Supporting Information

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SI Materials and Methods

Cell Culture. All mouse and human leukemia cell lines were cultured in RPMI 1640 supplemented with 10% (vol/vol) FBS (HyClone) and 1% (vol/vol) penicillin/streptomycin. Immortalized mouse embryonic fibroblasts were grown in DMEM with 10% (vol/vol) FBS and 2 mM L-glutamine (Gibco). 32D cells were cultured in RPMI supplemented with 10% (vol/vol) WEHI-conditioned media as a source of IL-3. G1E cells were cultured in Iscove's modified Dulbecco's media (IMDM) supplemented with Kit ligand (KitL) and erythropoietin. EML cells were cultured in IMDM supplemented with KitL. Mouse lung cancer line was maintained in RPMI 1640 supplemented with 10% (vol/vol) FBS, 1% (vol/vol) penicillin/streptomycin, and 1% (vol/vol) glutamine. OPM-1 cells were grown in RPMI 1640 with 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin. Ecotropic Plat-E Retroviral packaging cells were grown in DMEM with 10% (vol/vol) FBS, 2 mM L-glutamine, and selected with puromycin (1 μg/mL) and blasticidin (10 μg/mL). Lung adenocarcinoma cells were derived from a $Kras^{G12D}/p53^{-/-}$ mouse model, provided as a gift from Kwok Wong's Laboratory (Dana-Farber Cancer Institute, Boston, MA). Immortalized fibroblast line used here was described previously (1). 4T1 cells were a gift from Greg Hannon's Laboratory (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Human acute myeloid leukemia (AML) cell lines were all obtained through ATCC or DSMZ.

shRNA Sequences (97-mers). shRNA sequences (97-mers) were as follows:

Rnf20.1295: TGCTGTTGACAGTGAGCGACCGGAAGTTTGAGGAAATGAATAGTGAAGCCACAGATGTATTCATTTCCTCAAACTTCCGGGTGCCTACTGCCTCGGA;

Rnf20.3718: TGCTGTTGACAGTGAGCGACTGGAAGGTTTGAAGCCTTAATAGTGAAGCCACAGATGTATTAAGGCTTCAAACCTTCCAGGTGCCTACTGCCTCGGA;

Rnf20.3277: TGCTGTTGACAGTGAGCGCAGGCAAGCACTTACTCATTAATAGTGAAGCCACAGATGTATTAATGAGTAAGTGCTTGCCTATGCCTACTGCCTCGGA;

Rnf20.1944: TGCTGTTGACAGTGAGCGAAAGCAGGATTCTGAAGACCTATAGTGAAGCCACAGATGTATAGGTCTTCAGAATCCTGCTTCTGCCTACTGCCTCGGA;

Rnf20.2853: TGCTGTTGACAGTGAGCGACAGCTTGCAGATGACCTCAAATAGTGAAGCCACAGATGTATTTGAGGTCATCTGCAAGCTGGTGCCTACTGCCTCGGA;

Rnf20.2949: TGCTGTTGACAGTGAGCGCAAGGACTTGTTCAATTTCAAATAGTGAAGCCACAGATGTATTTGAAATTGAACAAGTCCTTTTGCCTACTGCCTCGGA;

Rnf20.3595: TGCTGTTGACAGTGAGCGATAGAGTGAACATATACTATTATAGTGAAGCCACAGATGTATAATAGTATATGTTCACTCTACTGCCTACTGCCTCGGA;

Rpa3.457: TGCTGTTGACAGTGAGCGCGCGACTCCTATAATTTCTAATTAGTGAAGCCACAGATGTAATTAGAAATTATAGGAGTCGCTTGCCTACTGCCTCGGA;

Rluc.713: TGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTATAGTGAAGCCACAGATGTATAGATAAGCATTATAATTCCTATGCCTACTGCCTCGGA;

RNF20.2710: TGCTGTTGACAGTGAGCGCAAAGGACATGTTCAATTTCAATAGTGAAGCCACAGATGTATTGAAATTGAACATGTCCTTTTTGCCTACTGCCTCGGA;

RNF20.2706: TGCTGTTGACAGTGAGCGAGTGGAGAACAGTGTTACCAAATAGTGAAGCCACAGATGTATTTGGTAACACTGTTCTCCACGTGCCTACTGCCTCGGA;

RNF20.1863: TGCTGTTGACAGTGAGCGAAACGAGAGAAGCAGAAGCTAATAGTGAAGCCACAGATGTATTAGCTTCTGCTTCTCTCGTTCTGCCTACTGCCTCGGA.

RT-qPCR Primers.

PCR amplicon	Forward	Reverse
Hoxa9	CCGAAAACAATGCCGAGAA	CCGGGTTATTGGGATCGAT
Hoxa10	ACAGGCCACTTCGTGTTCTTTT	TTGTCCGCAGCATCGTAGAG
Meis1	CCCTGGAATGCCAATGTCA	GAGCGTGAATGTCCATGACTTG
Mef2c	ACCCGGCAAGTGGTGACT	GTGAGCGTGCTTGGATGTTAG
Gapdh	TTCACCACCATGGAGAAGGC	CCCTTTTGGCTCCACCCT

ChIP-qPCR Primers.

