IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicant | Feng Zhang |
| Serial No. | 14/054,414 |
| For | CRISPR-CAS SYSTEMS AND METHODS FOR ALTERING EXPRESSION OF GENE PRODUCTS |
| Filed | October 15, 2013 |
| Examiner | LEITH, NANCY J |
| Art Unit | 1636 |
| Confirmation No. | 4798 |

1633 Broadway
New York, NY 10019

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ON January 30, 2014

**DECLARATION UNDER 37 C.F.R. §§ 1.132 AND 1.131**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Feng Zhang, declare and state that:

***Introduction And Qualifications.***

1. This Declaration is in connection with US application Serial No. 14/054,414 (the '414 application or the present application), on which I am the named inventor. I am familiar with the '414 application and its prosecution. My *Curriculum vitae* is attached as Exhibit 1. I am over 18 years of age and have no impediment to making this Declaration. As indicated on my attached *Curriculum vitae* (Exhibit 1), I received my A.B. in chemistry and physics from Harvard College and my Ph.D. in chemistry from Stanford University. I am an active researcher in the field of the '414 application and conduct this research on behalf of The Broad Institute of MIT and Harvard and the McGovern Institute for Brain Research, MIT. I am an assistant

professor at MIT with a joint appointment in the Departments of Brain and Cognitive Sciences and Biological Engineering and I am an author on seminal primary articles in reputable peer-reviewed journals including Nature, Science, Cell, Nature Biotechnology, Nature Communications, and Nature Protocols in the field of the '414 application. Thus, I have personal knowledge as to matters that I am stating in this Declaration. I respectfully submit that in view of my education, training and experience, I am considered by my peers to be an expert in the field to which the '414 application pertains, and qualified to knowledgeably opine on and characterize publications pertaining to '414 application, and to speak as to the '414 application, and the claims of '414 application (Exhibit 2). I particularly respectfully submit that I am qualified, in view of my education, training and experience, to present expert opinions about the claims of the '414 application (Exhibit 2), documents in support of those claims, and the documents I understand have been cited by the Examiner.

2. This Declaration is made in connection with my understanding that the Examiner of the '414 application alleges that claims 1-20 (as set forth in attached Exhibit 2) are anticipated by Jinek et al. PCT Patent Application Publication No. WO2013/176772 (the '772 publication) published November 28, 2013, claiming priority to US Provisional Patent Application No. 61/652,086 filed May 25, 2012 (the '086 priority application). This Declaration also addresses US Provisional Patent Application No. 61/716,256 filed October 19, 2012 (the '256 priority application) in the lineage of the '772 publication. I have familiarity with the '772 publication, the '086 priority application, the '256 priority application, and US Provisional Patent Application No. 61/757,640 filed January 28, 2013 (the '640 priority application). In addition, I have read and understand the Declaration of Le Cong that I understand is being concurrently submitted and concur therewith.

***The '772 Publication Via The '086 Priority Application And The '256 Priority Application Fails To Teach Or Suggest The Invention Of The '414 Application—And Especially Fails To Anticipate That Which Is Claimed In The '414 Application.***

**THE LITERATURE DEMONSTRATES THAT THE '256 PRIORITY APPLICATION FAILS TO TEACH OR SUGGEST THE PRESENT INVENTION**

3. I understand that the Examiner alleges that the '772 publication via the '086 priority application and the '256 priority application discloses each and every element of claims 1-20. I submit that the '772 publication does not via the '086 priority application and the '256

priority application does not and cannot disclose each and every element of claims 1-20, including because as evidenced by the literature, persons named as the inventors on the '772 publication state that the CRISPR-Cas system was not known to function in eukaryotic cells at the time of the '086 priority application and/or the '256 priority application.

