Sciencexpress Reports

Multiplex Genome Engineering Using CRISPR/Cas Systems

Le Cong,[1,2*] F. Ann Ran,[1,4*] David Cox,[1,3] Shuailiang Lin,[1,5] Robert Barretto,[6] Naomi Habib,[1] Patrick D. Hsu,[1,4] Xuebing Wu,[7] Wenyan Jiang,[8] Luciano A. Marraffini,[8] Feng Zhang[1†]

[1]Broad Institute of MIT and Harvard, 7 Cambridge Center, Cambridge, MA 02142, USA and McGovern Institute for Brain Research, Department of Brain and Cognitive Sciences, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [2]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA 02115, USA. [3]Harvard-MIT Health Sciences and Technology, Harvard Medical School, Boston, MA 02115, USA. [4]Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA. [5]School of Life Sciences, Tsinghua University, Beijing, 100084, China. [6]Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, NY 10032, USA. [7]Computational and Systems Biology Graduate Program and Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [8]Laboratory of Bacteriology, The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA.

*These authors contributed equally to this work.

†To whom correspondence should be addressed: zhang@broadinstitute.org

Functional elucidation of causal genetic variants and elements requires precise genome editing technologies. The type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats) adaptive immune system has been shown to facilitate RNA-guided site-specific DNA cleavage. We engineered two different type II CRISPR systems and demonstrate that Cas9 nucleases can be directed by short RNAs to induce precise cleavage at endogenous genomic loci in human and mouse cells. Cas9 can also be converted into a nicking enzyme to facilitate homology-directed repair with minimal mutagenic activity. Finally, multiple guide sequences can be encoded into a single CRISPR array to enable simultaneous editing of several sites within the mammalian genome, demonstrating easy programmability and wide applicability of the CRISPR technology.

Precise and efficient genome targeting technologies are needed to enable systematic reverse engineering of causal genetic variations by allowing selective perturbation of individual genetic elements. Although genome-editing technologies such as designer zinc fingers (ZFs) (*1–4*), transcription activator-like effectors (TALEs) (*4–10*), and homing meganucleases (*11*) have begun to enable targeted genome modifications, there remains a need for new technologies that are scalable, affordable, and easy to engineer. Here, we report the development of a new class of precision genome engineering tools based on the RNA-guided Cas9 nuclease (*12–14*) from the type II prokaryotic CRISPR adaptive immune system (*15–18*).

The *Streptococcus pyogenes* SF370 type II CRISPR locus consists of four genes, including the Cas9 nuclease, as well as two non-coding RNAs: tracrRNA and a pre-crRNA array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs) (Fig. S1) (*19*). We sought to harness this prokaryotic RNA-programmable nuclease system to introduce targeted double stranded breaks (DSBs) in mammalian chromosomes through heterologous expression of the key components. It has been previously shown that expression of tracrRNA, pre-crRNA, host factor RNase III, and Cas9 nuclease are necessary and sufficient for cleavage of DNA in vitro (*12*, *13*) and in prokaryotic cells (*20*, *21*). We codon optimized the *S. pyogenes Cas9 (SpCas9)* and *RNase III (SpRNase III)* and attached nuclear localization signals (NLS) to ensure nuclear compartmentalization in mammalian cells. Expression of these constructs in human 293FT cells revealed that two NLSs are most efficient at targeting SpCas9 to the nucleus (Fig. 1A). To reconstitute the non-coding RNA components of CRISPR, we expressed an 89-nucleotide (nt) tracrRNA (Fig. S2) under the RNA polymerase III U6 promoter (Fig. 1B). Similarly, we used the U6 promoter to drive the expression of a pre-crRNA array comprising a single guide spacer flanked by DRs (Fig. 1B). We designed our initial spacer to target a 30-basepair (bp) site (protospacer) in the human *EMX1* locus that precedes an NGG, the requisite protospacer adjacent motif (PAM) (Fig. 1C and fig. S1) (*22*, *23*).

