Expansion of the CRISPR–Cas9 genome targeting space through the use of H1 promoter-expressed guide RNAs

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The repurposed CRISPR-Cas9 system has recently emerged as a revolutionary genome-editing tool. Here we report a modification in the expression of the guide RNA (gRNA) required for targeting that greatly expands the targetable genome. gRNA expression through the commonly used U6 promoter requires a guanosine nucleotide to initiate transcription, thus constraining genomic-targeting sites to GN19NGG. We demonstrate the ability to modify endogenous genes using H1 promoter-expressed gRNAs, which can be used to target both AN19NGG and GN19NGG genomic sites. AN19NGG sites occur ~15% more frequently than GN19NGG sites in the human genome and the increase in targeting space is also enriched at human genes and disease loci. Together, our results enhance the versatility of the CRISPR technology by more than doubling the number of targetable sites within the human genome and other eukaryotic species.
Genome-editing technologies such as zinc-finger nucleases (ZFNs)\textsuperscript{1–4} and transcription activator-like effector nucleases (TALENs)\textsuperscript{4–10} have empowered the ability to generate targeted genome modifications and offer the potential to correct disease mutations with precision. While effective, these technologies are encumbered by practical limitations as both ZFNs and TALENs pair require synthesizing large and unique recognition proteins for a given DNA target site. Several groups have recently reported high-efficiency genome editing through the use of an engineered type II CRISPR/Cas9 system that circumvents these key limitations\textsuperscript{11–15}. Unlike ZFNs and TALENs, which are relatively time consuming and arduous to make, the CRISPR constructs, which rely upon the nuclease activity of the Cas9 protein coupled with a synthetic guide RNA (gRNA), are simple and fast to synthesize and can be multiplexed. However, despite the relative ease of their synthesis, CRISPRs have technological restrictions related to their access to targetable genome space, which is a function of both the properties of Cas9 itself and the synthesis of its gRNA.

Cleavage by the CRISPR system requires complementary base pairing of the gRNA to a 20-nucleotide DNA sequence and the requisite protospacer-adjacent motif (PAM), a short nucleotide motif found \textsuperscript{3′} to the target site\textsuperscript{16}. One can, theoretically, target any unique N\textsubscript{20}PAM sequence in the genome using the CRISPR/Cas9 system that circumvents these key limitations\textsuperscript{11–15}. Unlike ZFNs and TALENs, which are relatively time consuming and arduous to make, the CRISPR constructs, which rely upon the nuclease activity of the Cas9 protein coupled with a synthetic guide RNA (gRNA), are simple and fast to synthesize and can be multiplexed. However, despite the relative ease of their synthesis, CRISPRs have technological restrictions related to their access to targetable genome space, which is a function of both the properties of Cas9 itself and the synthesis of its gRNA.

An expanded CRISPR-targeting space. To determine the potential increase in targeting space, we performed bioinformatic analysis to determine the available CRISPR sites in the human genome. While AN\textsubscript{19}NGG sites might be predicted to occur roughly at the same frequency as GN\textsubscript{19}NGG sites, we found that they are actually 15% more common (Fig. 2; Supplementary Fig. 3); thus changing specificity from GN\textsubscript{19}NGG to RN\textsubscript{19}NGG more than doubles the number of available sites. With a few exceptions, (chr16, chr17, chr19, chr20 and chr22) AN\textsubscript{19}NGG sites are present at higher frequencies than GN\textsubscript{19}NGG sites on each chromosome. To compare the average genome-wide targeting densities, we calculated the mean distances between adjacent CRISPR sites in the genome for GN\textsubscript{19}NGG (59 bp), AN\textsubscript{19}NGG (47 bp) and RN\textsubscript{19}NGG sites (26 bp) (Fig. 2b). In addition, AN\textsubscript{19}NGG sites were even more enriched at relevant regions of targeting in the human genome. We found a 20% increase in AN\textsubscript{19}NGG sites in human genes, and a 21% increase at disease loci obtained from the OMIM database (Fig. 2c). We also examined 1,165 micro RNA genes from the human genome and found that 221 of these genes could be targeted through one or more AN\textsubscript{19}NGG sites, but not through a GN\textsubscript{19}NGG site (data not shown). Given that the efficiency of homologous recombination negatively correlates with increasing distance from cut sites, the increase in CRISPR-targeting sites by the use of the H1 promoter should facilitate more precise genomic targeting and mutation correction\textsuperscript{24}.

