Safeguarding gene drive experiments in the laboratory

Multiple stringent confinement strategies should be used whenever possible

By Omar S. Akbari, Hugo J. Bellen, Ethan Bier, Simon L. Bullock, Austin Burt, George M. Church, Kevin R. Cook, Peter Duchek, Owain R. Edwards, Kevin M. Esvelt, Valentino M. Gantz, Kent G. Golic, Scott J. Gratz, Melissa M. Harrison, Keith R. Hayes, Anthony A. James, Thomas C. Kaufman, Juergen Knoblich, Harmit S. Malik, Kathy A. Matthews, Kate M. O’Connor-Giles, Annette L. Parks, Norbert Perrimon, Fillip Port, Steven Russell, Ryu Ueda, Jill Wildonger

Gene drive systems promote the spread of genetic elements through populations by assuring they are inherited more often than Mendelian segregation would predict (see the figure). Natural examples of gene drive from Drosophila include sex-ratio meiotic drive, segregation distortion, and reproductive transposition. Synthetic drive systems based on selective embryonic lethality or homing endonucleases have been described previously in Drosophila melanogaster (1–3), but they are difficult to build or are limited to transgenic populations. In contrast, RNA-guided gene drives based on the CRISPR/Cas9 nuclease can, in principle, be constructed by any laboratory capable of making transgenic organisms (4). They have tremendous potential to address global problems in health, agriculture, and conservation, but their capacity to alter wild populations outside the laboratory demands caution (4–7). Just as researchers working with self-propagating pathogens must ensure that these agents do not escape to the outside world, scientists working in the laboratory with gene drive constructs are responsible for keeping them confined (4, 6, 7).

Two of us recently used a CRISPR/Cas9-based gene drive system to generate a Drosophila strain homozygous for a loss-of-function mutation [the mutagenic chain reaction (6)] (see the figure). Even though D. melanogaster ordinarily poses no threat to human health or agriculture, the accidental release of flies carrying gene drive constructs from the laboratory could have unpredictable ecological consequences. This study therefore used institutionally approved stringent barrier methods. Only one experimenter handled the flies, inside an Arthropod Containment Level 2 insectary suitable for work with mosquitoes carrying human pathogens. Because barrier protocols can be vulnerable to human error (8), these authors suggested (6) that additional molecular confinement methods described (4) and used by others of us in budding yeast (9) could further reduce risks. That these studies documented highly efficient RNA-guided gene drive in flies and yeast underscores the potential of the technology and the risk resulting from an accidental release.

As concerned scientists working in related areas, we engaged in collective discussions to identify and publicize interim safety recommendations for laboratory research involving potential gene drive systems while formal national guidelines are developed. Although we cannot claim to represent all researchers, we share a commitment to the safe and responsible development of gene drive technology. Although we differ in our assessments of the types of precaution needed, we recognize that any single confinement strategy could fail. We therefore unanimously recommend that future studies use a combination of stringent confinement strategies (see the table) whenever possible and always use safeguards adequate for preventing the unintentional release of synthetic gene drive systems into natural populations.

RECOMMENDATIONS. RNA-guided gene drive systems are created by delivering into the germline a DNA cassette encoding Cas9 and a single synthetic guide RNA (sgRNA) that is flanked by sequences matching those on either side of the sgRNA target site (4). Cas9 nucleic-stimulated copying of the cassette into the target allele leads to continued Cas9+sgRNA expression and subsequent copying of the cassette into the other allele (6, 9). The recurrent conversion of heterozygotes into homozygotes permits spread through populations (see the figure).

The vast majority of recent genome engineering approaches developed in model organisms neither involve nor risk the creation of gene drive systems. For example, Drosophila mutants can be readily generated by injecting sgRNAs or sgRNA-encoding plasmids into transgenic embryos expressing Cas9 (10–13) or by crossing

---

**Potentially stringent confinement strategies for gene drive research**

Multiple stringent confinement strategies should be used whenever possible.