To test whether heterologous expression of the CRISPR system (SpCas9, SpRNase III, tracrRNA, and pre-crRNA) can achieve targeted cleavage of mammalian chromosomes, we transfected 293FT cells with different combinations of CRISPR components. Since DSBs in mammalian DNA are partially repaired by the indel-forming non-homologous end joining (NHEJ) pathway, we used the SURVEYOR assay (Fig. S3) to detect endogenous target cleavage (Fig. 1D and fig. S2B). Co-transfection of all four required CRISPR components resulted in efficient cleavage of the protospacer (Fig. 1D and fig. S2B), which is subsequently verified by Sanger sequencing (Fig. 1E). Interestingly, SpRNase III was not necessary for cleavage of the protospacer (Fig. 1D), and the 89-nt tracrRNA is processed in its absence (Fig. S2C). Similarly, maturation of pre-crRNA does not require RNase III (Fig. 1D and fig. S4), suggesting that there may be endogenous mammalian RNases that assist in pre-crRNA maturation (*24–26*). Removing any of the remaining RNA or Cas9 components abolished the genome cleavage activity of the CRISPR system (Fig. 1D). These results define a minimal three-component system for efficient CRISPR-mediated genome modification in mammalian cells.

Next, we explored the generalizability of CRISPR-mediated cleavage in eukaryotic cells by targeting additional protospacers within the *EMX1* locus (Fig. 2A). To improve co-delivery, we designed an expression vector to drive both pre-crRNA and SpCas9 (Fig. S5). In parallel, we adapted a chimeric crRNA-tracrRNA hybrid (Fig. 2B, top) design recently validated in vitro (*12*), where a mature crRNA is fused to a partial tracrRNA via a synthetic stem-loop to mimic the natural crRNA:tracrRNA duplex (Fig. 2B, bottom). We observed cleavage of all protospacer targets when SpCas9 is co-expressed with pre-crRNA (DR-spacer-DR) and tracrRNA. However, not all chimeric RNA designs could facilitate cleavage of their genomic targets (Fig. 2C, Table S1). We then tested targeting of additional genomic loci in both human and mouse cells by designing pre-crRNAs and chimeric RNAs targeting the human *PVALB* and the mouse *Th* loci (Fig. S6). We achieved efficient modification at all three mouse *Th* and one *PVALB* targets using the crRNA:tracrRNA design, thus demonstrating the broad applicability of the CRISPR system in modifying different loci across multiple organisms (Table S1). For the same protospacer targets, cleavage efficiencies of chimeric RNAs were either lower than those of crRNA:tracrRNA

duplexes or undetectable. This may be due to differences in the expression and stability of RNAs, degradation by endogenous RNAi machinery, or secondary structures leading to inefficient Cas9 loading or target recognition.

Effective genome editing requires that nucleases target specific genomic loci with both high precision and efficiency. To investigate the specificity of CRISPR-mediated cleavage, we analyzed single-nucleotide mismatches between the spacer and its mammalian protospacer target (Fig. 3A). We observed that single-base mismatch up to 12-bp 5′ of the PAM completely abolished genomic cleavage by SpCas9, whereas spacers with mutations farther upstream retained activity against the protospacer target (Fig. 3B). This is consistent with previous bacterial and in vitro studies of Cas9 specificity (*12*, *20*). Furthermore, CRISPR is able to mediate genomic cleavage as efficiently as a pair of TALE nucleases (TALEN) targeting the same *EMX1* protospacer (Fig. 3, C and D).

Targeted modification of genomes ideally avoids mutations arising from the error-prone NHEJ mechanism. The wild-type SpCas9 is able to mediate site-specific DSBs, which can be repaired through either NHEJ or homology-directed repair (HDR). We engineered an aspartate-to-alanine substitution (D10A) in the RuvC I domain of SpCas9 to convert the nuclease into a DNA nickase (SpCas9n, Fig. 4A) (*12*, *13*, *20*), because nicked genomic DNA is typically repaired either seamlessly or through high-fidelity HDR. SURVEYOR (Fig. 4B) and sequencing of 327 amplicons did not detect any indels induced by SpCas9n. However, it is worth noting that nicked DNA can in rare cases be processed via a DSB intermediate and result in a NHEJ event (*27*). We then tested Cas9-mediated HDR at the same *EMX1* locus with a homology repair template to introduce a pair of restriction sites near the protospacer (Fig. 4C). SpCas9 and SpCas9n catalyzed integration of the repair template into *EMX1* locus at similar levels (Fig. 4D), which we further verified via Sanger sequencing (Fig. 4E). These results demonstrate the utility of CRISPR for facilitating targeted genomic insertions. Given the 14-bp (12-bp from the seed sequence and 2-bp from PAM) target specificity (Fig. 3B) of the wild type SpCas9, the use of a nickase may reduce off-target mutations.

