Cas9 Variants Expand the Target Repertoire in *Caenorhabditis elegans*

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ABSTRACT The proliferation of CRISPR/Cas9-based methods in *Caenorhabditis elegans* has enabled efficient genome editing and precise genomic tethering of Cas9 fusion proteins. Experimental designs using CRISPR/Cas9 are currently limited by the need for a protospacer adjacent motif (PAM) in the target with the sequence NGG. Here we report the characterization of two modified Cas9 proteins in *C. elegans* that recognize NGA and NGCG PAMs. We found that each variant could stimulate homologous recombination with a donor template at multiple loci and that PAM specificity was comparable to that of wild-type Cas9. To directly compare effectiveness, we used CRISPR/Cas9 genome editing to generate a set of assay strains with a common single-guide RNA (sgRNA) target sequence, but that differ in the juxtaposed PAM (NGG, NGA, or NGCG). In this controlled setting, we determined that the NGA PAM Cas9 variant can be as effective as wild-type Cas9. We similarly edited a genomic target to study the influence of the base following the NGA PAM. Using four strains with four NGAN PAMs differing only at the fourth position and adjacent to the same sgRNA target, we observed that efficient homologous replacement was attainable with any base in the fourth position, with an NGAG PAM being the most effective. In addition to demonstrating the utility of two Cas9 mutants in *C. elegans* and providing reagents that permit CRISPR/Cas9 experiments with fewer restrictions on potential targets, we established a means to benchmark the efficiency of different Cas9:PAM combinations that avoids variations owing to differences in the sgRNA sequence.

KEYWORDS alternate PAMs; *C. elegans*; CRISPR/Cas9; genome editing; VQR Cas9

CHARACTERIZATION of the type II CRISPR/Cas bacterial immunity system has provided a powerful and efficient means to tailor the genomes of a wide variety of organisms (Cong *et al.* 2013; DiCarlo *et al.* 2013; Dickinson *et al.* 2013; Friedland *et al.* 2013; Gratz *et al.* 2013; Li *et al.* 2013; Yang *et al.* 2014). The Cas9 endonuclease can be programmed by a readily modified single-guide RNA (sgRNA) to cleave a target DNA sequence (Jinek *et al.* 2012). *In vivo*, this activity can result in imperfect repair by nonhomologous end joining (NHEJ), often creating a null allele of a target gene. When a homologous donor template is provided, desired modifications can be precisely introduced through homologous recombination (HR). The affinity of Cas9 for targeted sequences also has been harnessed to directly influence gene expression. The use of

catalytically inactive Cas9 fused to transcriptional activators, repressors, or histone-modifying enzymes has shown that the full potential of CRISPR/Cas9 techniques is still being realized (Gilbert *et al.* 2013; Mali *et al.* 2013; Hilton *et al.* 2015; Kearns *et al.* 2015; Long *et al.* 2015).

All these methods share the requirement of a protospacer adjacent motif (PAM) in the target (Jinek *et al.* 2012). The NGG PAM required by the widely used Cas9 from *Streptococcus pyogenes* is relatively abundant in randomly composed DNA but is less common in AT-rich genomes, imposing a significant constraint. Recent studies have explored the utility of Cas9s from other bacterial species with diverse PAM specificities, as well as the directed evolution of the *S. pyogenes* Cas9 coding sequence to select mutants that recognize alternate PAMs (Esvelt *et al.* 2013; Kleinstiver *et al.* 2015; Ran *et al.* 2015).

Several groups have developed effective protocols using CRISPR/Cas9 and additional tools for the introduction of specific mutations into the *Caenorhabditis elegans* genome (Dickinson *et al.* 2013, 2015; Arribere *et al.* 2014; Kim *et al.* 2014; Paix *et al.* 2014, 2015; Zhao *et al.* 2014; Ward 2015). One particularly flexible procedure involves injection of the gonad with a Cas9 transgene, a sgRNA expression vector, and

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Figure 1 Construction of *C. elegans* transgenes expressing Cas9 mutants with alternative PAM preferences. (A) Partial amino acid sequences of the *S. pyogenes* Cas9 PAM-interacting domain and mutants that have been evolved (Kleinstiver *et al.* 2015) to recognize an NGA PAM or an NGCG PAM. Altered residues are numbered and highlighted in red. (B) A representation of the Cas9 transgene modified

to express each variant (Dickinson *et al.* 2013). The transgene was driven ubiquitously by the *eft-3* promoter and contains three synthetic introns and the 3' UTR from *tbb-2*. Asterisks denote the locations of the mutations introduced to generate the variants.