As CRISPR technology is increasingly utilized for genomic engineering across a wide array of model organisms, we sought to determine the potential impact of the use of the H1 promoter in other genomes. We carried out this analysis on five other vertebrate genomes that had high genomic conservation at the H1 promoter (mouse; rat; chicken; cow; and zebrafish). In all cases, we found a higher number of AN\textsubscript{19}NGG compared with GN\textsubscript{19}NGG sites: +9% cow; +14% chicken; +19% rat; +21% mouse; and +32% zebrafish (Fig. 2c). One explanation for this prevalence could be due to the higher AT content.
In the human genome, normalizing the GN_{19}NGG and AN_{19}NGG site occurrences to AT content brings the frequencies closer to parity, although this does not hold true for all genomes (Supplementary Fig. 4a,f). Nevertheless, this demonstrates the utility of using the H1 promoter, which more than doubles the currently available CRISPR-targeting space in the human genome, and similarly in all other genomes tested.

Targeting endogenous sites with the H1 promoter construct.

We next sought to demonstrate the ability to target an AN_{19}NGG site in an endogenous gene with the H1 promoter construct. Using H7 cells, we targeted the second exon of the MERTK locus, a gene involved with phagocytosis in the retinal pigment epithelium and macrophages and that when mutated causes retinal degeneration. 25 (Fig. 3a,b). To estimate the overall

Figure 1 | Evaluating the ability to direct CRISPR targeting via gRNA synthesis from the H1 promoter. (a) Schematic illustration depicting the gRNA expression constructs. Above, the U6 promoter only expresses gRNAs with a +1 guanosine nucleotide; below, the H1 promoter can drive expression of gRNAs initiating at either purine (adenosine or guanosine) nucleotide. On the right, a cartoon depiction of the Cas9 protein with gRNA targeting genomic sequence AN_{19}NGG. The location of the +1 A is indicated. (b) Schematic overview of the enhanced GFP (eGFP)-targeted disruption assay. eGFP fluorescence is disrupted by CRISPR targeting followed by error-prone NHEJ-mediated repair resulting in frameshift mutations that disrupt the coding sequence, resulting in loss of fluorescence. (c) Microscope images demonstrating successful CRISPR targeting by U6 or H1 promoter-expressed gRNAs. H7 ES cells were stained and colonies were visualized to show nuclei (left, magenta), eGFP fluorescence (middle, green) and merged images (right) indicating areas of GFP fluorescence mosaicism in the colony. To the right is shown the quantification of eGFP fluorescence loss by flow cytometry for the respective constructs. Below is a higher magnification of an H7 colony targeted by an H1-expressed gRNA showing expression mosaicism. Scale bar, 50 μm. (d) Surveyor assay-based quantitation of the frequency of NHEJ. Bioanalyzer gel image depicting control (first lane), U6-expressed gRNA (second lane), H1-expressed gRNA (third lane) and marker (fourth lane). The % indel (as calculated by the fraction of uncut (u) to cut (c) bands) is indicated below.
targeting efficiency, we harvested genomic DNA from a population of cells that were electroporated, and performed the Surveyor assay. We amplified the region surrounding the target sites with two independent PCR reactions and calculated a 9.5 and 9.7% indel frequency (Fig. 3b). Next, 42 randomly chosen clones were isolated and tested for mutation by Surveyor analysis.
Abcam ab52968 (1:10,000).

Figure 3 | CRISPR targeting of AN19NGG at an endogenous gene (MERTK) in H7 ES cells. (a) Schematic diagram of the MERTK locus and various protein domains. Target site in exon 2 is shown below in larger scale, indicating the CRISPR AN19NGG target site. (b) Quantification of CRISPR targeting at exon2 by the Surveyor assay. The CRISPR site in exon 2 is depicted above, with the various primers (arrows) used in the Surveyor assay; both F1:R1 and F2:R2 span the target site, while the control PCR product, F3:R3, is just outside the target site. The gel from the Surveyor assay is shown below with the three control products shown on the left, and targeting is shown on the right. Below the % indel frequency is indicated. (c) Sanger sequencing of mutant lines. Clonal lines were isolated and sequenced indicating that CRISPR targeting at the AN19NGG sites resulted in mutagenesis at this region. The aligned chromatograms show the six unique mutations that were cloned. (d) Western blot analysis for Mertk expression in H7-derived retinal pigment epithelium cells. Lanes 1, 3 and 4 indicate knockout lines and lane 2 indicates expression from heterozygous line. Rabbit monoclonal anti-MERTK IgG: Abcam ab52968 (1:10,000).