Finally, the natural architecture of CRISPR loci with arrayed spacers (Fig. S1) suggests the possibility of multiplexed genome engineering. Using a single CRISPR array encoding a pair of *EMX1*- and *PVALB*-targeting spacers, we detected efficient cleavage at both loci (Fig. 4F). We further tested targeted deletion of larger genomic regions through concurrent DSBs using spacers against two targets within *EMX1* spaced by 119-bp, and observed a 1.6% deletion efficacy (3 out of 182 amplicons; Fig. 4G), thus demonstrating the CRISPR system can mediate multiplexed editing within a single genome.

The ability to use RNA to program sequence-specific DNA cleavage defines a new class of genome engineering tools. Here, we have shown that the *S. pyogenes* CRISPR system can be heterologously reconstituted in mammalian cells to facilitate efficient genome editing; an accompanying study has independently confirmed high efficiency CRISPR-mediated genome targeting in several human cell lines (*28*). However, several aspects of the CRISPR system can be further improved to increase its efficiency and versatility. The requirement for an NGG PAM restricts the *S. pyogenes* CRISPR target space to every 8-bp on average in the human genome (Fig. S7), not accounting for potential constraints posed by crRNA secondary structure or genomic accessibility due to chromatin and DNA methylation states. Some of these restrictions may be overcome by exploiting the family of Cas9 enzymes and its differing PAM requirements (*22*, *23*) across the microbial diversity (*17*). Indeed, other CRISPR loci are likely to be transplantable into mammalian cells; for example, the *Streptococcus thermophilus* LMD-9 CRISPR1 can also mediate mammalian genome cleavage (Fig. S8). Finally, the ability to carry out multiplex genome editing in mammalian cells enables powerful applications across basic science, biotechnology, and medicine (*29*).

References and Notes

1. M. H. Porteus, D. Baltimore, Chimeric nucleases stimulate gene targeting in human cells. *Science* **300**, 763 (2003). doi:10.1126/science.1078395 Medline
2. J. C. Miller *et al*., An improved zinc-finger nuclease architecture for highly specific genome editing. *Nat. Biotechnol.* **25**, 778 (2007). doi:10.1038/nbt1319 Medline
3. J. D. Sander *et al*., Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CoDA). *Nat. Methods* **8**, 67 (2011). doi:10.1038/nmeth.1542 Medline
4. A. J. Wood *et al*., Targeted genome editing across species using ZFNs and TALENs. *Science* **333**, 307 (2011). doi:10.1126/science.1207773 Medline
5. M. Christian *et al*., Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* **186**, 757 (2010). doi:10.1534/genetics.110.120717 Medline
6. F. Zhang *et al*., Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. *Nat. Biotechnol.* **29**, 149 (2011). doi:10.1038/nbt.1775 Medline
7. J. C. Miller *et al*., A TALE nuclease architecture for efficient genome editing. *Nat. Biotechnol.* **29**, 143 (2011). doi:10.1038/nbt.1755 Medline
8. D. Reyon *et al*., FLASH assembly of TALENs for high-throughput genome editing. *Nat. Biotechnol.* **30**, 460 (2012). doi:10.1038/nbt.2170 Medline
9. J. Boch *et al*., Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* **326**, 1509 (2009). doi:10.1126/science.1178811 Medline
10. M. J. Moscou, A. J. Bogdanove, A simple cipher governs DNA recognition by TAL effectors. *Science* **326**, 1501 (2009). doi:10.1126/science.1178817 Medline
11. B. L. Stoddard, Homing endonuclease structure and function. *Q. Rev. Biophys.* **38**, 49 (2005). doi:10.1017/S0033583505004063 Medline
12. M. Jinek *et al*., A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816 (2012). doi:10.1126/science.1225829 Medline
13. G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U.S.A.* **109**, E2579 (2012). doi:10.1073/pnas.1208507109 Medline
14. J. E. Garneau *et al*., The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* **468**, 67 (2010). doi:10.1038/nature09523 Medline
15. H. Deveau, J. E. Garneau, S. Moineau, CRISPR/Cas system and its role in phage-bacteria interactions. *Annu. Rev. Microbiol.* **64**, 475 (2010). doi:10.1146/annurev.micro.112408.134123 Medline
16. P. Horvath, R. Barrangou, CRISPR/Cas, the immune system of bacteria and archaea. *Science* **327**, 167 (2010). doi:10.1126/science.1179555 Medline
17. K. S. Makarova *et al*., Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* **9**, 467 (2011). doi:10.1038/nrmicro2577 Medline
18. D. Bhaya, M. Davison, R. Barrangou, CRISPR-Cas systems in bacteria and archaea: versatile small RNAs for adaptive defense and regulation. *Annu. Rev. Genet.* **45**, 273 (2011). doi:10.1146/annurev-genet-110410-132430 Medline
19. E. Deltcheva *et al*., CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* **471**, 602 (2011). doi:10.1038/nature09886 Medline
20. R. Sapranauskas *et al*., The Streptococcus thermophilus CRISPR/Cas system provides immunity in Escherichia coli. *Nucleic Acids Res.* **39**, 9275 (2011). doi:10.1093/nar/gkr606 Medline
21. A. H. Magadán, M. E. Dupuis, M. Villion, S. Moineau, Cleavage of phage DNA by the Streptococcus thermophilus CRISPR3-Cas system. *PLoS ONE* **7**, e40913 (2012). doi:10.1371/journal.pone.0040913 Medline
22. H. Deveau *et al*., Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. *J. Bacteriol.* **190**, 1390 (2008). doi:10.1128/JB.01412-07 Medline
23. F. J. Mojica, C. Díez-Villaseñor, J. García-Martínez, C. Almendros, Short motif sequences determine the targets of the prokaryotic CRISPR defence system. *Microbiology* **155**, 733 (2009). doi:10.1099/mic.0.023960-0 Medline
24. M. Jinek, J. A. Doudna, A three-dimensional view of the molecular machinery of RNA interference. *Nature* **457**, 405 (2009). doi:10.1038/nature07755 Medline