an oligonucleotide repair template, resulting in gene conversion via HR with the donor template (Arribere *et al.* 2014; Paix *et al.* 2014; Zhao *et al.* 2014). Co-conversion with a dominant phenotypic marker mutation reduces the amount of screening necessary to isolate a desired mutant (Arribere *et al.* 2014; Kim *et al.* 2014; Ward 2015). In our application of this technique, we have observed that the recombination with the oligonucleotide donor is highly localized (Arribere *et al.* 2014). The portion of the donor incorporated into the genome often extends only 10 or fewer bases from the site of Cas9 cleavage. In addition, a scarcity of GG dinucleotides in many regions of the genome has confounded targeting certain types of functionally critical sequences, such as 3' UTRs. As a result, we were motivated to make more of the *C. elegans* genome accessible to efficient editing with oligonucleotide templates by expressing Cas9s with a variety of PAM requirements. In choosing an approach to extend the targetable genome, it was important to consider the substantial challenges that have been encountered in expressing foreign coding sequences in the nematode germline (Kelly *et al.* 1997). Expression of a “new” coding sequence in the germline can take several trials, with success or failure occurring as a complex function of the corresponding DNA, RNA, and protein sequences (Fire 2005). This provides a distinct advantage to approaches for extending editing technology that rely on coding regions that derive from established vectors that are active in the germline. In this work, we describe a set of variants of an effective *S. pyogenes* Cas9 germline expression vector.

To extend the functional editing repertoire in *C. elegans*, we investigated two recently described Cas9 variants that appeared particularly promising based on their reported specificities (Kleinstiver *et al.* 2015). One mutant, termed the *VQR variant*, possesses the amino acid substitutions D1135V, R1335Q, and T1337R, which results in a Cas9 protein that recognizes an NGA PAM. The second mutant, referred to as the *VRER variant*, has the mutated residues D1135V, G1218R, R1335E, and T1337R, which enable recognition of an NGCG PAM (Figure 1A). In the crystal structure of wild-type Cas9 in complex with a sgRNA and an NGG PAM, the R1335 residue, substituted in both the VQR and VRER variants, contacts the third PAM position (Anders *et al.* 2014). The T1337R substitution present in both variants is

also located in this conserved PAM-interacting domain. The mutant proteins recognize the same sgRNA scaffold (secondary structure) as wild-type *S. pyogenes* Cas9, a substantial advantage in working with these variants.

Materials and Methods

Strain construction and maintenance

The VC2010 N2 strain (Thompson *et al.* 2013) was used as an initial starting strain for all experiments described in this paper. The strain was used directly in experiments to test the VQR and VRER Cas9 variant transgenes in *dpy-10* and *unc-58* and to assess their PAM specificity via co-injection with the wild-type Cas9 transgene. When directly comparing the VQR transgene with wild-type Cas9 and the VRER variant using the same sgRNA vector, the VQR transgene was injected into VC2010 animals, whereas the other two were injected into mutant derivatives of VC2010 with modified PAMs (NGG or NGCG) at the *dpy-10* locus. The VC2010 variants needed for the wild-type and VRER assays were generated using a two-step oligonucleotide-templated CRISPR/Cas9 editing approach (Arribere *et al.* 2014). VC2010 animals were first altered to generate *dpy-10(cn64)* homozygotes, which have a *Dpy Rol* phenotype that is easily distinguished from *Rol* heterozygotes. Subsequent use of a sgRNA vector targeting the *dpy-10(cn64)* sequence facilitated recombination of oligonucleotides carrying silent mutations to alter the endogenous PAM and to revert the *dpy-10(cn64)* mutation, yielding *Rol* animals. From this group of *Rol* progeny, wild-type progeny were isolated and validated as the desired mutants by PCR and sequencing. All strains used to test each of the four NGAN PAMs with the VQR Cas9 variant were constructed in the same fashion. All strains were grown on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50 (Brenner 1974). All animals were grown at 16° prior to injection and at 23° after injection. Supporting Information, Table S1 lists all strains created and used in this study.

Plasmid construction

Plasmids have been deposited with Addgene with the following accession numbers: VQR Cas9 (pRB1080), 71309; VQR *dpy-10* sgRNA plasmid (pRB1081), 71479; VRER Cas9 (pRB1083),

