The research collaboration revealed that CRISPR/Cas9 is a ruthlessly effective viral assassin. CRISPR is the bacterial-coding mechanism that stores a snapshot of the bad guy, the viral DNA. A segment of RNA reads this snapshot of DNA and creates the complementary strand, like a seamstress who can make one side of an RNA zipper that custom matches its DNA complement. The RNA side of the zipper is mated with a pair of very sharp molecular scissors known as an endonuclease.

With amazing speed and specificity, the RNA/endonuclease assassin scans the zipper sections of DNA that it encounters inside the cell. If it finds viral DNA that is a match, this assassin uses the endonuclease scissors to cut the viral DNA in half, destroying its ability to infect the cell.

The resulting paper was published in Science in August 2012, with Jinek and Chylinski as the lead authors. On the first page, at th
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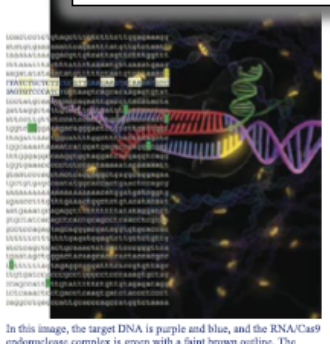
Says Doudna, "Our 2012 paper was a big success, but there was a problem. We weren't sure if CRISPR/Cas9 would work in eukaryotes—plant and animal cells." Unlike bacteria, plant and animal cells have a cell nucleus, and inside, DNA is stored in a tightly wound form, bound in a structure called chromatin.

The CRISPR/Cas9 system evolved to fight viral invaders inside the relatively simple cells of prokaryotes—bacteria and their closely related cousin, archaea. "My lab began to explore how well CRISPR/Cas9 would work in eukaryotic cells," says Doudna. "Meanwhile, the research groups of George Church at Harvard, and of Feng Zhang at MIT, were also working hard to see if they could get CRISPR/Cas9 to function in eukaryotic cells."



In this image, the target DNA is purple and blue, and the RNA/Cas9 endonuclease complex is green with a faint brown outline. The RNA/endonuclease is attracted to a three-base-pair DNA sequence called protospacer adjacent motif (yellow). The target DNA sequence is recognized when the RNA matches the DNA along a 20-base-pair

Ex. 2207, Catalyst at 3

One early form of CRISPR-based gene therapy could involve editing the genes responsible for blood disorders like sickle-cell anemia in bone marrow cells, growing them into mature blood cells and injecting them back into patients.

Little more than a year after **Doudna first described CRISPR (http://www.sciencemag.org/content/337/6096/816.short)** in the journal ***Science***, the cut-and-paste technology has yielded promising results in labs around the world. Last month, researchers from the Netherland's Utrecht institute reported in ***Cell Stem Cell*** that CRISPR **corrected the gene mutation responsible for cystic fibrosis (http://www.the-scientist.com/?articles**

Doudna experienced "many frustrations" getting CRISPR to work in human cells. But she knew if she succeeded, CRISPR would be "a profound discovery" — and maybe even a powerful gene therapy technique.

showing that **CRISPR can cut, delete and replace genes in human cells (https://newscenter.berkeley.edu/2013/01/07/cheap-and-easy-technique-to-snip-dna-could-revolutionize-gene-therapy/)**. University of Massachusetts biologist Craig Mello, who shared the 2006 Nobel Prize for another genome editing tool, hails **Doudna's CRISPR technique (http://www.independent.co.uk/news/science/exclusive-jawdropping-breakthrough-hailed-as-landmark-in-fight-against-hereditary-diseases-as-crispr-technique-heralds-genetic-revolution-8925295.html)** as a "tremendous breakthrough," even admitting that "in many ways it's better" than his own technique.

Other techniques can also edit genes at specific DNA regions. But they require scientists to engineer a separate protein for each target site. In contrast, CRISPR only needs the Cas9 protein, allowing it to correct multiple defects at once. Besides being cheaper and easier to use, CRISPR is also much more precise, reducing the risk of off-target modifications introducing dangerous mutations. As a result, it could help revive the gene therapy field, whose early clinical failures — including patient deaths — led some to dismiss it as overhyped.