25. C. D. Malone, G. J. Hannon, Small RNAs as guardians of the genome. *Cell* **136**, 656 (2009). doi:10.1016/j.cell.2009.01.045 Medline
26. G. Meister, T. Tuschl, Mechanisms of gene silencing by double-stranded RNA. *Nature* **431**, 343 (2004). doi:10.1038/nature02873 Medline
27. M. T. Certo *et al*., Tracking genome engineering outcome at individual DNA breakpoints. *Nat. Methods* **8**, 671 (2011). doi:10.1038/nmeth.1648 Medline
28. P. Mali *et al*., RNA-guided human genome engineering via Cas9. *Science*, published online 3 January 2013 (10.1126/science.1232033).
29. P. A. Carr, G. M. Church, Genome engineering. *Nat. Biotechnol.* **27**, 1151 (2009). doi:10.1038/nbt.1590 Medline
30. R. Barrangou, P. Horvath, CRISPR: new horizons in phage resistance and strain identification. *Annu Rev Food Sci Technol* **3**, 143 (2012). doi:10.1146/annurev-food-022811-101134 Medline
31. S. J. Brouns *et al*., Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* **321**, 960 (2008). doi:10.1126/science.1159689 Medline
32. D. Y. Guschin *et al*., A rapid and general assay for monitoring endogenous gene modification. *Methods Mol. Biol.* **649**, 247 (2010). doi:10.1007/978-1-60761-753-2_15 Medline

Acknowledgments: We thank the entire Zhang Lab for their support and advice; P.A. Sharp for generous help with Northern blot analysis; C. Jennings, R. Desimone, and M. Kowalczyk for helpful comments; and X. Ye for help with confocal imaging. L.C. and X.W. are HHMI International Student Research Fellows. D.C. is supported by MSTP. P.D.H is a James Mills Pierce Fellow. X.W. is supported by NIH grants R01-GM34277 and R01-CA133404 to Phillip A. Sharp, X.W.'s thesis advisor. L.A.M is supported by Searle Scholars, Rita Allen, an Irma T. Hirschl Award and a NIH Director's New Innovator Award (DP2AI104556). F.Z. is supported by a NIH Director's Pioneer Award (DP1MH100706), the Keck, McKnight, Gates, Damon Runyon, Searle Scholars, Klingenstein, and Simons Foundations, Bob Metcalfe, Mike Boylan, and Jane Pauley. The authors have no conflicting financial interests. A patent application has been filed relating to this work and the authors plan on making the reagents widely available to the academic community through Addgene and to provide software tools via the Zhang Lab website (http://www.genome-engineering.org).