<table>
<thead>
<tr>
<th>TYPE</th>
<th>STRINGENT CONFINEMENT STRATEGY</th>
<th>EXAMPLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular</td>
<td>Separate components required for genetic drive</td>
<td>sgRNA and Cas9 in separate loci (8)</td>
</tr>
<tr>
<td></td>
<td>Target synthetic sequences absent from wild organisms</td>
<td>Drive targets a sequence unique to laboratory organisms (3, 4, 8)</td>
</tr>
<tr>
<td>Ecological</td>
<td>Perform experiments outside the habitable range of the organism</td>
<td>Anopheles mosquitoes in Boston</td>
</tr>
<tr>
<td></td>
<td>Perform experiments in areas without potential wild mates</td>
<td>Anopheles mosquitoes in Los Angeles</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Use a laboratory strain that cannot reproduce with wild organisms</td>
<td>Drosophila with compound autosomes*</td>
</tr>
<tr>
<td>Barrier</td>
<td>Physical barriers between organisms and the environment</td>
<td>Triply nested containers, &gt;3 doors (6)</td>
</tr>
<tr>
<td></td>
<td>• Remove barriers only when organisms are inactive</td>
<td>Anesthetize before opening (6)</td>
</tr>
<tr>
<td></td>
<td>• Impose environmental constraints</td>
<td>Low-temperature room, air-blast fans</td>
</tr>
<tr>
<td></td>
<td>• Take precautions to minimize breaches due to human error</td>
<td>Keep careful records of organisms, one investigator performs all experiments (6)</td>
</tr>
</tbody>
</table>

*An example of reproductive confinement would be Drosophila laboratory strains with a compound autosomal, where both copies of a large autosomal are confined at a single centromere. These strains are sterile when crossed into any normal or wild-type strain because all progeny are monosomic or trisomic and die early in development.
The spread of RNA-guided gene drive systems. Unlike the population dynamics of normal genomic alterations, gene drive systems can spread changes through wild populations by converting heterozygotes into homozygotes in each generation.

sgRNA-expressing strains to Cas9-expressing strains (12–14). These approaches do not risk creating a gene drive system because cassettes encoding Cas9 and sgRNA are not inserted into the cut site or located adjacent to one another in the genome and can thus be safely used by researchers without additional precautions. Given the availability of efficient alternatives and the potential risks, we recommend that gene drive approaches to genome engineering be strictly reserved for cases that require their use.

The safest approach for using gene drives creates biallelic mutations with an sgRNA-only cassette that can spread only when combined with an unlinked Cas9 transgene (4). In such a “split gene drive system,” homozygous individuals lacking the Cas9 gene can be easily isolated in subsequent generations. The efficiency of gene drive exhibited by a split system in yeast is equivalent to that of a construct encoding both Cas9 and sgRNA (9). Split drive systems present a much lower risk if organisms are accidentally released because the population frequency of the Cas9 gene will be determined by normal, nondrive dynamics, consequently limiting the spread of the sgRNA cassette.

Nevertheless, any mutational event that moves the Cas9 gene into or directly adjacent to the sgRNA cassette could create an autonomous Cas9+sgRNA drive system by allowing the Cas9 gene to be copied into the target locus along with the sgRNA cassette upon repair of Cas9-induced DNA cleavage. Although the probability of such an event is extremely low, we recommend that at least one additional form of stringent confinement be used (see the table) and that the strains be continually monitored.

Other forms of stringent confinement include performing experiments in an area lacking wild populations (4) and, when the goal is to study gene drive systems in the laboratory, exclusively targeting synthetic sequences not found in natural populations (3, 4, 9). Because these strategies suffer from independent vulnerabilities, the safety improvements afforded by combining them will be multiplicative. Thus, the great majority of gene drive experiments can be performed with minimal risk of altering wild populations. Accordingly, we strongly recommend that

1) All work involving potential gene drive systems should be preceded by a thorough assessment by the relevant biosafety authorities of the risk of unwanted release from the laboratory. We encourage these authorities to seek guidance from external experts and make their evaluation available to others.

