Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains

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CRISPR-Cas9 genome editing technology holds great promise for discovering therapeutic targets in cancer and other diseases. Current screening strategies target CRISPR-Cas9–induced mutations to the 5′ exons of candidate genes1–4, but this approach often produces in-frame variants that retain functionality, which can obscure even strong genetic dependencies. Here we overcome this limitation by targeting CRISPR-Cas9 mutagenesis to exons encoding functional protein domains. This generates a higher proportion of null mutations and substantially increases the potency of negative selection. We also show that the magnitude of negative selection can be used to infer the functional importance of individual protein domains of interest. A screen of 192 chromatin regulatory domains in murine acute myeloid leukemia cells identifies six known drug targets and 19 additional dependencies. A broader application of this approach may allow comprehensive identification of protein domains that sustain cancer cells and are suitable for drug targeting.

The RNA-guided endonuclease Cas9 (CRISPR-associated protein 9), a component of the type II CRISPR (clustered, regularly interspaced, short palindromic repeats) system of bacterial host defense, is a powerful tool for genome editing6. Ectopic expression of Cas9 and a single guide RNA (sgRNA) is sufficient to direct the formation of a DNA double-strand break (DSB) at a specific region of interest7–9. In the absence of a homology-directed repair DNA template, these DSBs are repaired in an error-prone manner by means of the nonhomologous end-joining pathway to generate an assortment of short deletion and insertion mutations (indels) in the vicinity of the sgRNA recognition site7,8. This approach has been widely used to generate gene-specific knockouts in a variety of biological systems6. Recent studies have demonstrated the use of CRISPR-Cas9 mutagenesis for genetic screens in mammalian cell culture, which have relied on sgRNA libraries that target constitutive 5′ coding exons to achieve gene inactivation1–4. The capabilities of CRISPR-based genetic screens are particularly evident in the setting of positive selection, such as identifying mutations that confer drug resistance1–4. In negative selection screens, it has been shown that sgRNA hits are statistically enriched for essential gene classes (ribosomal, RNA processing and DNA replication factors); however, the overall accuracy of CRISPR-Cas9 for annotating genetic dependencies is currently unclear1,2.

We investigated the performance of CRISPR-Cas9 indel mutagenesis as a tool to identify essential genes in cancer cells. We employed a murine MLL-AF9/Netα1 acute myeloid leukemia cell line (RN2), which has been used extensively to identify genetic dependencies and therapeutic targets by RNA interference (RNAi)10,11. We derived a clonal Cas9-expressing RN2 cell line (RN2c), which is diploid and remains genomically stable during passaging (Fig. 1a and data not shown). Lentiviral transduction of RN2c cells with a vector expressing GFP and an sgRNA targeting the Rosa26 locus resulted in a high efficiency of indel mutagenesis near the predicted cut site, reaching >95% editing efficiency by day 7 post-infection (Fig. 1b). Next, we designed three sgRNAs targeting the first exon of Rpa3, which encodes a 17-kD protein required for DNA replication12. Unlike the effects of targeting Rosa26, we found that cells expressing Rpa3 sgRNAs were rapidly outcompeted by nontransduced cells over 8 days in culture, as shown by flow cytometry–based tracking of GFP expression (Fig. 1c). These effects were rescued by the presence of a human RPA3 cDNA that contains several mismatches with mouse Rpa3 sgRNAs, indicating that negative selection induced by CRISPR-Cas9 can be attributed to mutational effects at a single essential gene (Fig. 1c,d).

To further evaluate the performance of CRISPR-Cas9 mutagenesis as a negative-selection screening strategy, we targeted ten additional negative-control genes, chosen on the basis of having undetectable expression in RN2 (ref. 13). We also targeted five essential genes encoding chromatin regulators (Brd4, Smarca4, Eed, Suz12 and Rnf20) that were previously identified using short hairpin RNA–based knockdown10,13–15. For each gene we designed four to five sgRNAs that target constitutive 5′ coding exons, a strategy used in previous CRISPR-Cas9 screens1–4. None of the 49 sgRNAs targeting nonexpressed genes underwent significant negative selection, suggesting a low frequency of false-positive phenotypes conferred by off-target DNA cleavage (Fig. 1e–g). By contrast, a large fraction of the positive-control sgRNAs led to depletion of the percentage of GFP+ cells, with a subset exhibiting robust depletion that exceeded tenfold changes (Fig. 1c,h,i).