Supplementary Materials
www.sciencemag.org/cgi/content/full/science.1231143/DC1
Materials and Methods
Figs. S1 to S8
Tables S1 and S2
References (*30*–*32*)

05 October 2012; accepted 12 December 2012
Published online 03 January 2013; 10.1126/science.1231143

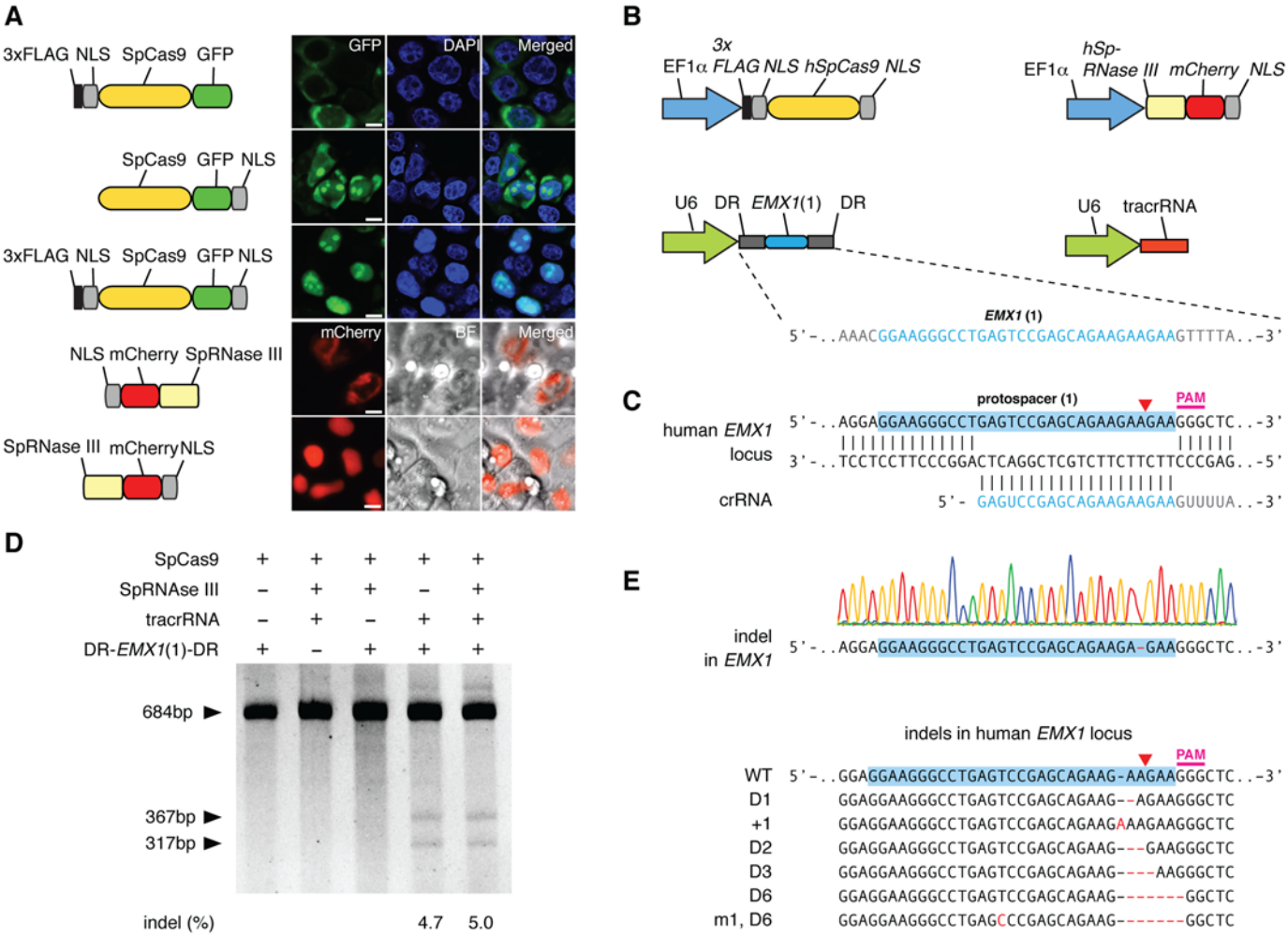

Fig. 1. The Type II CRISPR locus from *Streptococcus pyogenes* SF370 can be reconstituted in mammalian cells to facilitate targeted DSBs of DNA. (**A**) Engineering of SpCas9 and SpRNase III with NLSs enables import into the mammalian nucleus. (**B**) Mammalian expression of SpCas9 and SpRNase III are driven by the EF1a promoter, whereas tracrRNA and pre-crRNA array (DR-Spacer-DR) are driven by the U6 promoter. A protospacer (blue highlight) from the human *EMX1* locus with PAM is used as template for the spacer in the pre-crRNA array. (**C**) Schematic representation of base pairing between target locus and *EMX1*-targeting crRNA. Red arrow indicates putative cleavage site. (**D**) SURVEYOR assay for SpCas9-mediated