The CRISPR-Cas9 system is a powerful tool to edit eukaryotic genomes that has recently been adapted for functional screens. Several of its applications—including the disruption of genes using Cas9-nickase and the generation of large deletions—require co-expression of two distinct guide RNAs (gRNAs). However, the lack of experimental approaches to generate pools of paired gRNA vectors prevents these applications from being scalable. Here we report a simple, inexpensive, one-step method that allows for the rapid and efficient cloning of gRNA pairs into expression vectors. We show that this method can be used to generate pooled libraries and is therefore suitable for in vivo and in vitro functional screens.
The CRISPR-Cas9 system for genome editing is a powerful tool for functional screens in vitro and in vivo.

Co-expression of the bacterial Cas9 endonuclease and a short guide RNA molecule (gRNA) is sufficient to generate double-stranded DNA breaks in eukaryotic cells. These cuts occur at sites that have a short (~20 nucleotides) homology to the 5' end of the gRNA and are followed by an NGG sequence—known as the protospacer-adjacent motif. Because target recognition by Cas9 requires only a short homology between the gRNA and the genomic locus, the CRISPR-Cas9 system is suitable for large scale loss of function screens. Libraries of vectors expressing gRNAs designed to target genes of interest can be easily and inexpensively generated in one cloning step starting from on-chip synthesized oligonucleotides pools, a strategy that has been successfully applied to the generation of RNAi libraries.

However, a growing number of applications of the CRISPR-Cas9 system require the simultaneous expression of two guide RNAs transcribed from independent promoters. For instance, a strategy that significantly reduces off-target mutations while retaining on-target cutting efficiency relies on the expression of a mutant Cas9 having one of the two nuclease domains disrupted (Cas9n; nickase) together with two gRNAs targeting off-set sites on opposite DNA strands. Because the sequences encoding the gRNA pairs need to be cloned downstream of independent promoters in the same plasmid, available strategies to rapidly generate pooled libraries cannot be used. This severely limits the scalability of this approach for functional screens. To overcome this limitation, we have developed a simple one-step method that allows the rapid and efficient cloning of specific gRNA-pairs into virtually any CRISPR-expression vector starting from pools of short oligonucleotides. This methods uses an intermediate circularization-linearization step that ensures that the two gRNAs are cloned downstream of independent U6 promoters in the final plasmid. Importantly, because the sequence of the two gRNAs paired in the dual-expression construct is determined at the oligonucleotide design step, correct pairing of gRNAs for the same genomic locus in the resulting plasmid is ensured by default in our experimental scheme.

We also show that lentiviral vectors expressing gRNA pairs from two identical U6 promoters are prone to recombination and consequent loss of the proximal gRNA. We overcome this problem by generating a novel lentiviral vector containing a human U6 promoter and a modified murine U6 promoter in which key regulatory sequences are replaced by the human equivalents.

By providing a simple, fast and inexpensive way to generate pooled library of paired gRNAs expressing vectors this method greatly expands the potential applications of the CRISPR technology for functional genomic studies.

Results

A single vector for co-delivery of Cas9 and paired gRNAs. To determine whether a pair of gRNAs can be effectively expressed from a single vector, we tested the editing efficiency of a Cas9 expression plasmid containing two tandem U6 promoters, each followed by unique cloning sites and a gRNA-scaffold sequence.

We designed two guide RNAs targeting two sites 760 bp apart on the murine Trp53 locus and sequentially cloned them...
downstream of the two U6 promoters (Fig. 1a). Northern blot analysis showed that this plasmid configuration leads to expression of the two guides at approximately equimolar ratios (Fig. 1b). Importantly, gRNAs expressed from a single vector were able to generate on-target indels at approximately the same frequency as gRNAs co-expressed from two different plasmids (Fig. 1c) and could generate the desired genomic deletion (Fig. 1d).

These data, together with previous work from our lab showing that tandem gRNA expression from a single vector can be used to engineer chromosomal rearrangements in vitro and in vivo18, demonstrate that a single plasmid expressing Cas9 and two guide RNAs can be used for efficient genome editing.