In the experiments described above, we observed dramatic variability in the performance of individual sgRNAs targeting the same gene. For example, two of the Brd4 sgRNAs became depleted >20-fold whereas two were depleted only about twofold over 8 days in culture. Notably, Brd4 sgRNAs causing severe negative-selection phenotypes targeted sequences that encode bromodomain 1 (BD1), whereas the
Figure 1 Negative-selection CRISPR-Cas9 experiments in murine MLL-AF9/NrasG12D acute myeloid leukemia cells. (a) Experimental strategy. (top) Vectors used to derive clonal MLL-AF9/NrasG12D leukemia RN2c cells that express a human codon-optimized Cas9 (hCas9) and vectors used for sgRNA transduction. GFP or mCherry reporters were used where indicated to track sgRNA negative selection. LTR, long terminal repeat promoter; PGK, phosphoglycerate kinase 1 promoter; Puro, puromycin resistance gene; αEF1α, αEF1α-driven promoter; EFS, phosphoglycerate kinase 1 promoter; EFSsgRNA, EFS-driven sgRNA; EFS-LTR sgRNA, EFS-LTR-driven sgRNA; PGK terminal repeat promoter; PGK, phosphoglycerate kinase 1 promoter. (bottom) GFP+ mCherry+ cells over time following assay that plots the percentage of offspring of each vector. (b) GFP+ mCherry+ cells over time following assay that plots the percentage of offspring of each vector. (c) Negative-selection competition assay that plots the percentage of GFP+/mCherry+ cells over time following transduction of RN2c with the indicated sgRNAs. Experiments were performed in RN2c cells transduced with either an empty murine stem cell virus (MSCV) vector or MSCV expressing human RPA3, which are linked with a GFP reporter. The mCherry/GFP double-positive percentage is normalized to the 20th day measurement. Each bar represents an independent sgRNA targeting a unique genomic site. Red indicates mismatches. PAM, protospacer-adjacent motif. (d) Analysis of CRISPR-Cas9 editing efficiency at the Rosa26 locus in RN2c cells. Illumina sequencing was used to quantify the abundance of indel mutations occurring in the vicinity of the Rosa26 sgRNA cut site. (e) Negative-selection competition assay that plots the percentage of GFP+/mCherry+ cells over time following transduction of RN2c with the indicated sgRNAs. Experiments were performed in RN2c cells transduced with either an empty murine stem cell virus (MSCV) vector or MSCV expressing human RPA3, which are linked with a GFP reporter. The mCherry/GFP double-positive percentage is normalized to the 20th day measurement. Each bar represents an independent sgRNA targeting a unique genomic site. Red indicates mismatches. PAM, protospacer-adjacent motif. (e) Summary of negative selection experiments with sgRNAs targeting the indicated genes. Negative selection is plotted as the fold depletion of GFP+ cells (d2 GFP% divided by d10 GFP%) during 8 days in culture. Each bar represents an independent sgRNA targeting a 5′ exon of the indicated gene. The dashed line indicates a twofold change. The fold depletion for two Brd4 sgRNAs was >50, but the axis was limited to a 20-fold maximum for visualization purposes. The data shown are the mean value of three independent replicates.