indels. (**E**) An example chromatogram showing a micro-deletion, as well as representative sequences of mutated alleles identified from 187 clonal amplicons. Red dashes, deleted bases; red bases, insertions or mutations. Scale bar = 10μm.

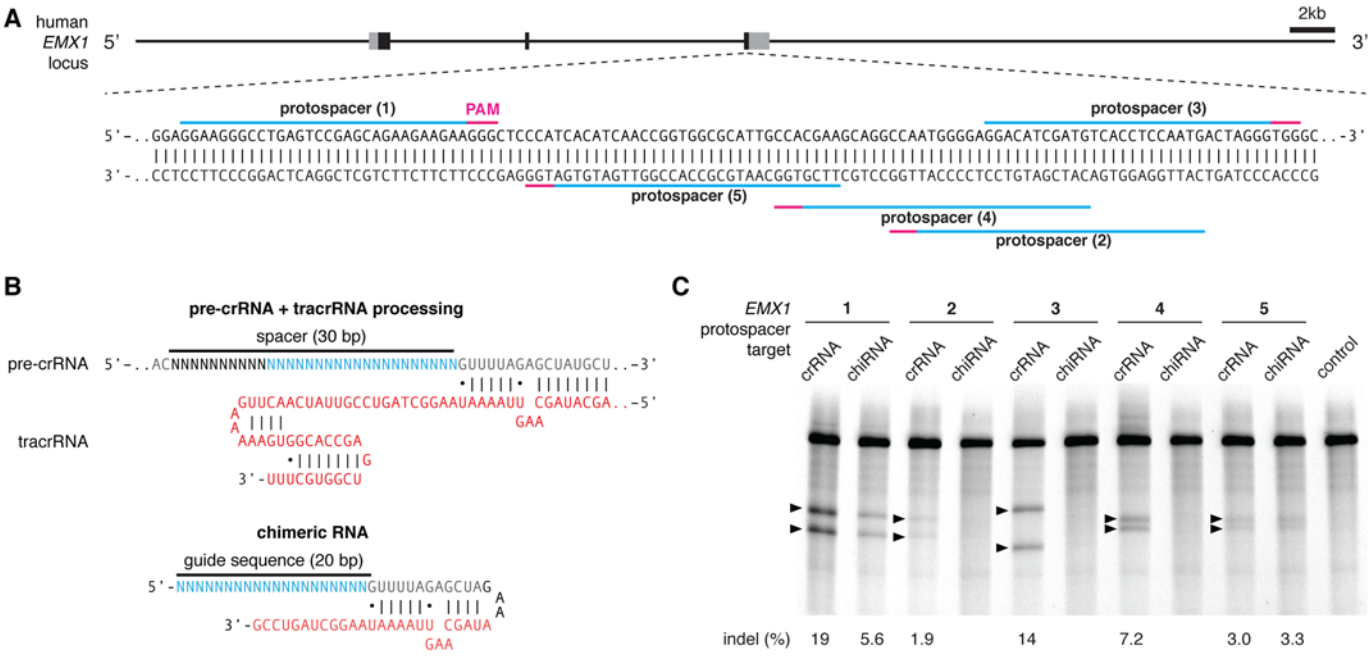

Fig. 2. SpCas9 can be reprogrammed to target multiple genomic loci in mammalian cells. (**A**) Schematic of the human *EMX1* locus showing the location of five protospacers, indicated by blue lines with corresponding PAM in magenta. (**B**) Schematic of the pre-crRNA/tracrRNA complex (top) showing hybridization between the direct repeat (gray) region of the pre-crRNA and tracrRNA. Schematic of a chimeric RNA design (*12*) (bottom). tracrRNA sequence is shown in red and the 20bp spacer sequence in blue. (**C**) SURVEYOR assay comparing the efficacy of Cas9-mediated cleavage at five protospacers in the human *EMX1* locus. Each protospacer is targeted using either processed pre-crRNA:tracrRNA complex (crRNA) or chimeric RNA (chiRNA).