Figure 3 Direct comparison of Cas9s recognizing three different PAMs using a single sgRNA by editing a *C. elegans* genomic target. (A) The endogenous NGA PAM used in the conversion of *dpy-10* by the VQR variant was edited to be an NGG or an NGCG PAM using mutations that did not alter the DPY-10 protein sequence. The resulting strains allowed a single sgRNA construct to be employed to target *dpy-10* for conversion with the three Cas9 transgenes (wild-type, VQR, and VRER). (B) Each Cas9 transgene was injected into a strain with its cognate PAM present in *dpy-10* adjacent to the sgRNA target. The break induced by each Cas9 using this sgRNA (marked with scissors) was the same distance from the mutated base that conferred the Rol phenotype. (C) The wild-type Cas9 and VQR variants were both highly competent

to produce Rol progeny, with many injected animals producing “jackpots” (>10 Rol progeny). The VRER Cas9 induced Rol animals through HR at a significantly lower frequency than the other two Cas9s. (D) In addition to Rol progeny, the injected worms generally yielded as many or more Dpy and Dpy Rol progeny, which derived largely from indels introduced by the NHEJ repair pathway but also could result from NHEJ repair on one chromosome and HR repair on the other. The number of injected animals with >10 Dpy or Dpy Rol progeny is explicitly indicated in the plots for the wild-type and VQR Cas9s, where that outcome was very frequent. The VRER Cas9, while producing fewer Rol progeny than wild-type Cas9 or the VQR variant (30 of 35 injected animals had 0 Rol progeny), displayed considerable activity, evidenced by the number of Dpy Rol and Dpy animals observed.

the obfuscating effect of Cas9 activity in somatic tissue. In our experience observing the progeny of these individuals, we have discerned that most carry heritable *dpy-10* null mutations but that a significant fraction possesses the *dpy-10(cn64)* mutation resulting from HR with the donor template, while others have no heritable phenotype. For our purposes, we scored any ambiguous phenotypes with partial or complete dumpiness and any degree of body shape dysfunction as Dpy Rol. When Dpy or Rol animals were also Unc, they were scored for consistency as a single class (Dpy Unc or Rol Unc).

Calculation of newly targetable sequences

The *C. elegans* annotated RefSeq genes were downloaded using the University of California–Santa Cruz (UCSC) Table Browser and the WS220/ce10 genome assembly. Each was downloaded with sufficient flanking sequence to determine the sgRNA sequence for Cas9 cleavage sites near the ends of each gene, but only bases within the coding sequence were checked for proximity to a potential Cas9 cleavage site. Intron sequences were included for sgRNA sequence determination but were not considered for the proximity calculation.

C. elegans 3' UTR sequences were obtained from the coordinates published in Jan *et al.* (2011). The sequences were downloaded using the UCSC Table Browser and the WS190/ce6 genome assembly and included sufficient flanking sequence to determine the sgRNA sequence for potential Cas9 cleavage sites near the ends of each 3' UTR, but only

bases within the 3' UTR sequence were considered for the proximity calculation. The analysis was performed using custom Python Scripts.

Results and Discussion

Two transgenes expressing Cas9 variants with alternate PAM specificities promote oligonucleotide-templated HR at multiple target loci

We introduced the sequence changes leading to the VQR and VRER Cas9 mutants into a construct that drives ubiquitous Cas9 expression in *C. elegans* with the promoter from *eft-3* (Figure 1B) (Dickinson *et al.* 2013). In the characterization of the VQR and VRER Cas9 variants by Kleinstiver *et al.* (2015), the assays employed to demonstrate function measured the disruption of genes by indel mutations resulting from NHEJ. Because homologous recombination using a defined template (HR) has been a key component in much of CRISPR/Cas9-mediated genome editing in *C. elegans*, we used a protocol that would detect both types of events. We co-injected each Cas9 variant plasmid with a sgRNA expression vector and an oligonucleotide to introduce mutations causing the easily scored Rol and Dpy phenotypes characteristic of *dpy-10(cn64)* and *dpy-10* loss of function, respectively (Figure 2, A and B) (Levy *et al.* 1993). We observed that each transgene was indeed capable of inducing both HR and NHEJ events at *dpy-10*. The editing process with these Cas9 variants in *C. elegans* was efficient: most injected worms produced