sgRNAs causing mild negative-selection phenotypes targeted regions that lie outside of BD1 (Fig. 2a). Prior studies have shown that the bromodomains of BRD4 are required for leukemia cell viability, as evidenced by the anti-leukemia activity of small-molecule inhibitors of BRD4 bromodomains. This prompted us to evaluate whether robust negative selection is generally correlated with the targeting of functionally important protein domains. Using 64 sgRNAs in total, we targeted every exon of Brd4 to evaluate the relative severity of negative selection (Fig. 2a). All of the sgRNAs that achieved over tenfold depletion after 4 days were found to target exons encoding BD1, BD2 or the C-terminal motif (CTM), which are domains of BRD4 involved in transcriptional regulation. At later time points, we also noted that sgRNAs targeting the BD4 extra-terminal (ET) domain became depleted more than tenfold (Supplementary Fig. 1). By contrast, many of the sgRNAs targeting regions outside of these domains caused only minimal negative-selection phenotypes (Fig. 2a and Supplementary Fig. 1). To further corroborate this relationship, we surveyed all of the exons of Smarca4 and found that sgRNAs targeting the ATPase domain generally produced more severe negative-selection phenotypes than sgRNAs targeting other regions (Fig. 2b). This finding is consistent with our prior demonstration that the catalytic function of SMARCA4 is essential in leukemia. In addition, CRISPR-Cas9 targeting of the catalytic domains of Aurora kinase A and B, the kinase domain of mToR, the AAA+ ATPase and winged helix DNA binding
CRISPR-Cas9 mutagenesis of functional protein domains leads to a higher proportion of null mutations and enhanced severity of negative selection. (a) Systematic evaluation of 64 Brd4 sgRNAs in negative selection experiments, targeting each Brd4 exon. The location of each sgRNA relative to the Brd4 protein is indicated along the x axis. Location of Brd4 sgRNAs used in Figure 1 is indicated. Plotted is the average of three biological replicates (independent lentivirally transduced RN2c cells). (b) Systematic evaluation of 88 Smarca4 sgRNAs in negative selection experiments, targeting each Smarca4 exon. The relative location of each sgRNA relative to the Smarca4 protein is indicated along the x axis. Location of Smarca4 sgRNAs used in Figure 1 is indicated. Indicated domains were obtained from the NCBI database. SNF_N and HELIC constitute the ATPase domain. Plotted is the average of three biological replicates. (c–h) Negative selection experiments evaluating sgRNAs targeting 5’ coding exons and domain locations for the indicated proteins. In a–h, the proteins are not drawn to the same scale. WH DBD: winged helix DNA binding domain. Plotted is the average of three biological replicates. (i–k) Deep sequencing analysis of mutation abundance following CRISPR-Cas9 targeting of different Brd4 regions. This analysis was performed on PCR-amplified genomic regions corresponding to the sgrNA cut site at the indicated time points. Indel mutations were categorized into two groups: in-frame (3n) or frameshift (3n + 1, 3n + 2). Nonsense mutations induced by CRISPR-Cas9 mutagenesis were included in the frameshift category; however, such mutations were rare. Blue and red numbers indicate the number of distinct in-frame and frameshift mutants that were tracked, respectively. Dots of the same color indicate the median normalized abundance at the indicated time point for all mutations within each group; shaded regions indicate the interquartile range of normalized abundance values. Significant differences between the enrichment values of the in-frame and frameshift mutations were assessed using a Mann-Whitney-Wilcoxon test; *** P < 0.005. The normalized abundance of each tracked mutation was defined as the ratio of the number of observed mutant sequences divided by the number of wild-type sequences, normalized by the value of this same quantity at day 3.

**Figure 2** CRISPR-Cas9 mutagenesis of functional protein domains leads to a higher proportion of null mutations and enhanced severity of negative selection. (a) Systematic evaluation of 64 Brd4 sgRNAs in negative selection experiments, targeting each Brd4 exon. The location of each sgRNA relative to the Brd4 protein is indicated along the x axis. Location of Brd4 sgRNAs used in Figure 1 is indicated. Plotted is the average of three biological replicates (independent lentivirally transduced RN2c cells). (b) Systematic evaluation of 88 Smarca4 sgRNAs in negative selection experiments, targeting each Smarca4 exon. The relative location of each sgRNA relative to the Smarca4 protein is indicated along the x axis. Location of Smarca4 sgRNAs used in Figure 1 is indicated. Indicated domains were obtained from the NCBI database. SNF_N and HELIC constitute the ATPase domain. Plotted is the average of three biological replicates. (c–h) Negative selection experiments evaluating sgRNAs targeting 5’ coding exons and domain locations for the indicated proteins. In a–h, the proteins are not drawn to the same scale. WH DBD: winged helix DNA binding domain. Plotted is the average of three biological replicates. (i–k) Deep sequencing analysis of mutation abundance following CRISPR-Cas9 targeting of different Brd4 regions. This analysis was performed on PCR-amplified genomic regions corresponding to the sgRNA cut site at the indicated time points. Indel mutations were categorized into two groups: in-frame (3n) or frameshift (3n + 1, 3n + 2). Nonsense mutations induced by CRISPR-Cas9 mutagenesis were included in the frameshift category; however, such mutations were rare. Blue and red numbers indicate the number of distinct in-frame and frameshift mutants that were tracked, respectively. Dots of the same color indicate the median normalized abundance at the indicated time point for all mutations within each group; shaded regions indicate the interquartile range of normalized abundance values. Significant differences between the enrichment values of the in-frame and frameshift mutations were assessed using a Mann-Whitney-Wilcoxon test; *** P < 0.005. The normalized abundance of each tracked mutation was defined as the ratio of the number of observed mutant sequences divided by the number of wild-type sequences, normalized by the value of this same quantity at day 3.