PCR amplicon	Forward	Reverse
Negative control	GAGGAGAATGACGCCCTTTCATAC	GTGTCACGAGTTTGTGCATATGTG
Hoxa9 promoter	GCACTGGACTTGAGCTGTAGTTTG	CCCCTGCCTTGGTTATCCTT
Hoxa9 + 2 kb	TCGCTGGGTTGTTTTTCTCT	CCACGCTTGACACTCACACT
Sfrs10 + 2 kb	TTGTACACATGGCCCAAAAA	CTCCTGCCTCTGGTTTCTGA
Meis1 + 5 kb	GGACTCTTGGCTCAAGTTCG	TCTAGCTGACCGGAGAGAGC

1. Zuber J, et al. (2011) Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. *Nat Biotechnol* 29:79–83.

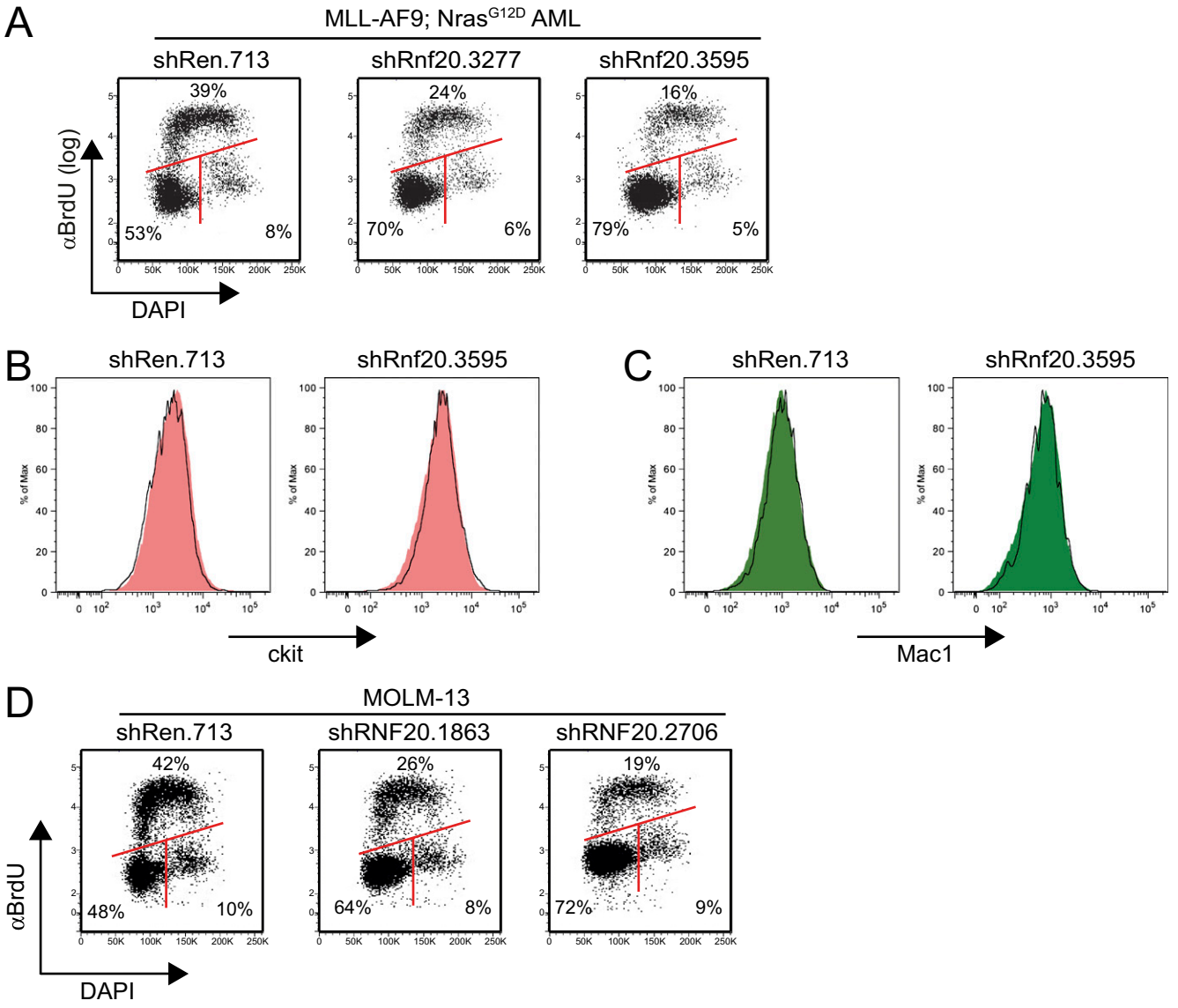

Fig. S1. Rnf20 knockdown leads to G_1/G_0 cell cycle arrest without evidence of myeloid maturation. (*A*) BrdU cell cycle flow cytometry analysis in murine leukemia cells after 4 d of doxycycline (dox) treatment. RN2 cells were transduced with indicated shRNAs in the TRMPV-Neo vector, followed by G418 selection. BrdU (BD; APC BrdU Flow kit; no. 552598) was pulsed for 30 min in the growth medium. Permeabilized cells were counterstained with DAPI to visualized DNA content. Gating was performed on dsRed+/shRNA+ cells. (*B*) Flow cytometry analysis of cell surface levels of c-kit (APC-conjugated antibody; Biolegend; no. 105811) following 4 d of dox treatment. Gating was performed on dsRed+/shRNA+ cells (histogram shaded in red) and on dsRed−/shRNA− cells as an internal negative control (histogram indicated with a black line). (*C*) Flow cytometry analysis of cell surface levels of and Mac1 (APC-conjugated antibody; Biolegend; no. 101211). Gating was performed on dsRed+/shRNA+ cells (histogram shaded in green) and on dsRed−/shRNA− cells as an internal negative control (histogram indicated with a black line). (*D*) BrdU analysis performed in the human cell line MOLM-13. This line was stably transduced with rtTA3 followed by human RNF20 TRMPV-Neo shRNAs. shRNAs were induced with dox (1 μg/mL) for 4 d. BrdU staining was performed as in *A*. All data analysis was performed with FlowJo software. Experiments throughout were performed with three independent biological replicates. A representative experiment is shown.