**A one-step cloning method for paired-gRNA vectors.** For the experiments described above, the two gRNA sequences were cloned sequentially in the recipient vector, a strategy that is incompatible with the generation of medium or large-scale pooled libraries. We therefore devised a method to simultaneously clone two guide RNAs starting from a short-(110 nt) DNA oligonucleotide (Fig. 2). The oligonucleotide contains the sequences corresponding to the two gRNAs separated by a short spacer harbouring two BbsI sites. At the 5’ and 3’ ends of the oligo are short sequences with homology to the U6 promoter and the gRNA scaffold, respectively. Amplification of the oligo by PCR using primers that bind to these regions generates a 148-bp dsDNA molecule that contains 40 bp homologies to the 3’ end of the U6 promoter and to the 5’ end of the gRNA scaffold (Fig. 2).

In addition, we generated a plasmid (pDonor) whose digestion with BbsI yields a 415 bp fragment consisting of a gRNA scaffold and a U6 promoter (Fig. 2, Supplementary Fig. 2). The presence of overlapping sequences at both ends of the Donor fragment and of the PCR product allows for their assembly into an intermediate circular molecule using the Gibson reaction20.

Digestion of this circular intermediate with BbsI produces a linear fragment with two distinct 5’ overhangs and allows for directional cloning into a variety of CRISPR-expression vectors9,13,21,22. This generates
a final plasmid in which the two gRNAs present in the oligonucleotide are cloned downstream of separate U6 promoters (Figs 1a, 2).

### Pooled cloning of gRNA pairs

To test whether this strategy was suitable for cloning pools of gRNA pairs, we designed oligos corresponding to nine different gRNA pairs (A-I; see Table 1) and pooled them at equimolar concentrations (Fig. 3a). We amplified the pool in a single-PCR reaction and ligated the resulting product to the U6:gRNA-scaffold fragment using the Gibson method\(^\text{20}\) (Fig. 3a). Linearization of the Gibson product with BbsI resulted in the expected 461 bp band (Fig. 3b), which was gel

### Table 1 | Guide RNA sequences encoded by the DNA oligos used in the pooled clonings.

| Oligo | Sequence of gRNA1 | Sequence of gRNA2 |
|-------|-------------------|-------------------|
| A     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| B     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| C     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| D     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| E     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| F     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| G     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| H     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |
| I     | 5'-GCACATGTCAGCTCTTCATATAAACTCTCA-3' | 5'-GATGCTTTAATCTACCTCTCA-3' |

gRNA, guide RNA.
purified and cloned into a linearized vector containing a U6 promoter and a gRNA scaffold. We reasoned that reducing the sequence identity between the two promoters should prevent recombination. We therefore generated two additional pDonor vectors carrying the murine U6 (mU6) promoter, or a synthetic mouse U6 (sU6) promoter harbouring regulatory sequence elements from hU6 (Supplementary Fig. 2). We then used these pDonor vectors to clone the gRNA pair into a recipient lentivirus containing the hU6 promoter, thus producing two new lentiviral constructs (Fig. 4a).

In contrast to cells infected with lentiviruses carrying two hU6 promoters, cells infected with lentiviruses expressing gRNAs from two different promoters displayed largely intact proviruses (Fig. 4c). Accordingly editing efficiency at the two cut sites was comparable to what observed when each gRNA was individually expressed (Fig. 4b), as were the expression levels of the gRNAs (Fig. 4d).

These results demonstrate that the use of two different promoters prevents lentiviral recombination and allows simultaneous editing at two sites. The series of pDonor plasmids we have generated can therefore be used to rapidly build paired gRNA libraries in a variety of currently available vectors.

**Discussion**

We describe here a novel method that allows the generation of pooled libraries of vectors containing paired gRNAs. The small size of the oligos that contain the gRNA sequences makes them compatible with ‘on-chip’ oligonucleotide synthesis, enabling...
the generation of large paired gRNA pooled libraries in a fast and cost-effective manner that is analogous to what has been previously described for shRNA and single gRNA screens.

