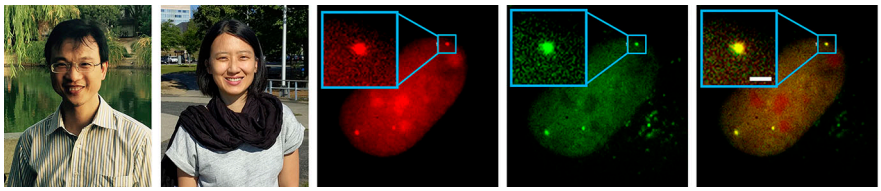
Text by Ben Short
bshort@rockefeller.edu

Tracking CRISPR targeting

Study examines how CRISPR complexes move through the nucleus to find their genomic target.

FOCAL POINT



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Hanhui Ma (left), Li-Chun Tu (right), Thoru Pederson, and colleagues investigate how CRISPR-Cas9 complexes assemble and find their genomic target in living cells. By fluorescently labeling both the Cas9 nuclease (red) and the guide RNA (gRNA, green) that directs it to its target, the researchers highlight the importance of expressing both components at stoichiometric levels for efficient gene targeting. gRNAs, for example, are extremely unstable in the absence of Cas9, whereas Cas9 accumulates in the nucleolus when it is present in excess. In addition, the researchers find that, in vivo, CRISPR complexes remain associated with their target for several hours. This residence time is reduced to just a few minutes when the gRNA contains a mismatch mutation, thus decreasing the efficiency of target cleavage.

Over the last few years, CRISPR has become a vital tool for scientists, as the bacterial acquired immunity system has been adapted to function in eukaryotic cells as a means to edit the genome, modulate gene expression, and label chromosomes for live cell imaging. The biochemical activity of CRISPR complexes—consisting of the Cas9 nuclease and an RNA that guides it to find and cleave a specific DNA sequence—has been well studied in vitro, but little is known about how the complexes function in living cells. Ma and Tu et al. now track the nuclear dynamics of CRISPR complexes to determine how they find their target sequences in vivo, and examine how this process is affected by mutations in the guide RNA (1).

Thoru Pederson, Hanhui Ma, and collaborators at the University of Massachusetts Medical School have successfully used CRISPR to fluorescently label chromosomes (2) and expanded the technology with their colleagues Li-Chun Tu and David Grunwald to visualize chromosome dynamics during interphase (3). "But as we enjoyed these successes, we were increasingly beguiled by how CRISPR is so able to meet our chromosome labeling and tracking goals," Pederson explains. "How does the complex move through the nucleus? How and how long does it interact with chromatin and other nuclear structures?"

To answer these questions, Pederson and colleagues devised a system in which both components of the CRISPR complex are fluorescently labeled in human cells (1). Catalytically dead Cas9 was fused to a conventional red fluorescent protein, while a guide RNA (gRNA) targeting a repetitive DNA sequence specific to chromosome 3 was tagged at its 3′ end with "Broccoli," a short RNA aptamer that binds to a small, cell-permeable, molecule that becomes fluorescent only upon binding to the aptamer (4).

When coexpressed, the two components colocalized as a distinct focus on the target. Surprisingly, however, when the researchers switched off Cas9 expression, the gRNA became virtually undetectable. "The gRNA is extremely unstable in the absence of Cas9," says Pederson. "gRNAs are similar to other small RNAs in the cell, but it seems that the RNA quality control machinery absolutely 'hates' them."

Conversely, when Cas9 was overexpressed compared with the gRNA, the nuclease accumulated in the nucleolus, perhaps because other small RNAs are enriched in this nuclear compartment. "So investigators using CRISPR for gene regulation or editing need to be mindful of the stoichiometric balance of the two components," Pederson advises.

> **"This was totally unexpected and very different from in vitro measurements."**

Ma and Tu et al. then performed a series of fluorescence recovery after photobleaching experiments to examine how the CRISPR complex interacts with its target. "The complex remains on the target for more than three hours," Pederson says. "This was totally unexpected and very different from in vitro measurements." It remains to be seen whether the CRISPR complex has to undergo some sort of additional step in between binding and cleaving its genomic target in vivo. Alternatively, Cas9 may cleave its target soon after binding, followed by a delay in its dissociation.

Even single point mutations in the gRNA reduced the complex's target residence time to less than two minutes and, using a cytological assay to measure cleavage in living cells, the researchers found that this correlated with a reduced efficiency of target cleavage. "So, when people are designing gRNAs, it's critical that the guide match with the target sequence is perfect," Pederson says. "Even one mismatch leads to inefficient cleavage. This could be inferred from important in vitro work that preceded our study but here we have established that this holds in vivo, the theater of CRISPR's envisioned applications."

The results suggest that, in vivo, CRISPR complexes can efficiently find their genomic target because they only interact briefly with even closely matched nontarget sequences. Nevertheless, Pederson says that it will be important to refine their experimental technique so that they can study the CRISPR complex's off-target DNA interactions. The researchers also hope to improve their system so that they can detect CRISPR's association with single-copy targets, rather than just the repetitive sequences they can currently observe.

1. Ma, H., et al. 2016. *J. Cell Biol.* http://dx.doi.org/10.1083/jcb.201604115
2. Ma, H., et al. 2015. *Proc. Natl. Acad. Sci. USA.* 112:3002–3007.
3. Ma, H., et al. 2016. *Nat. Biotechnol.* 34:528–530.
4. Filonov, G.S., et al. 2014. *J. Am. Chem. Soc.* 136:16299–16308.