50E-05
GO:0006378	mRNA polyadenylation	0.00069	0.00235
GO:0000349	generation of catalytic spliceosome for first transesterification step	0.00101	0.00286
Community:6			
ligh blue			
GO.id	Term	Pvalue	Adj.Pvalue

GO:0043983	histone H4-K12 acetylation	3.90E-09	5.90E-08
GO:0043981	histone H4-K5 acetylation	3.20E-08	1.60E-07
GO:0043982	histone H4-K8 acetylation	3.20E-08	1.60E-07
GO:0043984	histone H4-K16 acetylation	1.80E-07	6.70E-07
GO:0043966	histone H3 acetylation	2.70E-06	8.10E-06
GO:0006270	DNA replication initiation	0.00011	0.00028

Supplementary Table 2. sgRNA sequences targeting SETD2 and controls used in this study

target	sequence
Ren.38	GGATGATAACTGGTCCGCAGTGG
RPA3 e1.1	CCGGCGTTGATGCGCGACCTGGG
SETD2.e6.1	AGAGTTTAAAGCTCGAGTGAAGG
SETD2.e8.1	GGACTGTGAACGGACAAGTGAAGG
SETD2.e8.2	GACTGTGAACGGACAAGTGAAGG
SETD2.e8.3	GTGAACGGACAAGTGAAGGTTGG

target	sequence
Rosa26	GAAGATGGGCGGGAGTCTTC
Myb.33	GCTGAAGAAGCTGGTGGAAACAGAACGGAAC
Setd2_e1.1	AAATCCCCCATCTTCGGTGG
Setd2_e3.1	TCAAGTCGATTTTTGCCCAA
Setd2_e5.1	CTGGGGCTTAAGGGCTGCTA
Setd2_e6.1	GTACCTCTCCACAGTATTCC
Setd2_e8.1	GCCTGAAGGAAGTATTGG

Supplementary Table 3. shRNA sequences targeting SETD2 and controls used in this study

target	sequence
Ren.713	TGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGCCACAGATGTATAG ATAAGCATTATAATTCTATGCCTACTGCCTCGGA
MEN1.1105	TGCTGTTGACAGTGAGCGATAACAAGGAGTTCTTTGAAGTATAGTGAAGCCACAGATGTATAC TTCAAAGAACTCCTTGAGTGCCTACTGCCTCGGA
MLL.3710	TGCTGTTGACAGTGAGCGCAGAGAGCAGTGTGTGAAGAATAGTGAAGCCACAGATGTATT CTTCACAACACTGCTCTCTTTGCCTACTGCCTCGGA
MYB.721	CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGACTGGACGAACTGATAATGCTATAGTGAA GCCACAGATGTATAGCATTATCAGTTCGTCCAGGTGCCTACTGCCTCGGAATTC
SETD2.124	TGCTGTTGACAGTGAGCGCGAAGAAGAAGAAAATGAGGCATAGTGAAGCCACAGATGTATG CCTCATTTTCTTCTTCTTCATGCCTACTGCCTCGGA
SETD2.4318	TGCTGTTGACAGTGAGCGCCAGGACAGAAAGAAAGTTAGATAGTGAAGCCACAGATGTATC TAACTTTCTTTCTGTCCTGATGCCTACTGCCTCGGA
SETD2.4834	TGCTGTTGACAGTGAGCGAAAGGAGTATGCACGAAACAAATAGTGAAGCCACAGATGTATTT GTTTCGTGCATACTCCTTCTGCCTACTGCCTCGGA
SETD2.6417	TGCTGTTGACAGTGAGCGACCGGAAGTTGTTTGAGCAAGATAGTGAAGCCACAGATGTATCT TGCTCAAACAACCTCCGGCTGCCTACTGCCTCGGA
SETD2.6880	TGCTGTTGACAGTGAGCGCAACCAACAGTCTGTCAGTGTATAGTGAAGCCACAGATGTATAC ACTGACAGACTGTTGGTTTTGCCTACTGCCTCGGA
SETD2.7461	TGCTGTTGACAGTGAGCGCAAGCAAAGAAGTATTCAGAAATAGTGAAGCCACAGATGTATTT CTGAATACTTCTTTGCTTTTGCCTACTGCCTCGGA
Myb.670	CTCGAGAAGGTATATTGCTGTTGACAGTGAGCGACACAACCATTTGAATCCAGAATAGTGAA GC CACAGATGTATTCTGGATTCAAATGGTTGTGCTGCCTACTGCCTCGGAATTC
Setd2.1638	TGCTGTTGACAGTGAGCGCAACGGACTTCAGAGCATGAAATAGTGAAGCCACAGATGTATTT CATGCTCTGAAGTCCGTTTTGCCTACTGCCTCGGA
Setd2.4051	TGCTGTTGACAGTGAGCGACAGACTGAAGATTATAAGTGATAGTGAAGCCACAGATGTATCA CTTATAATCTTCAGTCTGCTGCCTACTGCCTCGGA
Setd2.6880	TGCTGTTGACAGTGAGCGCAACCAACAGTCTGTCAGTGTATAGTGAAGCCACAGATGTATAC ACTGACAGACTGTTGGTTTTGCCTACTGCCTCGGA

Supplementary Table 4. Primers used in this study

qPCR primers used in this study		
mSetd2_1	Forward	GATCAAACAGCTCCAAAGACTC
	Reverse	GGTGTATCATATCTGTCATCTGG
mSetd2_2	Forward	CTTATTCACAGACAACTCCACC
	Reverse	CATTTCAGATGGCTGCAAGG
mHoxA9	Forward	GCAGTGTATCATCACCACCA
	Reverse	TTTCTCTATCAACTGCCGTCC
hSETD2	Forward	AAACACAACAACCTGAACGAGG
	Reverse	GAGTTTGCTTGTCTGGGTCTC

ChIP qPCR primers used in this study		
mHoxA9	Forward	GCACTGGACTTGAGCTGTAGTTTG
	Reverse	CCCCTGCCTTGGTTATCCTT
mMeis1	Forward	CAGGCGGAGAGGTGGATTC
	Reverse	CCTGCCGAGGAGCCTACTTG

Primers used for genotyping of cells with CRISPR/Cas9-induced mutations in SETD2	
forward	GCACACACACACACACAC
reverse	CTCCTCAACTTCATATGGTCTACT