(data not shown). Sequencing revealed that 7/42 (16.7%) harboured mutations clustering within 3–4 nucleotides upstream of the target PAM site. Clones (6/7) had unique mutations (1 clone was redundant) and 3 of these were bi-allelic frame-shift mutations resulting in a predicted null MERTK allele that was confirmed by western blot analysis (Fig. 3c,d). Taken together, these results demonstrate the ability to effectively target an AN19NGG site located at an endogenous locus.

To quantitatively determine the extent of off-targeting that occurred from the GFP gRNA constructs, we used Surveyor analysis to examine three genomic loci that were bioinformatically predicted to be off-target sites (GFP_11-33, GFP_219-197 and GFP_315-293). Two of these constructs (GFP_219-197 and GFP_315-293) were GN19NGG target sites, allowing for expression with both promoters. One (GFP_11-33), an AN19NGG site, was expressed from the U6 promoter by appending a 5′-G nucleotide. In all three off-target loci we examined, we were unable to detect any off-target cleavage (data not shown). However, the lack of detectable off-targets could result from our initial selection of the GFP gRNA targets, in which sites were selected based upon low homology to other genomic loci. Thus, we reasoned that a more stringent challenge would be to compare gRNA expression from H1 and U6 promoters at targeting sites specifically known to elicit high levels of off-target hits.26–28. Furthermore, the 5′ nucleotide flexibility of the H1 promoter allowed for a direct comparison of identical gRNAs targeting GN19NGG sites between U6 and H1 promoters, and we tested two sites previously reported from Fu et al.26; VEGFA site 1 (T1) and VEGFA site 3 (T3) (Table 1; Supplementary Fig. 5).26,28 An additional benefit of the H1 promoter over the U6 promoter may be in increasing specificity by reducing spurious cleavage. Because increased gRNA and Cas9 concentrations have been shown to result in increased off-target hits,26,27,29 we reasoned that the lower gRNA expression level from the H1 promoter30–32 might also reduce off-target effects. Using quantitative (q) reverse transcriptase (RT)-PCR, we tested the levels of the VEGFA-T1 gRNA from either the H1 and U6 promoter, confirming the reduced level of expression of the gRNA (Supplementary Fig. 5a). For the VEGFA T1 site, we tested the efficiency of cutting at the on-target loci, as well as four off-target loci. In comparison with the U6 promoter, cutting at the on-target loci was comparable or slightly reduced; however, the H1 promoter-expressed gRNAs were notable more stringent at the examined off-target loci indicating greater specificity (off-target 1: 8 versus 25%; off-target 2: undetectable versus 20%; and off-target 4: 9 versus 26%) (Table 1; Supplementary Fig. 5). We detected equal targeting between the two promoter constructs at the VEGFA T3 site (26%), but again, lower levels of off-target cutting with the H1 promoter (Table 1; Supplementary Fig. 5). While further studies on H1 and U6 promoters expressed gRNAs need to be performed, our data suggest greater specificity from H1-expressed gRNAs.

Discussion

Accumulating evidence for S. pyogenes Cas9 targeting in vitro and in vivo, indicates that the Cas9:gRNA recognition extends throughout the entire 20-base pair targeting site. First, in testing >10^12 distinct variants for gRNA specificity in vitro, one study found that the +1 nucleotide plays a role in target recognition. Furthermore, positional specificity calculations from this data show that the 5′ nucleotide contributes a greater role in target recognition than its 3′ neighbour, indicating that the ‘seed’ model for CRISPR specificity might overly simplify the contribution of PAM-proximal nucleotides.27. Second, alternative uses such as CRISPR interference, which repurposes the CRISPR system for...
transcriptional repression, found that 5' truncations in the gRNA severely compromised repression, and 5' extensions with mismatched nucleotides—such as mismatched G bases for U6 expression—also reduce the repression efficiency, suggesting that both length (20 nt) and 5' nucleotide context are important for proper Cas9 targeting\(^{24,33-36}\). Finally, crystal structure data further supports the experimental data and importance of the 5' nucleotide in Cas9, as significant contacts are made with the 5' nucleotide of the gRNA and 3' end of the target DNA\(^{37,38}\).

