CRISPR/Cas9 Gene Drive: Growing Pains for a New Technology

Floyd A. Reed

Department of Biology, University of Hawai'i at Mānoa, Honolulu, Hawai'i 96822

ORCID ID: 0000-0003-4703-530X (F.A.R.)

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In this commentary, Floyd Reed discusses Unckless et al. (2017), "Evolution of resistance against CRISPR/Cas9 gene drive," which was published in the February issue of GENETICS.

The challenge in genetic pest management of designing gene drive systems that push desired genetic modifications to high frequency in wild populations is almost 50 years old (Curtis 1968). New and diverse types of gene drive systems continue to appear (see for reviews: Sinkins and Gould 2006; Champer et al. 2016). Once, chromosomal rearrangements resulting in underdominance were thought to be the answer, but over a decade of designing and testing this failed to provide a population transformation system that was robust against wild organisms (e.g., Foster et al. 1972; Boussy 1988; see also Buchman et al. 2016). Unanticipated nuclear architecture effects on gene regulation certainly played a role in reducing the fitness of these rearrangements (Harewood and Frasier 2014). In the last few years, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9-mediated gene drive (CGD) has arisen as a modern version of HEG (homing endonuclease genes) gene drive (Burt 2003). CGD has received a great deal of attention, perhaps with some hyperbole, but that is not to say that it is not potentially transformative (Esvelt et al. 2014; Hammond et al. 2016). Compared to many technologies, a high level of interdisciplinary expertise is required—from cell biology, molecular genetics, and evolutionary theory to ecology and the social sciences—to design and initially evaluate a novel gene drive system and its potential applications. Every new technology

goes through a series of revisions, testing, uncovering weaknesses, and development. This happened with gene drive via chromosomal rearrangements, and it should come as no surprise that CGD is undergoing the same process. The high frequency of off-target cutting by CRISPR/Cas9 recently became apparent, and developments are occurring to increase the specificity and accuracy of CRISPR/Cas9 (Fu et al. 2013; Anderson et al. 2015; Tycko et al. 2016). A new type of problem for CGD that needs to be addressed is now coming into focus. The Cas9 enzyme targets a highly specific 20-bp nucleotide sequence in the genome (see for review Ran et al. 2013). Individuals can be resistant to CGD if they have an alternative nucleotide sequence at the target site. Unckless et al. (2017) address this issue by considering the likelihood of both standing variation in the population and new mutations during the CGD application resulting in resistance, and estimate the probability that CGD will fail to stably transform the population.

Natural variation exists in every population; a DNA sequence used to design a target for CRISPR/Cas9 may have naturally occurring alleles that are resistant, and resistance alleles that did not exist prior to the initial release of a CGD system may arise as *de novo* mutations and increase in frequency by natural selection in response to CGD fitness effects. Even if a complete CGD transformation was achieved, mutations will continue to occur that might disrupt the system. Cells do not tolerate double-strand breaks in DNA and have machinery to quickly repair this type of damage, which can lead to cancer and/or sterility if unchecked (Burma et al. 2006; Allard and Colaiácovo 2010). Homology-directed repair (HDR) copies the corresponding sequence of an intact homologous chromosome to repair the break (i.e., allelic gene conversion). CGD takes advantage of this process to

convert heterozygotes into homozygotes to increase in frequency in a population (Gantz and Beir 2015). Cells can also reattach broken strands directly by nonhomologous end joining (NHEJ) and microhomology-mediated end joining (MMEJ). If multiple break sites exist in the genome this can lead to chromosomal rearrangements (e.g., Richardson and Jasin 2000), which are also associated with sterility and cancer (Lai *et al.* 2005; Bunting and Nussenzweig 2013). These processes have very high associated mutation rates, and a mutation in the target sequence is likely to render resistance to the targeted cutting of CRISPR/Cas9 (Cong *et al.* 2013; Gantz and Bier 2015; Hammond *et al.* 2016). The very act of Cas9 cutting the target sequence is likely to result in a disruptive mutation at the same site (Ran *et al.* 2013). Thus, CGD is a system that generates alleles resistant to the drive mechanism as it proceeds through the population. On the surface this predicts that, while gene drive may be able to initially increase in frequency and begin to spread through the population, CGD will be incomplete and possibly not capable of attaining frequencies close to fixation and/or will not be stable over subsequent generations. There will be non-CGD genomes in the wild that are altered by mutations, perhaps in a way that was not originally intended.