In addition to providing a simple method to generate Cas9 nickase-based pooled libraries, the method described here extends the use of CRISPR-Cas9 screenings to a wider range of biological questions. It allows for instance the generation of deletion libraries against long-noncoding RNA genes, whose functions are unlikely to be compromised by the short insertions/deletions introduced by conventional single-guide CRISPR libraries. It also allows the generation of pooled libraries to delete chromosomal regions that are recurrently lost in human cancers, as well as the systematic functional characterization of DNA-regulatory elements.

**Methods**

**DNA constructs.** pDonor plasmids were generated by cloning the Donor fragment (using BsaI and NotI restriction sites) into pBluescript KS+ vector (pBluescript KS+ vector). The u6 promoter was generated by replacing the regulatory elements of mU6 for those of hU6 (that is, the Octamer motif, the Proximal sequence element and the TATA-box). See Supplementary Fig. 2a for a schematic representation of these plasmids. The sequences contained by replacing the regulatory elements of mU6 for those of hU6 (that is, the Octamer motif, the Proximal sequence element and the TATA-box). See Supplementary Fig. 2a for a schematic representation of these plasmids. The sequences contained by replacing the regulatory elements of mU6 for those of hU6 (that is, the Octamer motif, the Proximal sequence element and the TATA-box).

**Paired gRNA cloning.** For the pooled cloning oligonucleotides were mixed at equilibrium concentrations and amplified with phusion polymerase (New England Biolabs) using primers that add homology regions to the 3'-region of the hU6 promoter (primer F1) and to the 5'-region of the hU6 scaffold (primer R1). The gene-purified 148-bp ampiclon was ligated to the 415-bp Donor fragment—generated by BstI digestion of the pDonor plasmid—in a 3:1 molar ratio, using the Gibson Assembly Master Mix (New England Biolabs; 1 h at 50 °C). The Gibson reaction was treated with Plasmid Safe exonuclease (Epicenter) for 1 h at 37 °C to remove unligated fragments, column purified (QiAquick PCR purification kit; Qiagen), and digested with BstI at 37 °C for 3 h. The linearized 461 bp fragment was gel purified and cloned into BsaI and NotI sites of the lentiCRISPR vector (Addgene plasmid 49535). For 10 bacterial clones, correct assembly was confirmed by digestion of plasmid DNA with NotI and EcoRI enzymes. Sequencing of vectors was done using the F2/R2 primer set. Cloning of the paired lentivirus vectors carrying distinct pol III promoters, was done as described above, but we used modified oligos and primers (Supplementary Fig. 2b and Supplementary Table 1). A list of all primer sequences and primer pairs (including corresponding amplicon sizes) used throughout this study is provided in Supplementary Tables 1 and 2.

**Cell culture.** Cells were cultured at 37 °C (5% CO2) in DMEM-HG supplemented with 10% FCS, L-glutamine (2 mM), penicillin (100 U ml−1) and streptomycin (100 μg ml−1). Cells were split after seeding (1 × 103 cells per well; 6-well plate), cells were transfected with 4 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. NIH3T3 cells (ATCC; CRL-1658) were collected for further analysis. A detailed step-by-step protocol for the generation of paired gRNA libraries is provided as a Supplemental Method.

**Northern blot analysis and detection of genomic editing.** To detect gRNA expression, transduced cells were collected in TRIZOL (invitrogen) and total RNA was isolated according to manufacturer’s protocols. For each sample, 10 μg of RNA were resolved in a 15% Urea-PAGE gel and blotted onto a Hybond-N+ nylon membrane (GE Healthcare). Membranes were ultraviolet-cross-linked and hybridized overnight with 32P-labelled probes against the 5'-region of each gRNA (gRNA1 probe, TTTGAGGCCTCTGCAGGGTGC: gRNA2 probe, CCTTTCGCACACTTCCTGCT) and against mU6 as a loading control (GCCAGGGCGATGCTCTTTCTGCTT). For detection of genomic deletions and indels, cells were collected in lysis buffer (100 μM Tris-HCl pH8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS and 100 ng ml−1 proteinase K) and incubated at 55 °C for 4 h. Genomic DNA was extracted with phenol-chloroform followed by ethanol precipitation and amplified by PCR with Phusion polymerase (New England Biolabs). Detection of the genomic deletion was done using primers that flank the gRNA target sites (F3/R3), which leads to the amplification of a ~1-kb band in cells carrying a wild-type locus, and a 340-bp band in cells carrying the deletion (Supplementary Fig. 3a). Sequencing of the genomic deletion was done after cloning the corresponding amplicon into Topo Blunt II vector (Invitrogen). For the detection of indel formation DNA was amplified with primers that flank the cut sites (5'cut with primers F3/R4; 3'cut with primers F4/R3) followed by generation of DNA heteroduplexes and DNA digestion with the mismatch-sensitive SURVEYOR nuclease (Transgenomic) according to manufacturer’s instructions. Digestion fragments were resolved on a 2.5% agarose gel (Supplementary Fig. 3d).