Domains of Orc1, the AAA+ ATPase domain of Orc4 and the AAA+ ATPase domain of Mcm4 all led to more severe negative-selection phenotypes than targeting of 5’ coding exons (Fig. 2c–h).

Using SURVEYOR assays and deep-sequencing analysis, we found that the differences in phenotypic severity between domain-targeting sgRNAs and 5’ exon Brd4 sgRNAs could not be explained by variation in the overall efficiency of mutagenesis (Supplementary Fig. 2). These phenotypic differences were also not attributable to off-target cutting at exons encoding homologous protein domains (Supplementary Fig. 3). Instead, CRISPR-Cas9-induced mutations within domains became depleted more rapidly from the cell population than mutations introduced outside of the domain (Supplementary Fig. 2). These findings raised the possibility that CRISPR-Cas9 targeting of functionally important domains resulted in a higher proportion of null mutations than targeting outside of critical domains.

We evaluated this hypothesis by deep sequencing of the CRISPR-Cas9–mutagenized Brd4 exons (PCR-amplified from genomic DNA) during the negative-selection time course, which is a means to track how individual mutations impair cellular fitness19. For these experiments, we directly compared BD1 mutations (introduced by sgRNAs e3.3 and e4.1) with mutations introduced outside of BD1 by sgRNA e3.1 (Fig. 2i–k). As expected, roughly two-thirds of the mutations generated by the three different sgRNAs resulted in a frameshift, which underwent negative selection when introduced at any of the three Brd4 locations (Fig. 2i–k). By contrast, we found that the in-frame mutations underwent negative selec-
tion only when generated within the BD1 region, and not when generated outside of BD1 (Fig. 2i–k). Indeed, the in-frame and frameshift mutations generated within BD1 displayed indistinguishable kinetics of negative selection, suggesting that both mutational classes are genetic nulls (Fig. 2j,k). These findings imply that in-frame mutations occurring within BD1 may compromise the

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Figure 3 A chromatin regulatory domain–focused CRISPR-Cas9 screen in MLL-AF9 leukemia validates known drug targets and reveals additional dependencies. (a–f) Summary of negative selection experiments with sgRNAs targeting the indicated domains plotted as fold depletion in percentage GFP+ cells. Each bar represents the mean value of three independent biological replicates for an independent sgRNA targeting the indicated domain. Red indicates domains for which prior pharmacological validation of the dependency has been performed. A 20-fold cutoff was applied for visualization purposes. Different time points of GFP measurements were chosen based on the severity of the strongest negative-selection phenotype in the screen.
acetyl-lysine recognition function of this domain to inactivate Brd4, whereas in-frame mutations occurring outside of BD1 retain full Brd4 functionality. A similar deep-sequencing analysis of Smarca4 also suggested that functional impairment of in-frame mutations contributes to the increased severity of negative selection when targeting the ATPase domain (Supplementary Fig. 4).

In a diploid cell line it is expected that random pairing of in-frame and frameshift CRISPR-Cas9 mutations will generate cell populations