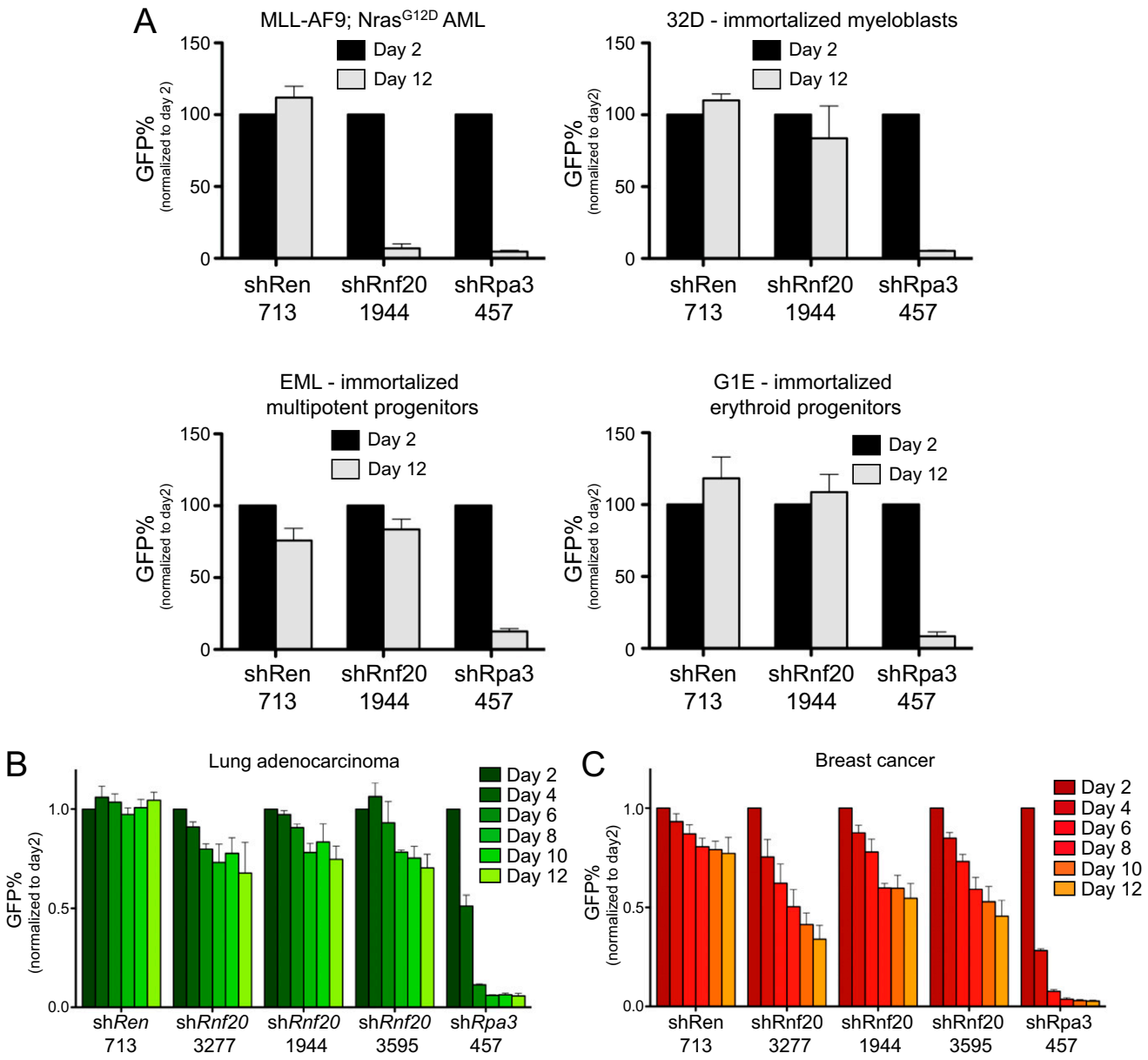

Fig. S2. Effect of Rnf20 shRNAs on proliferation of immortalized hematopoietic cell lines, lung cancer, and breast cancer cell lines. (*A*) Indicated cell lines were retrovirally transduced with constructs expressing the indicated shRNAs. The LMN vector was used (LTR-mir30 shRNA-Pgk promoter-NeoR-IRES-GFP). The GFP+ percentage was tracked using a Guava Easycyte (Millipore) at the indicated time points following retroviral infection. All percentages were normalized to those found at day 2. A *Renilla* luciferase-targeting shRNA (Ren.713) was used a negative control, and an Rpa3-targeting shRNA was included as a positive control. (*B* and *C*) Lung adenocarcinoma cells (derived from the KrasG12D/p53$^{-/-}$ model) and breast cancer cells (4T1) were retrovirally transduced with constructs expressing the indicated shRNAs.