**References**

1. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821 (2012).
2. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).
3. Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature 507, 62–67 (2014).
4. Mao, Z., Bozzella, M., Seluanov, A. & Gorbonova, V. DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells. Cell Cycle 7, 2902–2906 (2008).
5. Sanchez-Rivera, F. J. et al. Rapid modeling of cooperating genetic events in cancer through somatic genome editing. Nature 516, 428–431 (2014).
6. Wang, H. et al. One-step generation of mice carrying mutations in multiple genes by CRISPR/Cas-mediated genome engineering. Cell 153, 910–918 (2013).
7. Swiech, L. et al. In vivo interrogation of gene function in the mammalian brain using CRISPR-Cas9. Nat. Biotechnol. 33, 102–106 (2014).
8. Zhou, Y. et al. High-throughput screening of a CRISPR/Cas9 library for functional genomics in human cells. Nature 509, 487–491 (2014).
9. Shalem, O. et al. Genome-scale CRISPR-Cas9 knock out screening in human cells. Science 343, 84–87 (2014).
10. 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).
11. Platt, R. J. et al. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. Cell 159, 440–455 (2014).
12. Jao, L. E., Wente, S. R. & Chen, W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. Proc. Natl Acad. Sci. USA 110, 13904–13909 (2013).
13. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819–823 (2013).
14. Zuber, J. et al. Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. Nat. Biotechnol. 29, 79–83 (2011).
15. Ran, F. A. et al. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell 154, 1380–1389 (2013).
16. Izar, B. et al. Efficient genome modification by CRISPR/Cas9 nickase with minimal off-target effects. Nat. Methods. 11, 399–402 (2014).
17. Yang, H. et al. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. Cell 154, 1370–1379 (2013).
18. Maddalo, D. et al. In vivo engineering of oncogenic chromosomal rearrangements with the CRISPR/Cas9 system. Nature 516, 423–427 (2014).
19. Han, J. et al. Efficient in vivo deletion of a large imprinted IncRNA by CRISPR/Cas9. RNA Biol. 11, 829–835 (2014).
20. Gibson, D. G. et al. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods. 6, 343–345 (2009).
21. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308 (2013).
22. Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for CRISPR screening. Nat. Methods. 11, 783–784 (2014).
23. Cleary, M. A. et al. Production of complex nucleic acid libraries using highly parallel in situ oligonucleotide synthesis. Nat. Methods. 1, 241–248 (2004).

**Acknowledgements**

We are grateful to the members of the Ventura lab for helpful discussions, in particular Ciro Bonetti and Danilo Maddalo for insightful comments on the cloning strategy, Paul Ogrodowski for providing technical support and Jennifer Hollenstein for editing the manuscript. We would also like to acknowledge an anonymous reviewer of the manuscript for suggesting the possibility of recombination among identical U6 promoters. This work was funded by a grant from the Geoffrey Beene Cancer Research Foundation and by a NIH Core Grant (P30 CA008748).

**Author contributions**

J.A.V. and A.V. conceived the project. J.A.V. designed and performed the experiments. J.A.V. and A.V. wrote the manuscript.
Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Vidigal, J. A. & Ventura, A. Rapid and efficient one-step generation of paired gRNA CRISPR-Cas9 libraries. Nat. Commun. 6:8083 doi: 10.1038/ncomms9083 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/