For increased targeting space, the use of alternate Cas9 proteins has been shown to be effective, as in Neisseria meningitidis and S. thermophilus, yet PAM restrictions from other type II systems reported, so far have more stringent requirements and therefore reduce the sequence space available for targeting when used alone (data not shown and refs 11,17). In contrast, modified gRNA expression by use of the H1 promoter would be expected to greatly expand the targeting repertoire with any Cas9 protein irrespective of PAM differences. When we quantitated the great expansion of targeting space through use of the H1 promoter with several groups have reported that increased gRNA and Cas9 targeting densities coupled with high-fidelity target recognition will be paramount to delivering safe and effective therapeutics.

**Methods**

**Plasmid construction.** To generate the H1 gRNA-expressing construct, overlapping oligos were assembled to create the H1 promoter fused to the 76-bp gRNA sequence. The H1::gRNA scaffold::pol III terminator sequence was then TOPO cloned into pCR4-Blunt (Invitrogen), and sequenced verified; the resulting vector is

| Target | Promoter | Full-length target | Indel mutation frequency |
|--------|----------|--------------------|--------------------------|
| VEGFA-T1 | U6 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 24% |
| VEGFA-T1 | H1 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 16% |
| OT1-3 | U6 | 5'-GGGAGGAGGGAGTTGGCTCCGCGG-3' | 25% |
| OT1-3 | H1 | 5'-GGGAGGAGGGAGTTGGCTCCGCGG-3' | 8% |
| OT1-4 | U6 | 5'-GGGGAGGAGGGAGTTGGCTCCGCGG-3' | 20% |
| OT1-4 | H1 | 5'-GGGGAGGAGGGAGTTGGCTCCGCGG-3' | Not detected |
| OT1-6 | U6 | 5'-GGGGAGGAGGGAGTTGGCTCCGCGG-3' | Not detected |
| OT1-6 | H1 | 5'-GGGGAGGAGGGAGTTGGCTCCGCGG-3' | Not detected |
| OT1-11 | U6 | 5'-GGGAGGAGGGAGTTGGCTCCGCGG-3' | 26% |
| OT1-11 | H1 | 5'-GGGAGGAGGGAGTTGGCTCCGCGG-3' | 9% |
| VEGFA-T3 | U6 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 26% |
| VEGFA-T3 | H1 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 26% |
| OT3-1 | U6 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 20% |
| OT3-1 | H1 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 13% |
| OT3-4 | U6 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 16% |
| OT3-4 | H1 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | 11% |
| OT3-18 | U6 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | Not detected |
| OT3-18 | H1 | 5'-GGGGTGCGGAGTTGCCTCCGCGG-3' | Not detected |

\(^{gRNAs, guide RNAs. Mismatches between the on-target and off-target site are indicated in bold.}\)

\(^{\text{Table 1 | Frequency of indels induced at on-target and off-target sites by U6- or H1-expressed gRNAs.}}\)
Gene targeting of H7 cells. hESCs were cultured in 10 mM Rho Kinase inhibitor (DDDS003325 EMD Millipore) 24 h before electroporation. Electroporation were performed using the Neon kit (Invitrogen), according to the manufacturer’s instruction. Briefly, on the day of electroporation, hESC were digested with Accutase (Sigma) for 1–2 min until colonies lifted. Importantly, colonies were not dissociated into a single-cell suspension. After colonies were harvested, wet pellets were kept on ice for 15 min, and then resuspended in electroporation buffer containing 200 mM GlutaMAX. Electroporation parameters were as following: voltage: 1,400 ms; interval: 30 ms; 1 pulse. Following electroporation, cell colonies were slowly transferred to mTeSR1 medium containing 10 mM Rho Kinase inhibitor, and then kept at room temperature for 20 min before plating on Matrigel-coated dishes and further cultured.

For analysis of double-derived colonies, electroporated hESCs were grown to sub-confluence, passaged as described in the previous paragraph and plated at a density of 500 cells per 35 mm dish. Subsequently, single colonies were isolated by manual picking and further cultured.

For 293T cell transfection, ~100,000 cells per well were seeded in 24-well plates (Falcon) 24 h before transfection. Cells were transfected in quadruplicate using Lipofectamine LTX Plus Reagent (Invitrogen) according to the manufacturer’s recommended protocol. For each well of a 24-well plate, 400 ng of the Cas9 plasmid and 200 ng of the gRNA plasmid were mixed with 0.5 µl of Plus Reagent and 1.5 µl of Lipofectamine LTX reagent.