**Figure 4** CRISPR-Cas9 targeting of enzymatic domains consistently outperforms targeting of 5′ coding exons in negative selection experiments. (a) Evaluation of 32 Ezh2 sgRNAs in negative selection experiments, targeting each Ezh2 exon. The location of each sgRNA relative to the Ezh2 protein is indicated along the x axis. (b–j) Evaluation of 5′ coding exon and enzymatic domain-focused sgRNAs in negative selection experiments. The relative location of each sgRNA relative to the protein is indicated along the x axis. Owing to the large size of Mll4/Kmt2d, we have cropped out amino acids 2,000 to 15,000 for visualization purposes. For a–j, plotted is the average fold depletion in GFP% of three biological replicates. KMT, lysine methyltransferase domain. The proteins are not drawn to the same scale. (k–o) Deep sequencing analysis of mutation abundance following CRISPR-Cas9-targeting of different Ezh2 and Dot1l regions. This analysis was performed on PCR-amplified genomic regions corresponding to the sgRNA cut site at the indicated time points. Indel mutations were categorized into two groups: in-frame (3n) or frameshift (3n + 1, 3n + 2). Nonsense mutations induced by CRISPR-Cas9 mutagenesis were also included in the frameshift category; however, such mutations were rare. Blue and red numbers indicate the number of in-frame and frameshift mutants that were tracked, respectively. Dots of the same color indicate the median normalized abundance at the indicated time point for all mutations within each group; shaded regions indicate the interquartile range of normalized abundance values. Significant differences between the enrichment values of the in-frame and frameshift mutations were assessed using a Mann-Whitney-Wilcoxon test; **P < 0.01; ***P < 0.005. The normalized abundance of each tracked mutation was defined as the ratio of the number of observed mutant sequences divided by the number of wild-type sequences, normalized by the value of this same quantity at day 3.
of varying genotypes. If in-frame CRISPR-Cas9 mutations are functional and occur at the expected frequency of around 33% (Supplementary Fig. 5), then ~56% of cells in the population will possess at least one functional allele of the essential gene, thereby limiting the overall severity of observed phenotypes (Supplementary Fig. 6). By contrast, if in-frame CRISPR-Cas9 mutations are non-functional, it is anticipated that nearly all cells in the population are capable of experiencing a robust cellular phenotype. Random allele pairing with in-frame variants also explains why frameshift mutations undergo stronger negative selection when occurring within a domain than outside of a domain (Supplementary Discussion). Finally, we also noted that the deep sequencing–based measurement of allele functionality also provided a means of excluding off-target effects when validating hits obtained from CRISPR-Cas9-based screens (Supplementary Discussion).

The major implication of the experiments described above is that the severity of negative selection in CRISPR-Cas9 screens reflects, at least in part, the functional importance of the protein region being targeted. Therefore, a CRISPR-Cas9 screening strategy that exclusively targets exons encoding protein domains could be used to nominate individual domains as cancer dependencies and potentially as drug targets. To evaluate this hypothesis, we designed an sgRNA library that targeted 192 domains involved in chromatin regulation. These domains included methyltransferase, demethylase, acetyltransferase, deacetylase, ATPase, and histone acetyltransferase activities of Dot1l, Ezh2, Ehmt1/2 and the lysine demethylase and histone deacetylase families, respectively. To maximize the likelihood of targeting domains, we focused on 3′ coding exons in our CRISPR-Cas9 frameshift screens (Fig. 4a–j). Hence, a screening strategy that exclusively targets 5′ coding exons in negative selection experiments (Fig. 4a–j). Therefore, a screening strategy that exclusively targets 5′ coding exons would have failed to identify most of these regulators as cancer cell dependencies. Finally, deep sequencing analyses of Ezh2 and Dot1l mutation abundance was performed at both methyltransferase domain and 5′ exon locations. This analysis confirmed the contribution of functionally defective in-frame mutations at domain regions as the underlying basis for the increased severity of negative selection (Fig. 4k–o and Supplementary Fig. 10).

The overall performance of CRISPR-Cas9 for genetic screening is influenced by several experimental parameters affecting the overall efficiency of mutagenesis, including the level of Cas9 expression25 and sgRNA sequence features.2,5 Here we have shown that the performance of CRISPR-Cas9 in generating null mutations is substantially improved when Cas9 cutting is directed to sequences that encode functionally important protein domains. This leads to a potentially useful approach to identify cancer dependencies suitable for pharmacological inhibition. sgRNA libraries should be designed to target exons that encode ‘druggable’ protein domains. In such screens the severity of negative selection phenotypes would directly indicate the functional importance of the domain being targeted. We expect this approach to be critical when targeting genes that encode large multidomain proteins, but less important for small proteins, like Rpa3. It would also be expected that domain-focused CRISPR-Cas9 screens might be most effective for probing discrete enzymatic active sites, but potentially less amenable for probing extended protein–protein interaction surfaces. Nonetheless, in this study we have targeted a diverse collection of protein to highlight the general utility of domain-focused CRISPR-Cas9 screening as a tool for drug target discovery.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. SRA: SRP057117. The source code for performing all these computations is available at https://github.com/jkinney/14_crispr.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
J.S. designed experiments; J.S., E.W. and J.P.M. carried out experiments; S.R.A., J.S. and C.R.V. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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1. Shalem, O. et al. Genome-scale CRISPR-Cas9 knockout screening in human cells. Science 343, 84–87 (2014).
2. Wang, T., Wei, J.J., Sabatini, D.M. & Lander, E.S. Genetic screens in human cells using the CRISPR-Cas9 system. Science 343, 80–84 (2014).
3. Koike-Yusa, H., Li, Y., Tan, E.P., Velasco-Herrera Mollé, C. & Yusa, K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. Nat. Biotechnol. 32, 267–273 (2014).