2) All laboratory gene drive experiments should employ at least two stringent confinement strategies (see the table) whenever possible to minimize the risk of altering wild populations. Using one form of confinement may be justified only if relevant biosafety authorities determine that

---

1Department of Entomology, Univ. of California, Riverside, CA 92507, USA. 2Center for Disease Vector Research, Institute for Integrative Genome Biology, Univ. of California, Riverside, CA 92507, USA.
2Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA. 3Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030, USA. 4Section of Cell and Developmental Biology, Univ. of California, San Diego, La Jolla, CA 92095, USA. 5Division of Cell Biology, Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 0QH, UK.
6Department of Life Sciences, Imperial College London, Silwood Park, Ascot, Berks SL5 7PY, UK. 7Wyss Institute for Biologically Inspired Engineering, Harvard Medical School, Boston, MA 02115, USA.
8Department of Genetics, Harvard Medical School, Boston, MA 02115, USA. 9Bloomington Drosophila Stock Center, Department of Biology, Indiana Univ., Bloomington, IN 47405, USA.
10Institute of Molecular Biotechnology of the Austrian Academy of Sciences, 1030 Vienna, Austria. 11CSIRO Centre for Environment and Life Sciences, Underwood Avenue, Forest, WA 6014, Australia. 12Department of Biology, Univ. of Utah, Salt Lake City, UT 84112, USA. 13Laboratory of Genetics, Univ. of Wisconsin-Madison, Madison, WI 53706, USA. 14Department of Biomedical Chemistry, Univ. of Wisconsin-Madison, Madison, WI 53706, USA. 15CSIRO Biosecurity Flagship, General Post Office Box 1538, Hobart, Tasmania, 7001, Australia. 16Departments of Microbiology & Molecular Genetics and Molecular Biology & Biochemistry, Univ. of California at Irvine, Irvine, CA 92697, USA.
17Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA. 18Howard Hughes Medical Institute, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA. 19Laboratory of Cell and Molecular Biology, Univ. of Wisconsin-Madison, Madison, WI 53706, USA. 20Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA. 21Department of Genetics, Univ. of Cambridge, Cambridge, Cambridgeshire CB2 2EH, UK. 22Department of Genetics, Graduate Univ. for Advanced Studies, Mishima, Shizuoka 411-8540, Japan. 23NIG-Fl Stock Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan. 24Department of Biochemistry, Univ. of Wisconsin-Madison, Madison, WI 53706, USA. 25Corresponding author. E-mail: kevin.esvelt@wyss.harvard.edu (K.E.); eber@ucsd.edu (E.B.)
it will reduce the probability of release to a level that is acceptably low. This probability must be defined on a case-by-case basis. The analyses necessary to confidently predict the efficacy of confinement strategies for gene drive systems are in a nascent form. Therefore, any proposal to use one rather than multiple forms of confinement requires even greater scrutiny and extensive deliberation between regulatory authorities and scientists.

3) Organisms carrying gene drive constructs that could spread if the reproductively capable life stages were to escape in transit should not be distributed to other institutions until formal biosafety guidelines are established. Whenever possible, laboratories should instead send DNA constructs or information sufficient to reconstruct the gene drive. Protocols for distributing materials should be established in discussion with the wider research community and other relevant stakeholders.

Broadly inclusive and ongoing discussions among diverse groups concerning safeguards, transparency, proper use, and public involvement should inform expert bodies as they develop formal research guidelines for gene drive research in the laboratory and potential transitions to open field trials. We applaud the U.S. National Academy of Sciences for committing to provide recommendations for responsible gene drive research (15). By recommending strong safeguards and encouraging discussion of this technology, we hope to build a foundation of public trust for potential future applications in public health, sustainable agriculture, and ecological conservation.

REFERENCES AND NOTES
13. F. Port et al., G3 (Bethesda) 5, 1493 (2015).

ACKNOWLEDGMENTS
The authors are grateful for conversations with T. Wu, J. Lunshof, and A. Birnbaum. V.M.G., E.B., G.M.C., and K.M.E. are inventors on relevant provisional and nonprovisional patents filed by the University of California and Harvard University.