Figure 4 Co-conversion by each Cas9 mutant and wild-type Cas9 demonstrates PAM specificity. (A) A schematic of the reagents injected to assess PAM stringency. *dpy-10(cn64)* and *unc-58(e665)* donor oligonucleotides were included in all injection mixes, as well as sgRNA vectors with a target in *unc-58* with an NGG PAM. A sgRNA vector with a target in *dpy-10* with an NGA or an NGCG PAM was present if the VQR or VRER Cas9 variant was to be tested, respectively. (B) One variant (VQR or VRER) Cas9 transgene and the wild-type Cas9 transgene were injected together or individually, always co-injected with both donor oligonucleotides and sgRNA expression vectors. (C) The VQR and VRER variants should cleave *dpy-10* and produce Rol progeny in the F₁ generation, while the wild-type Cas9 should cleave the *unc-58* locus and produce Unc progeny. An abundance of both Rol and Unc phenotypes was expected when variant and wild-type Cas9s were present, in addition to Dpy Unc and Rol Unc animals with mutations at both loci. When only one Cas9 transgene was included in the injection mix, a preponderance of the corresponding phenotype (Rol for a variant and Unc for wild-type) should result if the Cas9s were specific to their PAMs. (D) When either variant transgene was injected with the wild-type Cas9 transgene, the progeny exhibited an abundance of Rol, Dpy Unc, Rol Unc, and Unc phenotypes, indicating robust activity at both loci. (E) When the wild-type transgene was not included, Rol progeny were a substantial fraction, although some Dpy Unc, Rol Unc, and Unc animals were observed. Worms injected with the VRER Cas9 variant alone produced relatively few Rol animals, but additional Dpy Rol and Dpy worms were present among their progeny (Figure S1). Many injected animals had no progeny with a given mutant phenotype. For the VQR transgene, these counts were 6 (0 Rol), 12 (0 Unc), and 12 (0 Dpy Unc or Rol Unc). For the VRER transgene, these counts were 12 (0 Rol), 14 (0 Unc), and 14 (0 Dpy Unc or Rol Unc). (F) When the wild-type Cas9 alone was injected, the result was an anticipated excess of Unc convertant progeny, but a small number of animals with Dpy Unc or Rol Unc phenotypes also were present. Many injected animals had no progeny with a given mutant phenotype. When the wild-type transgene was injected with an NGA PAM sgRNA expression vector, these counts were 10 (0 Rol), 7 (0 Unc), and 12 (0 Dpy Unc or Rol Unc). When the wild-type transgene was injected with an NGCG PAM sgRNA expression vector, these counts were 15 (0 Rol), 2 (0 Unc), and 11 (0 Dpy Unc or Rol Unc).

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animals with modified targets among their progeny. These events were heritable, as expected for germline modification of the locus. The confirmation of germline activity was important because somatic NHEJ events at *dpy-10* often can produce a nonheritable Dpy phenotype. In these experiments, the efficiency of the VQR variant at *dpy-10* appeared comparable to that of wild-type Cas9 acting at a similar site. While the VRER variant was able to generate both HR and NHEJ events, it appeared to be somewhat less active than either wild-type Cas9 or the VQR variant in the *dpy-10* assay. Additional support for the function of the VQR and VRER variants was provided by HR assays with the *unc-58* gene (Figure 2, A and C) (Arribere *et al.* 2014). Although the efficiency at *unc-58* was low for both variants compared to the wild-type Cas9 transgene, the appearance of worms with the unique shaking phenotype characteristic of the intended point mutant (*unc-58(e665)*) provided a confirmation of effective HR.

Editing of an endogenous PAM allows a direct comparison of three Cas9 transgenes using a single sgRNA target

We used a CRISPR/Cas9 genome editing approach (Arribere *et al.* 2014) to build a set of target sites at an endogenous genomic location that were designed to allow parallel evaluation of efficacy for the wild-type, VQR, and VRER proteins. The new alleles were engineered with synonymous substitutions in *dpy-10* that convert an endogenous NGA PAM to

either NGG or NGCG (Figure 3A). The strains, in conjunction with their wild-type ancestor, allowed the same sgRNA expression vector to be used with all three Cas9 transgenes (wild-type, VQR, and VRER) to introduce the *dpy-10(cn64)* mutation by HR with the donor template (Figure 3B). Therefore, we eliminated the variables of sgRNA sequence and distance of the marker mutation from the site of Cas9 cleavage. In this controlled assay, the effectiveness of VQR and wild-type Cas9 was comparable, while the VRER Cas9 exhibited lower efficiency (Figure 3, C and D).

Co-conversion and PAM specificity of wild-type and mutant Cas9s

The VQR and VRER Cas9 mutants differ only slightly from the wild-type protein; therefore, we considered an evaluation of their PAM preferences to be important. To characterize PAM stringency, we used the *dpy-10(cn64)* and *unc-58(e665)* phenotypes to assay for HR events. We injected a series of six mixtures that contained donor oligonucleotides for the *dpy-10(cn64)* and *unc-58(e665)* marker mutations. The injection mixes also all included two sgRNA expression vectors, one targeting a sequence in *unc-58* with an NGG PAM and one targeting a sequence in *dpy-10* with either an NGA or an NGCG PAM (Figure 4A).