Quantitative real-time PCR. 293T cells were seeded at 250,000 cells per well in 12-well plates (Falcon) 24 h before transfection. Cells were transfected in triplicate using Lipofectamine LTX with Plus Reagent (Invitrogen) according to the manufacturer’s recommended protocol with a six-dose titration of the gRNA plasmid: 0, 31.25, 62.5, 125, 250 or 500 ng in each well. Forty-eight hours post transfection, total RNA was isolated using RNAzol RT (Molecular Research Center), and purified using direct zol RNA MiniPrep (Zymo). Total RNA (500 ng) was used to remove residual genomic DNA contamination and reverse transcribed in a 20-µl reaction using Superscript III reverse transcriptase (Invitrogen) following the manufacturer’s recommendations. For each reaction, 0.1 µl of the following oligo-dT primers were used to prime each reaction; gRNA scaffold-5’-CTCCTGATGTGACCTGACGACTGTCGACAC3’ and Untranslated RNA Reference-5’-ACGCTGTCGACACACATATACATCTC-3’ (U6 snoRNA-5’-AAATATGGACGCTCAGGAATTGTG-3’). The underlined scaffold sequence anchors an end sequence added for transcript stability. Each qPCR reaction was carried out in a Bio-Rad CFX 96 real-time PCR machine in a 10-µl volume using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) containing 250 nM of an oligonucleotide primer and 1 µl of a 1:5 dilution of the RT reaction product from above. Reactions were carried out for 40 cycles with 95°C denaturation, 54°C annealing temperature and 60°C extension steps. The following primers were used for detecting the gRNA and reference gene, respectively: F1505-GTTTGGAGAGGAGGTGAATCTTG and R1501-CTTCCGTGCAGGCACATATACATCTC. Relative normalized expression for each gRNA sample and the s.e.m. was calculated using Bio-Rad’s integrated CFX manager software.

Flow cytometry. Following blebbistatin treatment, sub-confluent hESC colonies were harvested by Accutase treatment, dissociated into a single-cell suspension and pelleted cells were then resuspended in Live Cell Solution (Invitrogen) containing Vybrant DyeCycle ruby stain (Invitrogen) and analysed on an Accuri C6 flow cytometer.

Biographies. To determine all the potential CRISPR sites in the human genome, we used a custom Perl script to search both strands and overlapping occurrences of the 23-mer CRISPR sequence sites on the human genome. To calculate the mean and median distance values, we first defined the predicted CRISPR cut site as occurring between the third and fourth bases upstream of the PAM sequence. After sorting the sequences, we then calculated the distances between all adjacent gRNAs in the genome. This data were imported into R to calculate the mean and median statistical values, and to plot the data. To calculate the mean density, the gRNA cut sites were binned across the genome and calculated for the frequency of occurrences. These data were plotted in R using the ggplot2 package, or used Circos to generate a circular plot. To calculate the occurrences in human genes or at disease loci, we used BEDTools utility intersectBed to find the occurrence of overlaps with either a ReSeq BED file retrieved from the UCSC Genome Browser or a BED file from OMIM (Online Mendelian Inheritance in Man, OMIM). McKeon-Niethans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD, USA). 2013. World Wide Web URL: http://omim.org/). As a reference, on average, TALEN targeting sites are expected to occur every 35–38 base pairs, and RNAi sites occur every 100–120 base pairs. The genomes used in this study were human (hg19), mouse (mm10), rat (rn6), cow (bosTa7), chicken (galG4), zebrafish (drf), drosophila (dm3), C. elegans (ce10) and S. cerevisiae (SacCer3).

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Author contributions
V.R. conceived the study, designed the experiments and analysed the data with input from D.J.Z. V.R. generated the constructs and performed the biochemistry. V.R. and J.M. performed the cell-culture work and flow cytometry. K.W. generated and validated the integrated reporter lines used in this study and performed the qRT-PCR experiment with V.R. V.R. performed the bioinformatics and statistical analysis. V.R. wrote the paper with input from D.J.Z.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: Johns Hopkins University has filed a patent application on use of the technology described in this manuscript. V.R. and D.J.Z. are listed as inventors on this application.

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