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4. Zhou, Y. et al. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. *Nature* **509**, 487–491 (2014).
5. Doench, J.G. et al. Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nat. Biotechnol.* **32**, 1262–1267 (2014).
6. Hsu, P.D., Lander, E.S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262–1278 (2014).
7. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819–823 (2013).
8. Mali, P. et al. RNA-guided human genome engineering via Cas9. *Science* **339**, 823–826 (2013).
9. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
10. Zuber, J. et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* **478**, 524–528 (2011).
11. Zuber, J. et al. Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. *Nat. Biotechnol.* **29**, 79–83 (2011).
12. McJunkin, K. et al. Reversible suppression of an essential gene in adult mice using transgenic RNA interference. *Proc. Natl. Acad. Sci. USA* **108**, 7113–7118 (2011).
13. Shi, J. et al. Role of SWI/SNF in acute leukemia maintenance and enhancer-mediated Myc regulation. *Genes Dev.* **27**, 2648–2662 (2013).
14. Wang, E. et al. Histone H2B ubiquitin ligase RNF20 is required for MLL-rearranged leukemia. *Proc. Natl. Acad. Sci. USA* **110**, 3901–3906 (2013).
15. Shi, J. et al. The Polycomb complex PRC2 supports aberrant self-renewal in a mouse model of MLL-AF9;Nras(G12D) acute myeloid leukemia. *Oncogene* **32**, 930–938 (2013).
16. Mertz, J.A. et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. *Proc. Natl. Acad. Sci. USA* **108**, 16669–16674 (2011).
17. Dawson, M.A. et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. *Nature* **478**, 529–533 (2011).
18. Shi, J. & Vakoc, C.R. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. *Mol. Cell* **54**, 728–736 (2014).
19. Findlay, G.M., Boyle, E.A., Hause, R.J., Klein, J.C. & Shendure, J. Saturation editing of genomic regions by multiplex homology-directed repair. *Nature* **513**, 120–123 (2014).
20. Xu, B. et al. Selective inhibition of EZH2 and EZH1 enzymatic activity by a small molecule suppresses MLL-rearranged leukemia. *Blood* **125**, 346–357 (2015).
21. Daigle, S.R. et al. Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell* **20**, 53–65 (2011).
22. Harris, W.J. et al. The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells. *Cancer Cell* **21**, 473–487 (2012).
23. Lehner, B. et al. The methyltransferase G9a regulates HoxA9-dependent transcription in AML. *Genes Dev.* **28**, 317–327 (2014).
24. Kim, W. et al. Targeted disruption of the EZH2-EED complex inhibits EZH2-dependent cancer. *Nat. Chem. Biol.* **9**, 643–650 (2013).
25. Hsu, P.D. et al. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat. Biotechnol.* **31**, 827–832 (2013).
ONLINE METHODS

Plasmid construction and sgRNA design. The constitutive Cas9 expression construct was derived by subcloning the 5′ 3xFLAG tagged human-codon optimized Cas9 cDNA from Streptococcus pyogenes (Addgene: #49535) into the MSCV-PGK-Puro vector (Clonetech: #634401). The U6-sgRNA-EFS-GFP and the U6-sgRNA-EFS-mCherry vectors were derived from the lentCRISPR-Cas9 plasmid (Addgene: #49535) by removing the hCas9 cDNA and replacing the Puro cassette with GFP or mCherry. The wild-type Rpa3 was cloned by PCR directly from human cDNA into the MSCV-IRES-GFP (MigR1) vector. All cloning procedures were done using the In-Fusion cloning system (Clonetech: #638909). sgRNAs were cloned by annealing two DNA oligos and ligating into a BsmB1-digested U6-sgRNA-EFS-GFP/mCherry vectors, as described26. To improve U6 promoter efficiency, we added an extra 5′ G nucleotide to all of the sgRNAs that did not start with a 5′ G.