For each of the Cas9 variants, three mixes were injected. In one, both the variant (VQR or VRER) and the wild-type Cas9 transgenes were present, while in the other two, only one type was included (Figure 4B). If the proteins discriminated

Figure 5 Direct comparison of cleavage efficiency by the VQR Cas9 with a single sgRNA using the four NGAN PAMs by editing a *C. elegans* genomic target. (A) A genomic target sequence with an NGG PAM used previously to convert *dpy-10* as a marker for Cas9 expression (Arribere *et al.* 2014) was edited by CRISPR/Cas9 to make an alanine-to-serine substitution (CCG to TCG), alter the wobble position of the upstream valine codon (GTN), and generate all four NGAN PAMs. (B) The NGAG PAM was highly efficient in recruiting the VQR Cas9 to convert *dpy-10* using this sgRNA, with most injected worms yielding >10 Rol progeny. The NGAA and NGAT PAMs also were able to induce conversion, but to a more modest extent. The NGAC PAM was significantly less proficient but still competent. Wild-type worms with an NGG PAM injected with the wild-type Cas9 transgene produced a number of Rol progeny comparable to the NGAG strain injected with the VQR variant transgene. The number of injected animals with >10 Rol progeny is explicitly indicated in the plots where that outcome was very frequent.

effectively between their preferred PAMs, the expectation when both transgenes were present in the injection mix was to observe an abundance of both *Rol* and *Unc* phenotypes in the F₁ progeny. We also predicted the presence of *Dpy Unc* and *Rol Unc* animals with mutations at both loci (resulting from NHEJ or HR at *dpy-10* and HR at *unc-58*) (Figure 4C). When the wild-type Cas9 was omitted from the injection mix, we expected to observe a majority of *Rol* animals (Figure 4C). Conversely, in the absence of either Cas9 variant, we anticipated a preponderance of *Unc* animals (Figure 4C).

We found that each of the classes of homologous recombinants was most efficiently recovered in experiments in which the matched Cas9 was present (Figure 4, D–F). Leaving out the cognate Cas9 resulted in a consistent and dramatic decrease in insertion activity (Figure 4, E and F). Selectivity, although high, was not without exception. The VQR and VRER variants could occasionally promote HR with an NGG PAM (Figure 4E). Wild-type Cas9 could induce HR with an NGA PAM (albeit rarely) and also exhibited a low level of activity using an NGCG PAM (Figure 4F). These results are consistent with previous observations of strong but not perfect PAM dependence for both wild-type Cas9 (Hsu *et al.* 2013; Pattanayak *et al.* 2013; Fu *et al.* 2014; Wu *et al.* 2014) and the VQR/VRER variants (Kleinstiver *et al.* 2015).

Editing of an endogenous PAM elucidates the cleavage efficiency of the VQR variant with different NGAN PAMs

Kleinstiver *et al.* (2015) reported differential effectiveness of the VQR variant depending on the base following the NGA PAM. Their description of the VQR variant summarized the influence as NGAG > NGAA = NGAT > NGAC, with NGAA and NGAT having approximately 75% of the effectiveness of NGAG, while NGAC was about 50% effective. When converting *dpy-10* and *unc-58*, we used NGAG and NGAA PAMs, respectively, and observed much greater efficiency with

NGAG. However, the contributing factors of distinct sgRNA sequences, different distances of marker mutations from the Cas9 cleavage site, and disparate genomic locations underscored the value of a more controlled evaluation. We therefore developed a common sgRNA assessment strategy similar to the means by which we compared the three Cas9 variants. Using CRISPR/Cas9, we edited the genome near the site of the *dpy-10(cn64)* mutation (Arribere *et al.* 2014), creating four different strains. In each, an NGG PAM adjacent to a sgRNA target we had previously verified with wild-type Cas9 was converted to four different NGAN PAMs with each of the four possible nucleotides at the fourth position. This approach allowed us to use a single proven sgRNA to accurately test the VQR Cas9 cleavage efficiency employing each of the NGAN PAMs (Figure 5A). The modifications involved making a nonsynonymous substitution converting an alanine codon to four different serine codons (all the resulting Ala → Ser variants were wild-type in phenotype).

We observed that injected animals derived from all four strains gave rise to *Rol* progeny, confirming effective HR using each of the NGAN PAMs. Our results strongly support NGAG as the most effective PAM and substantiate the report that NGAA and NGAT are both more effective than NGAC. When injected with the wild-type Cas9 transgene, wild-type animals with an NGG PAM adjacent to the same sgRNA target produced *Rol* progeny in quantities similar to worms injected with the VQR transgene with an NGAG PAM in the same position (Figure 5B).