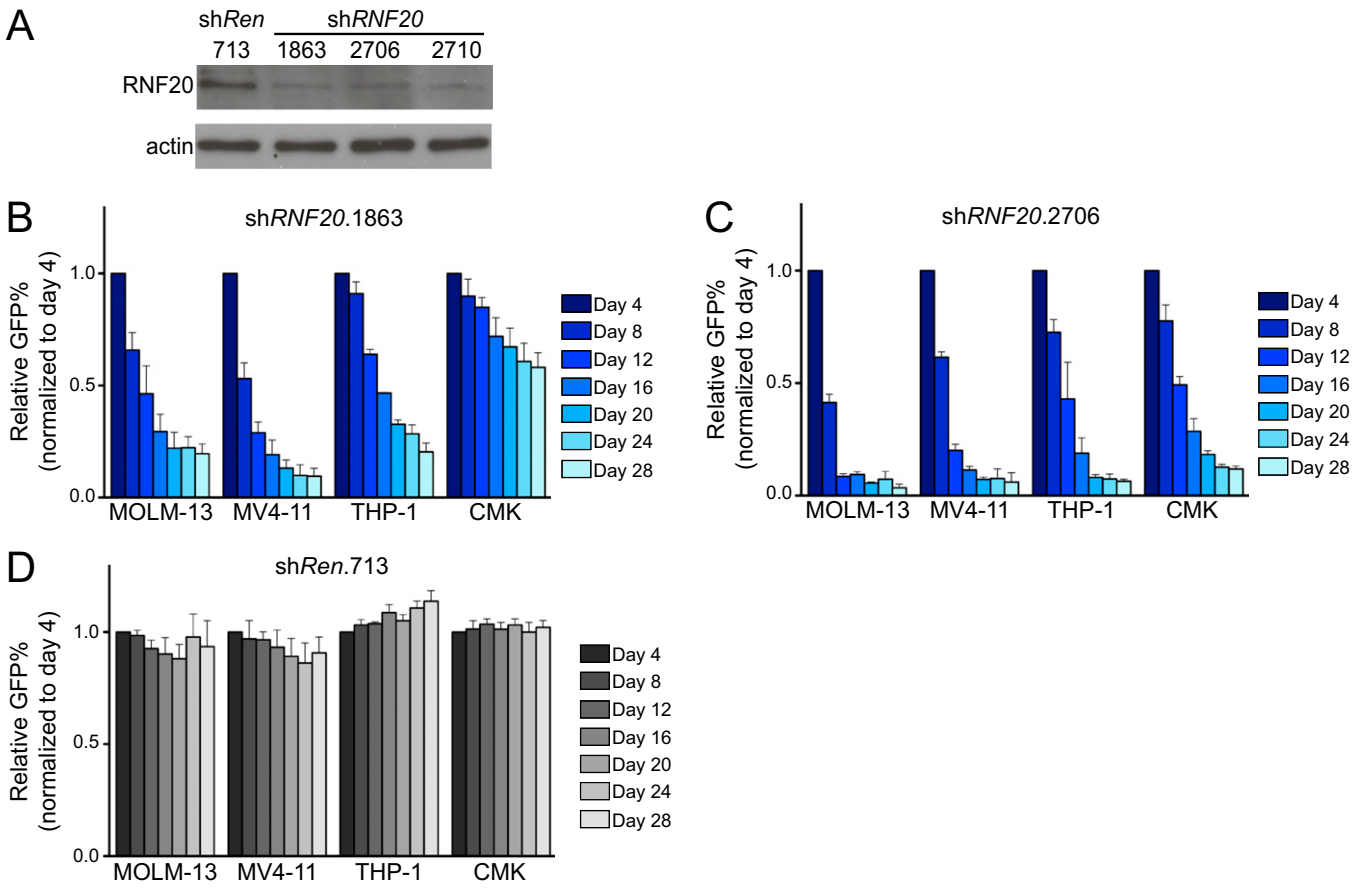

Fig. S3. Effect of RNF20 shRNAs on growth of the indicated human AML cell lines. (*A*) Western blotting of RNF20 and β-actin in whole-cell lysates prepared from OPM1 cells transduced with the indicated MLS-shRNA constructs. Cells were >95% GFP+/shRNA+ following retroviral infection. Representative experiment of three independent repeats is shown. (*B–D*) Impact of indicated shRNAs on proliferation of human AML cell lines. The percentage of shRNA/GFP-positive cells at the indicated time points is shown, normalized to day 4. Mean of three independent experiments is shown. All error bars denote SEM. The MLS vector was used (LTR-mir30shRNA-SV40 promoter-GFP). The GFP+ percentage was tracked using a Guava Easycyte (Millipore) at the indicated time points following retroviral infection. Note: the RNF20.2706 shRNA leads to a stronger phenotype than the other two RNF20 shRNAs and consequently a less dramatic difference between MLL-rearranged cell lines (MOLM-13, MV4-11, THP-1) and a non–MLL-rearranged line (CMK) is observed. This would indicate that potent RNF20 knockdown will have broader effects on AML proliferation extending beyond the MLL-rearranged subtype of disease.