Published online 30 July 2015

10.1126/science.aac7932

MARTIPOSA

Microbiota RORgulates intestinal suppressor T cells

Gut microbes influence the balance of regulatory T cell subtypes to control inflammation

By Ahmed N. Hegazy1,2 and Fiona Powrie1,2

The immune system in the intestine is highly adapted to resist invading pathogens while residing peacefully with the abundant and diverse commensal bacteria that colonize the gastrointestinal tract. In turn, bacterial signals shape immunity in the intestine, promoting intestinal homeostasis in part by inducing and expanding specialized regulatory T (Treg) cells that prevent aberrant inflammatory responses to self and environmental stimuli (1). On pages 989 and 993 of this issue, Ohnmacht et al. (2) and Sefik et al. (3), respectively, report the development and function of a subgroup of Treg cells found primarily in the large intestine, and characterized by expression of the nuclear hormone receptor retinoic acid receptor-related orphan receptor γ (RORγt). This is surprising because RORγt classically promotes the differentiation of T helper 17 (Th17) cells, a population associated with tissue inflammation in many inflammatory diseases (4). Both studies show that microbiota-derived signals induce the expression of RORγt in Treg cells that control intestinal inflammation (see the figure). These findings highlight the diversity of colonic Treg cells, and their important role in the intestine. Treg cells express the forkhead transcription factor Foxp3, which promotes their differentiation, maintenance, and function (5). Alongside anti-inflammatory functions, they control nonimmunological processes including tissue repair and metabolism in the parenchyma (6). Treg cells also adapt to environmental cues by expressing canonical effector T cell–associated transcription factors to control pathogenic immune responses (7).

Both Ohnmacht et al. and Sefik et al. found that in mice, a large fraction of intestinal Treg cells express RORγt. These cells were distinct from colonic Treg cells that express the transcription factor GATA3 and are poised to respond to the cytokine interleukin (IL)–17 after tissue damage (8, 9). However, RORγt-expressing Treg cells had an activated phenotype similar to that of GATA3-expressing Treg cells, and bore markers related to Treg cells residing in lymphoid and non-lymphoid tissues (6). Strikingly, the microbiota was an absolute requirement for the induction and maintenance of RORγt-expressing Treg cells in these animals. This Treg cell population was markedly reduced in germ-free mice, and colonization with a diverse microbiota or consortia of symbionts was sufficient for the induction of RORγt-expressing Treg cells. Sefik et al. went further and reconstituted germ-free mice with 22 different bacterial species, and showed that a number of them (not belonging to any specific phylum or genus) elicited RORγt-expressing Treg cells at comparable frequencies to a diverse microbiota. Short-chain fatty acids, which are common bacterial metabolites, can selectively expand intestinal Treg cells (10). Ohnmacht et al. could increase RORγt-expressing Treg cells by feeding mice a diet rich in the short-chain fatty acid butyrate.

“...These studies... are an important stepping stone to deciphering the complex dynamics of different tissue-resident Treg cell subsets...”

Which signals promote RORγt expression in Treg cells? The Th17-favoring cytokines IL-6 and IL-23 were required for accumulation of RORγt-expressing Treg cells, which raises the question of what tips the balance toward these T cells rather than Th17 cells. The vitamin A metabolite retinoic acid promotes Treg cell generation in vivo and RORγt-expressing Treg cells in vitro (11, 12). Consistent with this, Ohnmacht et al. show that vitamin A metabolism influences the differentiation equilibrium by favoring the development of RORγt-expressing Treg cells in vivo. Although both Treg cells and Th17 cells express RORγt, analysis of all the transcripts expressed by each population revealed marked differences, suggesting that the transcriptional footprint of RORγt is context-dependent in different T cells. What is the function of RORγt-expressing
Safeguarding gene drive experiments in the laboratory


Science 349 (6251), 927-929.
DOI: 10.1126/science.aac7932 originally published online July 30, 2015