Conclusions

We have demonstrated that precise genome engineering can be extended to a broader range of target loci in the *C. elegans* genome by using two Cas9 transgenes expressing modified proteins with alternative PAM specificities. We undertook a

careful comparison of the altered Cas9 proteins to the wild-type by editing a PAM in a genomic target, *dpy-10*. Our engineering permitted experimental comparisons in which a single sgRNA could be used in multiple tester strains in which its constant target sequence was adjacent to the PAM variants recognized by the different Cas9s. Such assays showed that the VQR Cas9, when targeted to an endogenous NGAG PAM, can promote HR at levels comparable to wild-type Cas9. We similarly probed the performance of the VQR variant by editing another PAM in *dpy-10* to test the effect of the downstream base. Our results provided corroboration of a gradation in activity (Kleinstiver *et al.* 2015), with NGAC at this site being the least efficient and NGAG being the most effective, extending these results to a meiotic system and to *C. elegans*. This work demonstrates the potential of genome engineering to allow efficient and accurate testing of Cas9 transgenes and characterization of their PAM requirements. In addition, the reagents we developed can serve as a foundation to generate *C. elegans* expression vectors for Cas9 fusion proteins with alternate PAM recognition.

C. elegans has an AT-rich genome composition, leading to frequent challenges in identifying NGG PAMs with suitable proximity for efficient oligonucleotide-templated editing. The availability of reagents that allow the use of alternative NGA PAMs should greatly expand the regions of the genome accessible to efficient editing by such methods. Examining coding sequences (Figure S2, A and B) and 3' UTRs (Figure S2, C and D), we indeed see substantial expansion of the targetable repertoires. For example, the addition of sequences within five bases of NGA PAM cleavage sites to those within five bases of NGG cleavage sites would expand the number of efficiently targetable bases in coding sequence by 44% and more than double the number in 3' UTR sequence.

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GENETICS

Supporting Information

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Cas9 Variants Expand the Target Repertoire in *Caenorhabditis elegans*

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Figure S1: Dpy Rol and Dpy animals among the F1 progeny of worms injected with only the VRER Cas9 in the PAM stringency co-conversion experiment

These observations provided evidence for the activity of the VRER Cas9 in addition to the appearance of the Rol and Dpy Unc progeny depicted in Figure 4e.

Expansion of target repertoire in coding sequence

Figure S2a: The fraction of all bases in the annotated *C. elegans* RefSeq genes which have Cas9 cleavage sites within 10 bp induced using NGG, or NGA, or either NGG or NGA PAMs.

The bases depicted meet the following criteria: 1. A distance of ≤ 10 bp from a Cas9 induced cleavage site, and 2. The associated sgRNA sequence would have no runs of more than 4 T bases and a GC content between 20-80%.

Enlargement of target repertoire in coding sequence

Figure S2b: The fraction of all bases in the annotated *C. elegans* RefSeq genes which have Cas9 cleavage sites within 5 bp induced using NGG, or NGA, or either NGG or NGA PAMs.

The bases depicted meet the following criteria: 1. A distance of ≤ 5 bp from a Cas9 induced cleavage site, and 2. The associated sgRNA sequence would have no runs of more than 4 T bases and a GC content between 20-80%.

Expansion of 3' UTR target repertoire

Figure S2c: The fraction of all bases in *C. elegans* 3' UTRs which have Cas9 cleavage sites within 10 bp induced using NGG, or NGA, or either NGG or NGA PAMs.

The bases depicted meet the following criteria: 1. A distance of ≤ 10 bp from a Cas9 induced cleavage site, and 2. The associated sgRNA sequence would have no runs of more than 4 T bases and a GC content between 20-80%.

Expansion of 3' UTR target repertoire

Figure S2d: The fraction of all bases in *C. elegans* 3' UTRs which have Cas9 cleavage sites within 5 bp induced using NGG, or NGA, or either NGG or NGA PAMs.

The bases depicted meet the following criteria: 1. A distance of ≤ 5 bp from a Cas9 induced cleavage site, and 2. The associated sgRNA sequence would have no runs of more than 4 T bases and a GC content between 20-80%.

Table S1: Strains used and created in this study.

Strain name	Genotype	Partial <i>dpy-10</i> sequence	Description	Reference
PD1074	VC2010	...GTACCGCTCGTGGTGCCTATGGTAGC...	Wild type	(THOMPSON <i>et al.</i> 2013)
PD3439	<i>dpy-10(cc3439)</i> II	...GTACCG CATG CGGTGCCTATGGTAGC...	<i>dpy-10(cn64)</i> with silent mutations	This study
PD3440	<i>dpy-10(cc3440)</i> II	...GTACCG CCCG TGGTGCCTATGGTAGC...	NGG PAM added	This study
PD3441	<i>dpy-10(cc3441)</i> II	...GTACCG GCG TGGTGCCTATGGTAGC...	NGCG PAM added	This study
PD3442	<i>dpy-10(cc3442)</i> II	...G TTT CGCGCGTGGTGCCTATGGTAGC...	NGAA PAM added	This study
PD3443	<i>dpy-10(cc3443)</i> II	...GT GTC GCGCGTGGTGCCTATGGTAGC...	NGAC PAM added	This study
PD3444	<i>dpy-10(cc3444)</i> II	...GT CTC GCGCGTGGTGCCTATGGTAGC...	NGAG PAM added	This study
PD3445	<i>dpy-10(cc3445)</i> II	...GTAT CGC GCGTGGTGCCTATGGTAGC...	NGAT PAM added	This study

Table S2: Plasmids, oligonucleotides, and gBlocks used in this study.