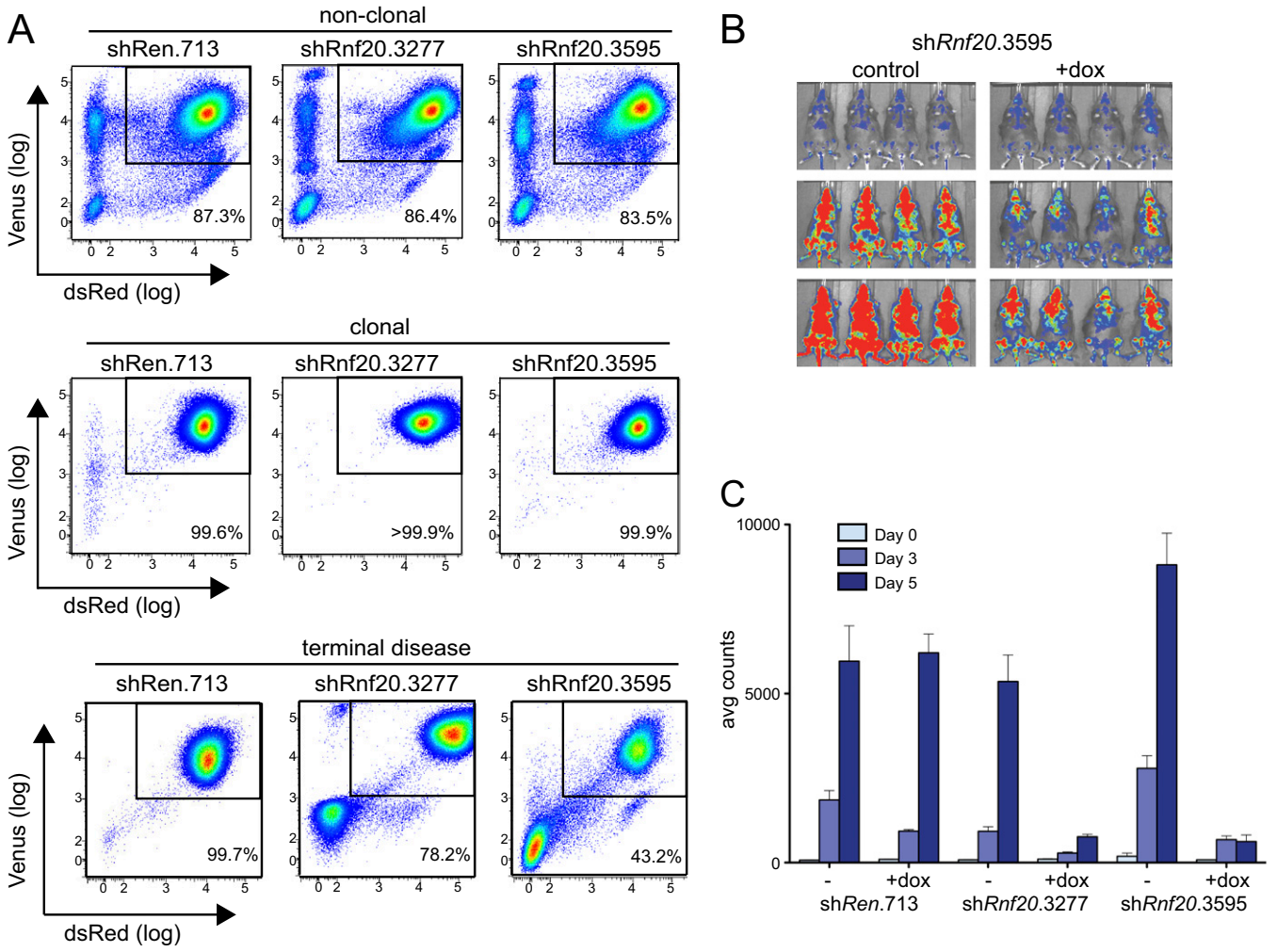

Fig. S4. Additional data for in vivo mouse experiments. (*A*) Flow cytometry analysis of clonal TRMPV-Neo leukemia cells used for in vivo trials. (Top row) RN2 (rtTA3+, luciferase+, MLL-AF9/Nras^{G12D}+) cells were retrovirally transduced with the indicated TRMPV-Neo shRNA vectors, followed by G418 selection. Cells were then treated with dox (1 μg/mL) for 2 d and analyzed by flow cytometry. The Venus reporter is expressed from a constitutive promoter and dsRed is linked to the shRNA, expressed from a dox-inducible TRE promoter. Note: following G418 selection, only ∼85% of cells become dsRed+/shRNA+, likely indicating variability in retroviral integration sites in the nonclonal population. (Middle row) Analysis performed as described in *A*, in clonal lines derived by limiting dilution. Note: the dsRed+/shRNA+ population is now >99% in the indicated clones. These clones were used for the in vivo experiments shown in Fig. 2. (Bottom row) Similar analysis as above, performed in bone marrow obtained from mice transplanted with indicated clones following disease reaching terminal endpoint. Gating was performed donor-derived leukemia cells (using anti-CD45.2 antibody; Biolegend; no. 109820). Note: the Ren.713 control disease remains largely clonal (>99% dsRed+), whereas Rnf20 shRNA clones now have an emergence of dsRed-/shRNA- population, indicating a competitive advantage for subclones that have bypassed shRNA expression. (*B*) Bioluminescent imaging. Dox was administered upon disease onset, 5–6 d after transplant. Day 0 indicates the time of dox treatment. (*C*) Quantification of bioluminescent imaging responses after dox treatment. Mean values of 8–10 replicate mice are shown. (–) indicates animals where no dox was administered.

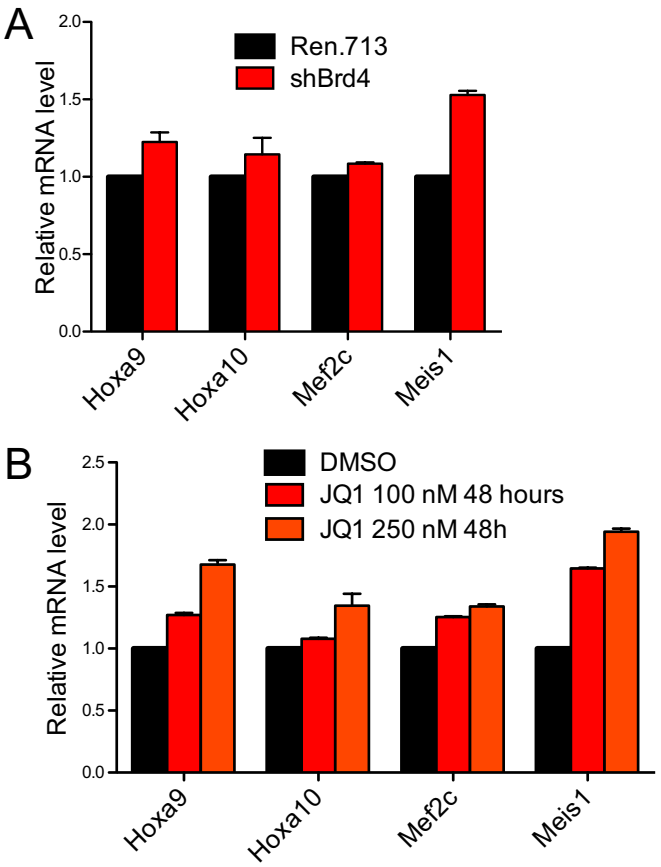

Fig. S5. BRD4 inhibition does not lead to down-regulation of MLL-AF9 target genes. (*A*) RT-qPCR analyzing the mRNA level of the indicated genes following BRD4 knockdown. Results were normalized to levels of *Gapdh*. Results are averaged from three independent Brd4 shRNAs. (*B*) RT-qPCR analysis of JQ1-treated cells as described in *A*.