All sgRNAs in this study were designed using http://crispr.mit.edu/25. The majority of sgRNAs used in this study had a quality score above 70 to minimize off-target effects. In Figure 1, sgRNAs were designed targeting 5′ constitutive coding exons of each target gene. For the chromatin regulatory domain-focused CRISPR-Cas9 screen, sgRNAs were designed to target the catalytic domain or bromodomain of each protein based on the NCBI database annotation.

All sgRNA sequences used in this study are provided in Supplementary Table 1.

Cell culture, virus production and sgRNA competition assays to measure negative selection. All of the cell lines used in this study were tested for mycoplasma and were negative. RN2c cells were derived by retroviral transduction of a murine MLL-AF9/NrasG12D acute myeloid leukemia cell line (RN2)11 with MSCV-hCas9-PGK-Puro, followed by puromycin selection and serial dilution to derive single cell–derived clones. Clones were screened by anti-flag western blot analysis for high levels of stable Cas9 expression. Multiple independent clones displayed a similar CRISPR-Cas9 editing efficiency as RN2c. RN2c cells were cultured in RPMI1640 supplemented with 10% FBS and penicillin/streptomycin. 38B9 cells were cultured in RPMI1640 supplemented with 10% FBS and 0.055 mM 2-mercaptoethanol. NIH3T3 cells were cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin. Ecotropic Plat-E cells and HEK293T cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. Plat-E cells were used for retroviral delivery of hCas9 or RPA3 cDNA expression vectors, following standard procedures27.

sgRNA competition assays were done using the U6-sgRNA-EFS-GFP or the U6-sgRNA-EFS-mCherry plasmids, where indicated. These plasmids were used to generate lentivirus by transfecting HEK293T cells with sgRNA:pVSVg:U6-sgRNA-EFS-mCherry vectors, where indicated. These plasmids were prepared by PCR amplification using the 3′ primer set specific for the sgRNA targeted region with 2X Phusion Master Mix (Thermo Scientific #F-548) following the manufacturer’s instructions. All of the primers were optimized to ensure their selectivity for a single genomic region. The PCR products were subjected to SURVEYOR assay (Transgenicom #706020) following the manufacturer’s protocol. To calculate the cleavage efficiency, we quantified the intensity of each DNA band using the ImageJ software (http://imagej.nih.gov/ij/) resolved by agarose gel electrophoresis, stained with ethidium bromide. SURVEYOR assay–based calculation of indel frequency was done using a binomial probability distribution of random duplex formation, as described previously26. All PCR primers for SURVEYOR analysis are provided in Supplementary Table 1.

MiSeq library construction to evaluate mutation abundance. To quantify the abundance of individual indel mutations induced by CRISPR-Cas9, we prepared gDNA as above and PCR-amplified a 100- to 200-bp amplicon centered on the sgRNA recognition region. 100 ng gDNA was amplified for 20 cycles with 2X Phusion Master Mix. The PCR product was end repaired with T4 DNA polymerase (New England Biolabs, NEB), DNA polymerase I (NEB) and T4 polynucleotide kinase (NEB). An A overhang was added to the end-repaired DNA using Klenow DNA Pol Exo− (NEB). The DNA fragment was then ligated with diversity-increased barcoded Illumina adaptors followed by five precapture PCR cycles. Barcoded libraries were pooled at equal molar ratio and subjected to massively parallel sequencing using a MiSeq instrument (Illumina) using paired-end 150-bp sequencing (MiSeq Reagent Kit v2; Illumina MS-102-2002).

All primer sequences are listed in Supplementary Table 1.

MiSeq analysis of abundance of indel mutations induced using CRISPR-Cas9. Custom Python scripts were used to reconstruct mutant allele sequences from paired-end reads, to analyze these sequences. The scripts for performing these computation and generation of figures are available at https://github.com/jkbinney/14_crispr.

Paired-end reads were stitched together to form observed sequences as follows. Customized barcodes were used to separate different samples. Next, the 5′-most 15 bp of each primer sequence was used to identify which genome region the paired-end read came from. Forward and reverse reads that overlapped by at least 15 bp were then stitched together and classified as “observed sequences.”