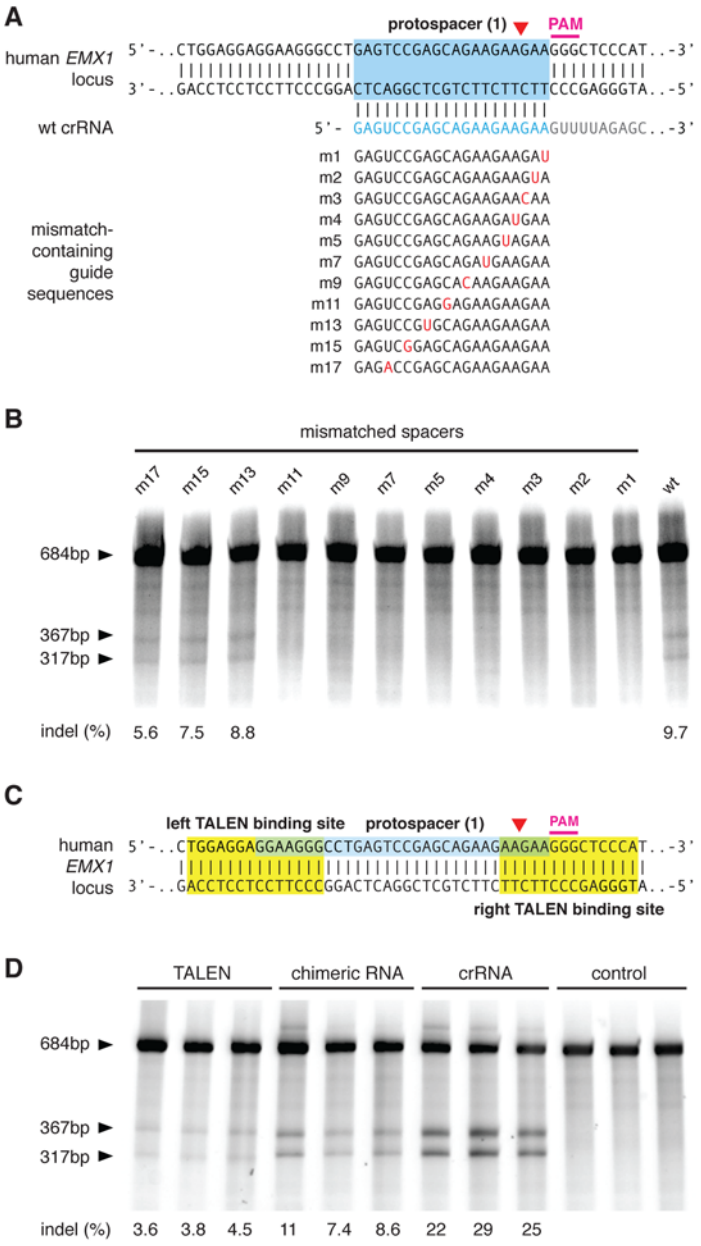

Fig. 3. Evaluation of the SpCas9 specificity and comparison of efficiency with TALENs. (**A**) *EMX1*-targeting chimeric crRNAs with single point mutations were generated to evaluate the effects of spacer-protospacer mismatches. (**B**) SURVEYOR assay comparing the cleavage efficiency of different mutant chimeric RNAs. (**C**) Schematic showing the design of TALENs targeting *EMX1*. (**D**) SURVEYOR gel comparing the efficiency of TALEN and SpCas9 (N = 3).