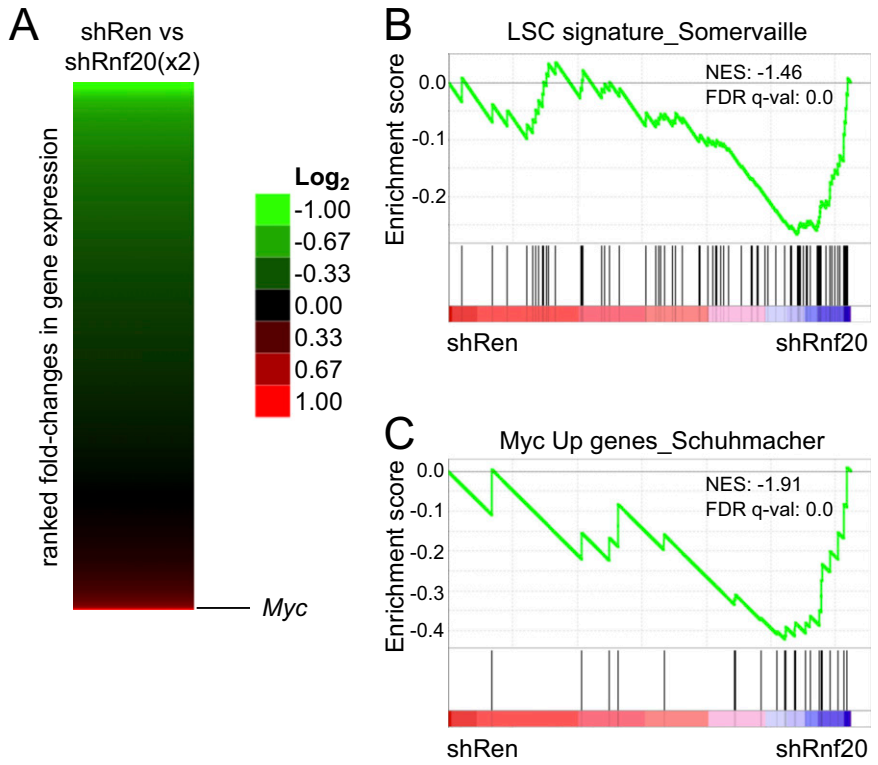

Fig. S6. Rnf20 knockdown leads to up-regulation of Myc expression and associated gene expression signatures. (*A*) Ranked fold changes in gene expression following Rnf20 knockdown. Two independent Rnf20 shRNAs were averaged and compared with a Ren.713 control. *Myc* is one of the few up-regulated genes identified. (*B*) GSEA analysis of a leukemia stem cell gene signature (1), which is known to governed by MYC (2). The negative enrichment score indicates LSC-related genes are up-regulated following Rnf20 knockdown. (*C*) GSEA analysis of a MYC up-regulated gene signature (3). The negative enrichment score indicates LSC-related genes are up-regulated following Rnf20 knockdown.

1. Somervaille TC, et al. (2009) Hierarchical maintenance of MLL myeloid leukemia stem cells employs a transcriptional program shared with embryonic rather than adult stem cells. *Cell Stem Cell* 4(2):129–140.
2. Kim J, et al. (2010) A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. *Cell* 143(2):313–324.
3. Schuhmacher M, et al. (2001) The transcriptional program of a human B cell line in response to Myc. *Nucleic Acids Res* 29(2):397–406.

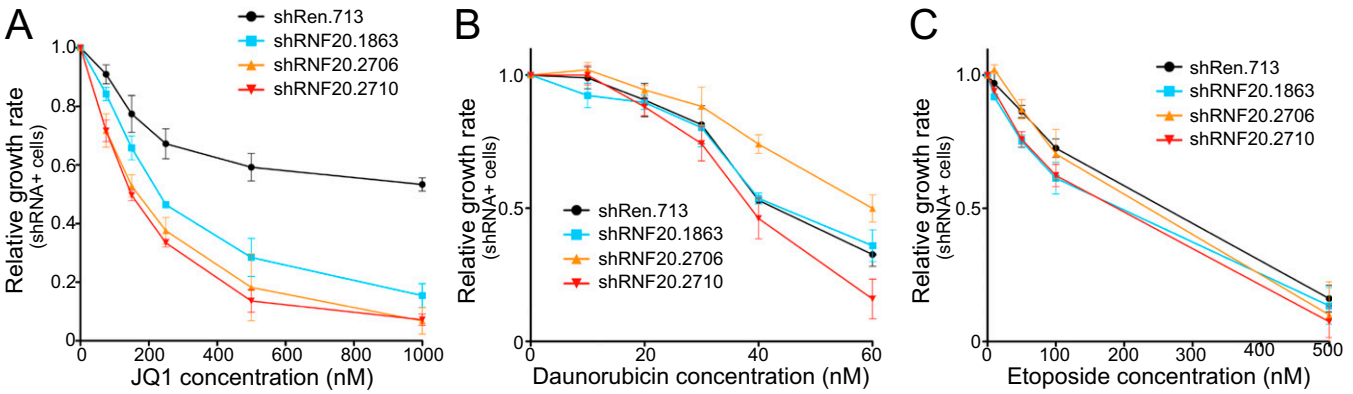

Fig. S7. RNF20 knockdown sensitizes MOLM-13 cells to JQ1 but not to DNA-damaging agents etoposide and daunorubicin. Tet-On competent MOLM-13 cells were transduced with TRMPV-Neo constructs containing the indicated shRNAs, followed by neomycin selection. Cells were treated with 1 μg/mL dox for 48 h to deplete RNF20, followed by addition of the indicated concentrations of JQ1 (*A*), daunorubicin (*B*), or etoposide (*C*) for an additional 48 h (96 h total). The net increase in viable dsRed+ cells from 48 to 96 h was fit to a logarithmic growth curve to derive the relative proliferation rate, which was normalized to the rate of cells grown in the presence vehicle (DMSO), set to 1.0.