Plasmids used in this study

Name	Description	Reference
pJA50	U6:: <i>unc-58</i> gRNA (NGG PAM)	(ARRIBERE <i>et al.</i> 2014)
pJA58	U6:: <i>dpy-10</i> gRNA (NGG PAM)	(ARRIBERE <i>et al.</i> 2014)
pRB1017	Empty U6::gRNA vector used for cloning	(ARRIBERE <i>et al.</i> 2014)
pRB1080	<i>eft-3</i> ::VQR Cas9:: <i>tbb-2</i> 3' UTR	This study
pRB1081	U6:: <i>dpy-10</i> gRNA (NGAG PAM)	This study
pRB1082	U6:: <i>unc-58</i> gRNA (NGAA PAM)	This study
pRB1083	<i>eft-3</i> ::VRER Cas9:: <i>tbb-2</i> 3' UTR	This study
pRB1084	U6:: <i>dpy-10</i> gRNA (NGCG PAM)	This study
pRB1085	U6:: <i>dpy-10</i> gRNA (NGCG PAM)	This study
pRB1086	U6:: <i>dpy-10(cc3439)</i> gRNA (NGG PAM)	This study

Oligonucleotides used in this study

Name	Sequence (5' to 3')	Purpose	Associated plasmid
AF-KLA-292	CAATTCCTTAAGTCCTGGTGG	<i>dpy-10</i> sequencing primer	N/A
AF-RB-308	TCTTGTCGCTACCATAGGCACCA	VQR <i>dpy-10</i> gRNA oligo	pRB1081
AF-RB-309	AAACTGGTGCCTATGGTAGCGGAC	VQR <i>dpy-10</i> gRNA oligo	pRB1081
AF-RB-323	TCTTGCCACGCACATGGTCACTAC	VQR <i>unc-58</i> gRNA oligo	pRB1082
AF-RB-324	AAACGTAGTGACCATGTGCGTGCC	VQR <i>unc-58</i> gRNA oligo	pRB1082
AF-RB-327	TCTTGCGCTACCATAGGCACACG	VRER <i>dpy-10</i> gRNA oligo	pRB1084
AF-RB-328	AAACCGTGGTGCCTATGGTAGCGC	VRER <i>dpy-10</i> gRNA oligo	pRB1084
AF-RB-339	TCTTGCTACCATAGGCACCGCATG	<i>dpy-10(cc3439)</i> gRNA oligo	pRB1086
AF-RB-340	AAACCATGCGGTGCCTATGGTAGC	<i>dpy-10(cc3439)</i> gRNA oligo	pRB1086
AF-RB-347	TCTTGCTTTCCGTAGTGACCATG	VRER <i>unc-58</i> gRNA oligo	pRB1085

AF-RB-348	AAACCATGGTCACTACGGAAAGAC	VRER <i>unc-58</i> gRNA oligo	pRB1085
AF-RB-349	TCAAGAAGACCGAGGTCCAAAC	pRB1080 and pRB1083 sequencing primer	
AF-ZF-831	GTCAGATGATCTACCGGTGTGTCAC	<i>dpy-10</i> PCR primer	N/A
AF-ZF-832	GTCTCTCCTGGTGCTCCGTCTTCAC	<i>dpy-10</i> PCR primer	N/A

Donor oligonucleotides used in this study (5' to 3')

AF-ZF-827 <i>dpy-10(cn64)</i> donor	CACTTGAAC TTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCATGCGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT
AF-RB-342 <i>dpy-10</i> NGG PAM donor	CACTTGAAC TTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCCCGTGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT
AF-RB-343 <i>dpy-10</i> NGCG PAM donor	CACTTGAAC TTCAATACGGCAAGATGAGAATGACTGGAAACCGTACCGCGCGTGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT
AF-RB-356 <i>dpy-10</i> NGAT PAM donor	CACTTGAAC TTCAATACGGCAAGATGAGAATGACTGGAAACCGTATCGCTCGTGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT
AF-RB-357 <i>dpy-10</i> NGAC PAM donor	CACTTGAAC TTCAATACGGCAAGATGAGAATGACTGGAAACCGTGTCGCTCGTGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT