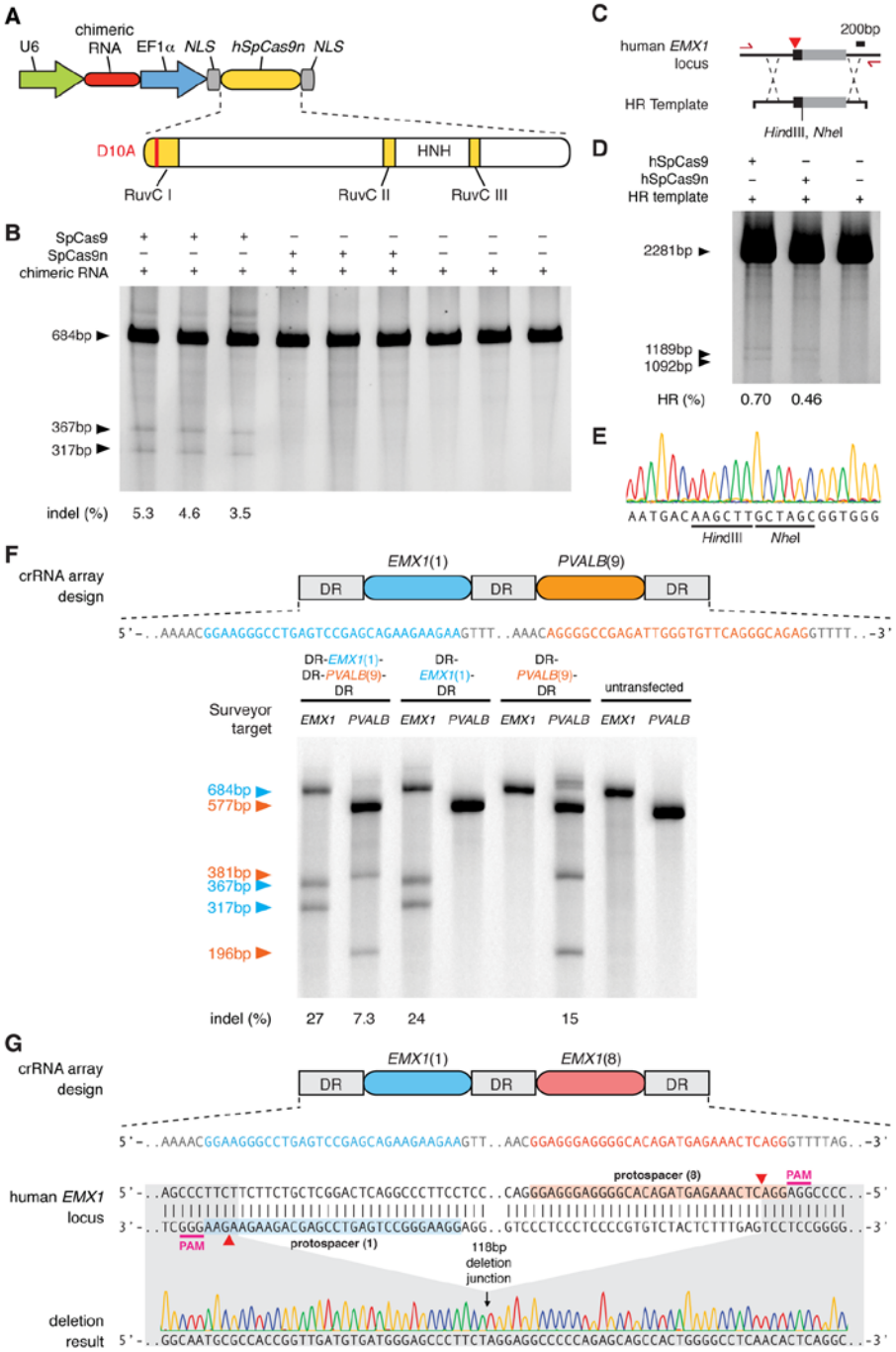

Fig. 4. Applications of Cas9 for homologous recombination and multiplex genome engineering. (**A**) Mutation of the RuvC I domain converts Cas9 into a nicking enzyme (SpCas9n) (**B**) Co-expression of *EMX1*-targeting chimeric RNA with SpCas9 leads to indels, whereas SpCas9n does not (N = 3). (**C**) Schematic representation of the recombination strategy. A repair template is designed to insert restriction sites into *EMX1* locus. Primers used to amplify the modified region are shown as red arrows. (**D**) Restriction fragments length polymorphism gel analysis. Arrows indicate fragments generated by *Hin*dIII digestion. (**E**) Example chromatogram showing successful recombination. (**F**) SpCas9 can facilitate multiplex genome modification using a crRNA array containing two spacers targeting *EMX1* and *PVALB*. Schematic showing the design of the crRNA array (top). Both spacers mediate efficient protospacer cleavage (bottom). (**G**) SpCas9 can be used to achieve precise genomic deletion. Two spacers targeting *EMX1* (top) mediated a 118bp genomic deletion (bottom).