Unckless *et al.* (2017) rigorously modeled the dynamics of CGD allele conversion, fitness costs, mutations, and genetic drift under three scenarios: CGD resistance from standing genetic variation; CGD resistance from standard *de novo* mutations; and CGD resistance from NHEJ and similar mutations. They found that the initial release frequency of a CGD system, the conversion rate of CGD, and its associated fitness cost have very little effect on the likelihood of resistance evolving. Most importantly, Unckless *et al.* (2017) found that the mutation rate resulting in CGD resistance is the dominant force and that, even with very conservative assumptions, the evolution of gene drive resistance is essentially inevitable. The authors point out that the evolution of CGD resistance may not be undesirable, depending on the goals and applications of gene drive in specific cases. Transient and incomplete population transformations can still be achieved. CGD resistance may be thought of as incorporating a natural braking mechanism to limit the rate of establishment of gene drive in the wild. One can also imagine utilizing CGD to transiently boost a coreleased designed resistance allele with a desired “cargo” to higher frequency (compare with Gould *et al.* 2008).

In applications where a complete, stable, efficient transformation is desired, mutations are a serious obstacle. There are already several ideas for addressing this problem. One approach is to target a highly-conserved sequence that cannot tolerate disruption by mutations so that non-HDR repair is removed by selection; however, this is also likely to inhibit CGD for the same reasons (Noble *et al.* 2016a). Furthermore, evolution is rife with fascinating examples of selfish genes that dramatically lower organismal fitness, becoming rapidly inactivated by the host genome via a range of mechanisms (e.g., Lyttle 1981; Charlat *et al.* 2007; Josse *et al.* 2007; Tao

et al. 2007). Another approach is to multiplex several targets so that the mutation of one or a few targets to resistant alleles does not block the progression of gene drive (Marshall *et al.* 2016). Noble *et al.* (2016a) propose to combine these two approaches using multiple targets within a gene that induce a strong fitness disadvantage when disrupted. Importantly, the inserted gene drive “cargo” repairs the gene to be functional, but with an altered sequence to evade additional CRISPR/Cas9 cutting. In this way, random mutations are likely to be removed by selection and only fully homology-directed repaired sequences will persist in the population. As these approaches become more and more complex one cannot help but be reminded of the criticism of Curtis (1985): “these methods... possess great appeal to the geneticist who feels that he or she ought to be doing something useful... this led us to start work on some intellectually delightful but impractical schemes... there may be a danger that the intellectual appeal of [new genetic technologies] may lead applied entomologists to waste time on baroque schemes, without thinking whether their aims could be achieved more simply and quickly by old-fashioned [methods].”

At the risk of an additional baroque elaboration, an alternative path forward may be to synergistically combine the relative advantages of multiple gene drive systems (compare with Gokhale *et al.* 2014). For example, CGD could be combined with underdominance. Underdominance cannot increase in frequency when rare but can proceed to fixation once a “threshold” allele frequency is surpassed (Altrock *et al.* 2010). CGD can increase in frequency when rare but cannot achieve stable fixation once non-HDR mutation effects are factored in. In a combined system, CGD could be used for the initial drive to dramatically lower the threshold for release to result in a transformation, then underdominance could continue to drive the system at higher frequencies [perhaps ideally in a stepwise replacement using the “daisy drive” system (Noble *et al.* 2016b), with an initial nondriving synthetic target to limit the final geographic spread]. Underdominance at sufficient frequency can also replace CGD-resistant mutations at the same locus in the population created by the initial CGD process to truly achieve 100% fixation in a local population. This renders the final system geographically stable and potentially reversible (with the release of sufficient numbers of unmodified organisms to bring the frequency below the transformation threshold) due to the nature of underdominance (Altrock *et al.* 2010), enhancing the safety of the system, which is likely also to have positive effects on both regulation and public acceptance.

These new proposals also need to be rigorously modeled and safely tested to elucidate the path forward for gene drive development, risk assessment, and the evaluation of potential applications.

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