AF-RB-358 <i>dpy-10</i> NGAA PAM donor	CACTTGAACCTCAATACGGCAAGATGAGAATGACTGGAAACCGTTTCGCTCGTGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT
AF-RB-359 <i>dpy-10</i> NGAG PAM donor	CACTTGAACCTCAATACGGCAAGATGAGAATGACTGGAAACCGTCTCGCTCGTGGTGCCTATGGTAGCGGAGCTTCACATGGCTTCA GACCAACAGCCTAT
AF-JA-76 <i>unc-58(e665)</i> donor for wild type Cas9	ATTTTGTGGTATAAAATAGCCGAGTTAGGAAACAAATTTTCTTTCAGGTTTCTCAGTAGTGACCATGTGCGTGGATCTTGCGTCCACA CATCTCAAGGCGTACTT
AF-RB-341 <i>unc-58(e665)</i> donor for VQR Cas9	ATTTTGTGGTATAAAATAGCCGAGTTAGGAAACAAATTTTCTTTCAGGTTTTAGTGTAGTGACCATGTGCGTGGATCTTGCGTCCAC ACATCTCAAGGCGTACTT
AF-RB-352 <i>unc-58(e665)</i> donor for VRER Cas9	ATTTTGTGGTATAAAATAGCCGAGTTAGGAAACAAATTTTCTTTCAGGTTTTAGTGTAGTGACCATGTGTGTGGATCTTGCGTCCACA CATCTCAAGGCGTACTT

gBlocks used in this study

AF-RB-VQR

CAAAGAAGTACGGAGGATTCgtcTCCCCAACCGTCGCCTACTCCGTCCTCGTCGTCGCCAAGGTCGAGAAGGGAAAGTCCAAGAAGCTCAAGTCCGT

CAAGGAGCTCCTCGGAATCACCATCATGGAGCGTTCCTCCTTCGAGAAGAACCCAATCGACTTCCTCGAGGCCAAGGGATACAAGGAGGTCAAGAA
GGACCTCATCATCAAGCTCCCAAAGTACTCCCTCTTCGAGCTCGAGAACGGACGTAAGCGTATGCTCGCCTCCGCCGGAGAGCTCCAAAAGGGAAAC
GAGCTCGCCCTCCCATCCAAGTACGTCAACTTCCTCTACCTCGCCTCCCACTACGAGAAGCTCAAGGGATCCCCAGAGGACAACGAGCAAAAGCAAC
TCTTCGTCGAGCAACACAAGCACTACCTCGACGAGATCATCGAGCAAATCTCCGAGTTCTCCAAGCGTGTATCCTCGCCGACGCCAACCTCGACAA
GGTCCTCTCCGCCTACAACAAGCACCGTGACAAGCCAATCCGTGAGCAAGCCGAGAACATCATCCACCTCTTCACCCTCACCAACCTCGGAGCCCCA
GCCGCCTTCAAGTACTTCGACACCACCATCGACCGTAAGcaaTACcgtTCCACCAAGGAGGTCCTCGA

AF-RB-VRER

CAAAGAAGTACGGAGGATTcgtTCCCCAACCGTCGCCTACTCCGTCCTCGTCGTCGCCAAGGTCGAGAAGGGAAAGTCCAAGAAGCTCAAGTCCGT
CAAGGAGCTCCTCGGAATCACCATCATGGAGCGTTCCTCCTTCGAGAAGAACCCAATCGACTTCCTCGAGGCCAAGGGATACAAGGAGGTCAAGAA
GGACCTCATCATCAAGCTCCCAAAGTACTCCCTCTTCGAGCTCGAGAACGGACGTAAGCGTATGCTCGCCTCCGCCcgtGAGCTCCAAAAGGGAAACG
AGCTCGCCCTCCCATCCAAGTACGTCAACTTCCTCTACCTCGCCTCCCACTACGAGAAGCTCAAGGGATCCCCAGAGGACAACGAGCAAAAGCAACT
CTTCGTCGAGCAACACAAGCACTACCTCGACGAGATCATCGAGCAAATCTCCGAGTTCTCCAAGCGTGTATCCTCGCCGACGCCAACCTCGACAAG
GTCCTCTCCGCCTACAACAAGCACCGTGACAAGCCAATCCGTGAGCAAGCCGAGAACATCATCCACCTCTTCACCCTCACCAACCTCGGAGCCCCAGC
CGCCTTCAAGTACTTCGACACCACCATCGACCGTAAGgagTACcgtTCCACCAAGGAGGTCCTCGA

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