That doesn't mean CRISPR is perfect, though. While it's extremely precise, it occasionally modifies DNA at similar sites elsewhere in the genome instead of the target gene. Understanding and exploiting how Cas9 avoids these close matches "is an active area of investigation," Doudna said. Still, CRISPR is "a real game-changer

Ex. 2230, Pandika at 3

eLIFE elife.elifesciences.org RESEARCH ARTICLE

RNA-programmed genome editing in human cells

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National Laboratory, Berkeley, United

Abstract Type II CRISPR immune system Cas9, to cleave foreign DNA at specific site RNAs in human cells and can induce the for complementary to the guide RNA sequen both Cas9 and the complementary binding transfected cells show that RNA expression

eLIFE Research article Genomics and evolutionary biology | Human biology and medicine

eLife digest The ability to make specific changes to DNA—such as changing, inserting or deleting sequences that encode proteins—allows researchers to engineer cells, tissues and organisms for therapeutic and practical applications. Until now, such genome engineering has required the design and production of proteins with the ability to recognize a specific DNA

These findings suggested the exciting possibility that Cas9:sgRNA complexes might constitute a simple and versatile RNA-directed system for generating DSBs that could facilitate site-specific genome editing. However, it was not known whether such a bacterial system would function in eukaryotic cells.

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We show here that Cas9 can be expressed and localized to the nucleus of human cells, and that it assembles with sgRNA in vivo.These complexes can generate double stranded breaks and stimulate non-homologous end joining (NHEJ) repair in genomic DNA at a site complementary to the sgRNA sequence, an activity that requires both Cas9 and the sgRNA. Extension of the RNA sequence at its 3′ end enhances DNA targeting activity in vivo. Further, experiments using extracts from transfected cells show that sgRNA assembly into Cas9 is the limiting factor for Cas9-mediated DNA cleavage. These results demonstrate the feasibility of RNA-programmed genome editing in human cells.

Results

To test whether Cas9 could be programmed to cleave genomic DNA in vivo, we co-expressed Cas9 together with an sgRNA designed to target the human clathrin light chain (CLTA) gene. The CLTA genomic locus has previously been targeted and edited using ZFNs (*Doyon et al., 2011*). We first tested the expression of a human-codon-optimized version of the *Streptococcus pyogenes* Cas9 protein and sgRNA in human HEK293T cells. The 160 kDa Cas9 protein was expressed as a fusion protein bearing an HA epitope, a nuclear localization signal (NLS), and green fluorescent protein (GFP) attached to the C-terminus of Cas9 (*Figure 1A*). Analysis of cells transfected with a vector encoding the GFP-fused Cas9 revealed abundant Cas9 expression and nuclear localization (*Figure 1B*). Western blotting confirmed that the Cas9 protein is expressed largely intact in extracts from these cells (*Figure 1A*). To program Cas9, we expressed sgRNA bearing a 5′-terminal 20-nucleotide sequence complementary to the target DNA sequence, and a 42-nucleotide 3′-terminal stem loop structure required for Cas9 binding (*Figure 1C*). This 3′-terminal sequence corresponds to the minimal stem-loop structure that has previously been used to program Cas9 in vitro (*Jinek et al., 2012*). The expression of this sgRNA was driven by the human U6 (RNA polymerase III) promoter (*Medina and Joshi, 1999*). Northern blotting analysis of RNA extracted from cells transfected with the U6 promoter-driven sgRNA plasmid expression vector showed that the sgRNA is indeed expressed, and that their stability is enhanced by the presence of Cas9 (*Figure 1D*).

Next, we investigated whether site-specific DSBs are generated in HEK293T cells transfected with Cas9-HA-NLS-mCherry and the CLTA1 sgRNA. To do this, we probed for minor insertions and deletions in the locus resulting from imperfect repair by DSB-induced NHEJ using the Surveyor nuclease

Ex. 1057, Jinek 2013 at 1-2