Observed sequences were aligned with the corresponding wild-type sequence starting from both ends and moving inward. Forward and reverse breakpoints were called as the positions at which the wild-type sequence and observed sequence became substantially different (defined as two consecutive nucleotide mismatches). “Deletions” were defined as the region of wild-type sequence between these two breakpoints. “Insertions” were defined as the region of observed sequence between these breakpoints.

After alignment with the wild-type sequence, each observed sequence was classified as follows. If the sequence exactly matched the endogenous locus, it was classified as “wild-type.” If the sequence contained an insertion and/or deletion mutation that altered the exon reading frame or generated a stop codon, it was classified as a “frameshift” mutant. If the sequence contained an insertion and/or deletion that preserved the reading frame and did not generate a stop codon, it was classified as an “in-frame” mutant. Observed sequences with insertions and/o deletions that disrupted an exon boundary were discarded.

To ensure sufficient counts, only sequences occurring at least 100 times in each day 3 sample were tracked. To remove common PCR-induced mutations from the analysis, observed sequences that occurred more than ten times in the corresponding control sample (Rosa26 sgRNA) were discarded. At each time point t, the enrichment ratio for a sequence s at time point t was defined as

\[ P(s, t) = \frac{N(s, t)}{N(wt, t)} \]  

where \( N(s, t) \) is the number of observations of sequence s at time point t, \( wt \) denotes the wild-type sequence and \( d3 \) is the day-3 time point. Solid colored dots in the figures (Figs. 2–k, 4k–o and Supplementary Fig. 4) show the median enrichment ratio for each class of mutations at each time point. Shaded region indicates the corresponding interquartile range of these enrichment ratios.
The efficiency of CRISPR-Cas9 mutagenesis in each sample was estimated from the following measured quantities:

- $M$: the fraction of observed sequences containing mutations
- $I$: the fraction of cells that were infected and observed to express sgRNA
- $m$: the probability of mutation due to PCR artifacts

We estimated the efficiency $E$ of CRISPR-Cas9 mutagenesis from these quantities using the relationship

$$E = \frac{M - m}{(1-m)I}$$

The rationale for this relationship is as follows. If we obtain $N$ observed sequences in a given sample, $MN$ of these observed sequences will have mutations. Of these mutant sequences, $NIE$ will come from CRISPR-Cas9–mutagenized alleles inside infected, $NI(1-E)m$ will come from nonmutated alleles within infected cells and contain PCR-generated mutations, and $N(1-I)m$ will come from wild-type alleles within noninfected cells and contain PCR-generated mutations. Solving for $E$, we obtain

$$E = \frac{M - m}{(1-m)I}$$

Due to the uncertainties in $M$, $m$ and $I$, some of these efficiency estimates are greater than 100%. This issue is particularly evident in samples having low values for $I$ (due to strong negative selection). Nevertheless, these estimates all suggest true CRISPR-Cas9 mutagenesis efficiencies that are very close to 100%.

**Pooled sgRNA screening and data analysis.** The lysine methyltransferase U6-sgRNA-EFS-GFP library was pooled at equimolar ratio and used to generate a lentiviral supernatant as described above. The total number of RN2c cells was chosen to achieve at least 500-fold representation of each sgRNA in the initially infected cell population. To ensure that a single sgRNA was transduced per cell, the viral volume for infection was chosen to achieve a multiplicity of infection (MOI) of 0.3–0.4. Genomic DNA was extracted at the indicated time points using QiAamp DNA mini kit (Qiagen #51304), following the manufacturer’s instructions. To maintain >500× sgRNA library representation, 16–20 independent PCR reactions were used to amplify the sgRNA cassette, which were amplified for 20 cycles with 100–200 ng of starting gDNA using the 2X Phusion Master Mix (Thermo Scientific #F-548). PCR products were pooled and subjected to Illumina MiSeq library construction and sequencing, as described above. The sequence data were trimmed to contain only the sgRNA sequence then were mapped to the reference sgRNA library without allowing any mismatches. The read counts were then calculated for each individual sgRNA. To compare the differential representation of individual sgRNAs between day-2 and day-12 time points, the read counts for each sgRNA were normalized to the counts of the negative control Rosa26 sgRNA.

The PCR primers and conditions are listed in **Supplementary Table 1**.

26. Ran, F.A. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. **8**, 2281–2308 (2013).
27. Morita, S., Kojima, T. & Kitamura, T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. **7**, 1063–1066 (2000).