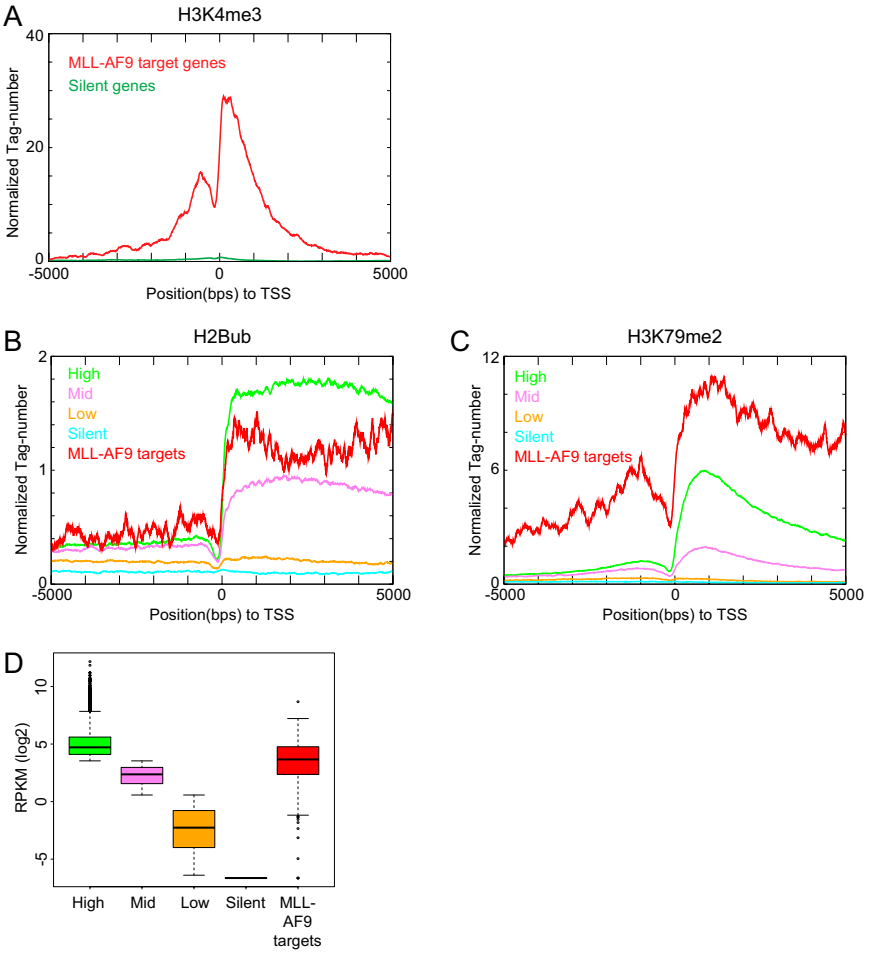

Fig. S8. Additional ChIP-seq data analysis. (*A*) Profile of H3K4me3 at MLL-AF9 target genes. A metaprofile of H3K4me3 levels at 139 MLL-AF9 target genes compared with 6,221 silent genes. The *x*-axis shows the relative distance to each TSS, whereas the *y*-axis shows the normalized read count of the histone modification (*B*–*D*) Metagene profile of ChIP-seq data obtained from the indicated gene sets, categorized based on absolute expression (RPKM) or based on being an MLL-AF9 target. Shown in *D* is a box-plot representation of the absolute expression level of the five analyzed gene categories. Importantly, MLL-AF9 target genes have a median expression level between that of "Mid" and "High" gene categories. The levels of H2Bub at MLL-AF9 targets similarly lies between Mid and High, indicating the levels correlate with expression. In contrast, median H3K79me2 levels are significantly above even the most highly expressed genes in the genome (*C*). This indicates that H3K79me2 is hypermethylated beyond simply the expected level of any expressed gene, consistent with prior observations (1). As outlined in the model presented in Fig. 5*D*, these results are consistent with DOT1L being recruited through an active MLL-AF9–mediated mechanism, whereas RNF20 is recruited through a cotranscriptional process.

1. Bernt KM, et al. (2011) MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. *Cancer Cell* 20(1):66–78.

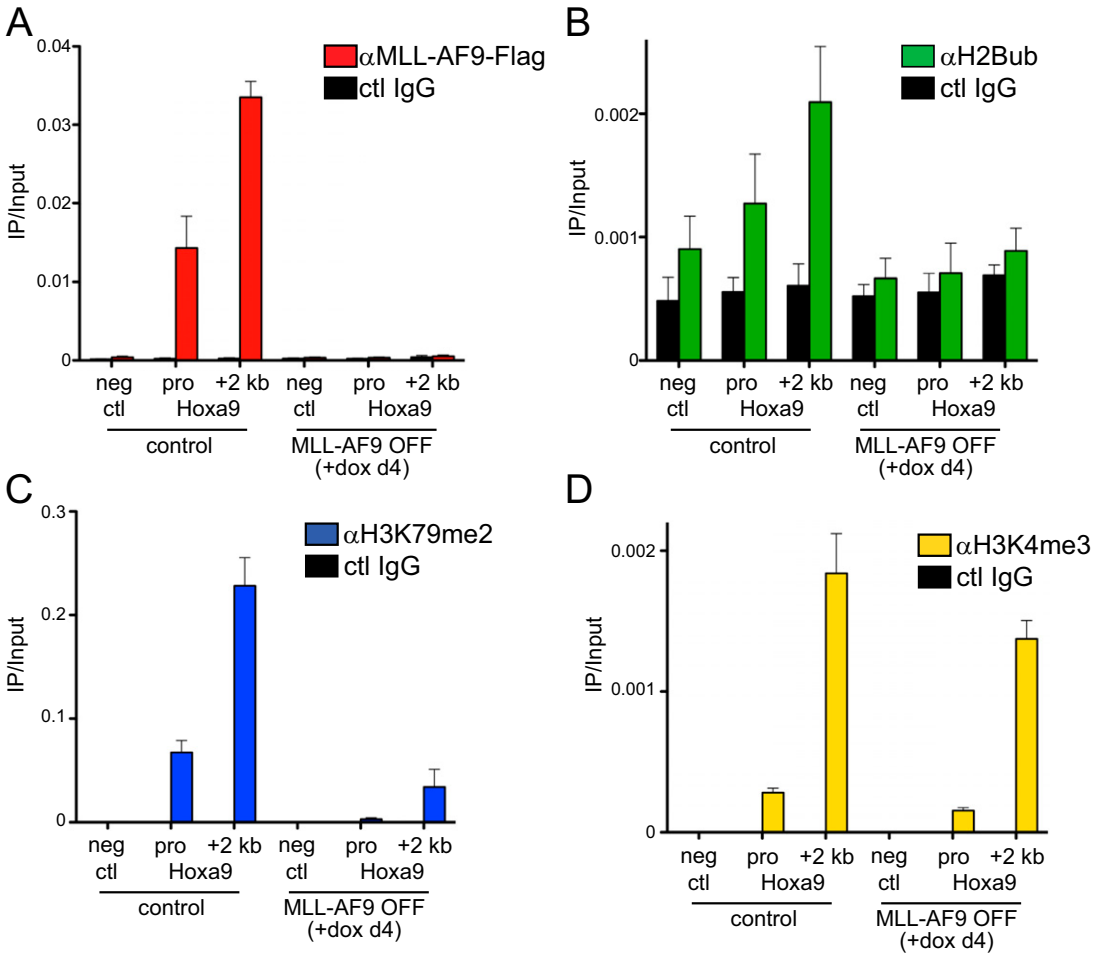

Fig. S9. MLL-AF9 knockdown leads to reduced H2Bub and H3K79 methylation at *Hoxa9*. ChIP-qPCR was performed in Tet-Off MLL-AF9 leukemia cells modified with five flag tags at the C terminus of MLL-AF9. Cells were treated with dox for 4 d to knock down MLL-AF9 expression, verified in *A*. ChIP was performed with the anti-flag (*A*), anti-H2Bub (*B*), anti-H3K79me2 (*C*), anti-H3K4me3 (*D*) antibodies. The negative control region lies downstream of the Hoxa locus in a nontranscribed location.

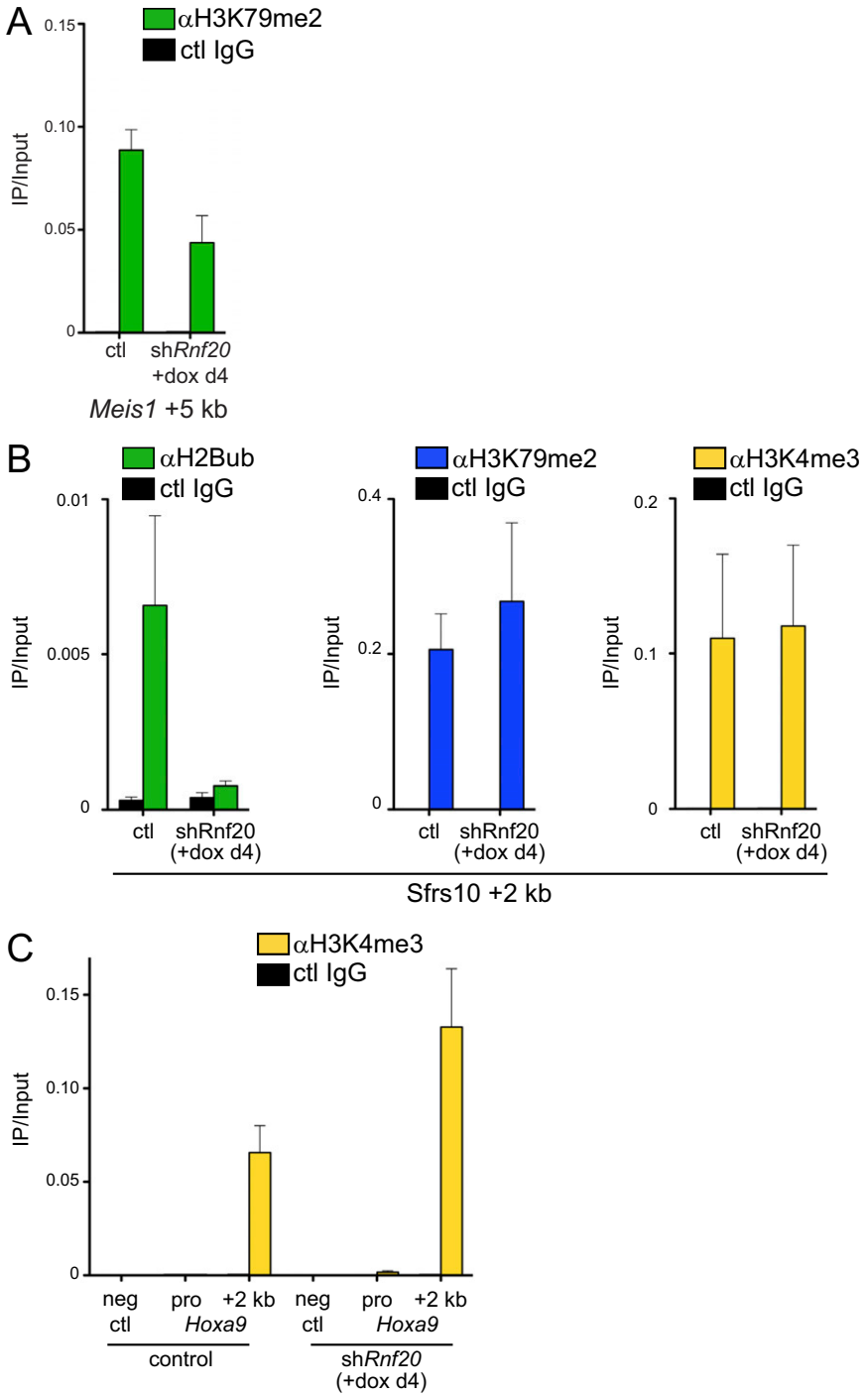

Fig. S10. Additional ChIP-qPCR data. (*A*) Rnf20 is required to maintain H3K79me2 at *Meis1*. ChIP-qPCR evaluation with anti-H3K79me2 antibodies following conditional RNF20 knockdown. "+5 kb" is relative to the *Meis1* TSS. (*B*) Rnf20 is required to maintain H2Bub but is dispensable for H3K79me2 and H3K4me3 at the highly expressed *Sfrs10* gene. ChIP-qPCR performed in the gene body of *Sfrs10* with the indicated antibodies. A clonal RN2 line expressing the Rnf20.3595 shRNA was treated with dox for days and compared with untreated control cells. Control cells were not treated with dox. (*C*) Rnf20 is dispensable for maintaining H3K4me3 at *Hoxa9*. ChIP-qPCR evaluation with anti-H3K4me3 antibodies following conditional RNF20 knockdown. Different PCR amplicons are labeled along the x-axis. Neg ctl refers to negative control primers that amplify a nontranscribed region downstream of the Hoxa locus. Pro, Hoxa9 promoter. "+2 kb" is relative to the *Hoxa9* TSS. Control cells were not treated with dox. Mean of three independent experiments is shown. All error bars denote SEM.