3.1 First, a clear indication that neither the '086 priority application nor the '256 priority application teaches or suggests the present invention is the text at Example 2 of the '772 publication (which Example does NOT appear until the '640 priority application), namely, "Data provided below demonstrate that Cas9 can be expressed and localized to the nucleus of human cells and that it assembles with single-guide RNA ('sgRNA'; encompassing the features required for both Cas9 binding and DNA target site recognition) in a human cell." That is, this sentence states that the text of the '086 priority application and the '256 priority application did NOT have "the features required for both Cas9 binding and DNA target site recognition [] in a human cell." (The '640 priority application corresponds to the Jinek et al eLife article of Exhibit 3 hereto, discussed *infra*.)

3.2 Second, the text at Example 2 of the '772 publication (which Example does NOT appear until the '640 priority application) parallels that which is stated in Jinek et al, eLife 2013;2:e00471, published January 29, 2013 (the eLife article, Exhibit 3), that "Research into genome defense mechanisms in bacteria showed that CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) loci encode RNA-guided adaptive immune systems that can destroy foreign DNA" and cites findings in publications by Bhaya et al., 2011, Gasiunas et al., 2012, Deltcheva et al., 2011, Jinek et al., 2012, Terns and Terns, 2011, Wiedenheft et al., 2012 and Sapranauskas et al., 2011, further stating:

> 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** [emphasis added].

This clearly indicates that the information in the '772 publication prior to the incorporation of the information from the eLife article does NOT relate to a functional CRISPR-Cas system that exhibits actual genomic cleavage in a eukaryotic cell.

3.3 Third, in the recent article "Right on target: New era of fast genetic engineering" by Colin Barras, New Scientist, 28 January 2014, Magazine issue 2953 (Exhibit 4), it is stated,

> **But Charpentier and Doudna's study involved targeting bits of DNA floating loose in a tube.** Would the bacterial system work inside the complex cells of animals and plants? **Doudna's team** set out to test this but they **were beaten to it by Zhang's team** and that of George Church at Harvard University, both of which reported in the first week of 2013 that the CRISPR-Cas9 system worked beautifully in human cells (*Science*, vol 339, p 819 and p 823). **This was a vital demonstration says Doudna**, whose own study appeared just three weeks later.

This clearly indicates that the '086 priority application and the '256 priority application did NOT provide evidence that the CRISPR-Cas system could function in a eukaryotic cell, particularly a human cell (*see also* discussions below of difficulties in extrapolating to eukaryotic cells and concerning Example 1 of the '086 priority application).

3.4 And further, despite the several overstatements and misrepresentations in the article "Jennifer Doudna, CRISPR Code Killer" published on-line at http://www.ozy.com/rising-stars-and-provocateurs/jennifer-doudna/4690.article on January 7, 2014 (Exhibit 5), it is stated that (with emphasis added) "**Doudna experienced 'many frustrations' getting CRISPR to work in human cells**." Hence, there is no doubt that the '086 priority application and the '256 priority application lack the necessary evidence to show that the CRISPR-Cas system could function in a eukaryotic cell.

3.4.1 Also noteworthy in the article "Jennifer Doudna, CRISPR Code Killer" is the following statement: "Thrilled, **Doudna** immediately **contacted Church. They shared their results**, and both published studies in January 2013 showing that CRISPR can cut, delete and replace genes in human cells" (emphasis added). In this regard, I also note the acknowledgement section of the Jinek eLife publication: "**We thank** David Druhin, Barbara Meyer and Te-Wen Lo for helpful discussions and expert advice; Jamie Cate, Andy May and Rachel Haurwitz for comments on the manuscript; **George Church for sharing unpublished data**; and Kaihong Zhou and Alison Smith for excellent technical support." (emphasis added). I respectfully question the origin of the Example that only first appears in January 28, 2013-filed '640 priority application in the lineage of the '772 publication.

3.5 Clearly, in view of the difficulties in extrapolating to eukaryotic cells (*see, e.g.,* discussion below), and the literature, including all the admissions by named inventors on the '772 publication: the '086 priority application, the '256 priority application and the '772 publication via the '086 priority application and the '256 priority application do not anticipate and could not have even suggested the instantly claimed invention. I submit that any reference to CRISPR-Cas system functioning in a eukaryotic cell in the '086 priority application or the '256 priority application is mere conjecture as there was no conception or reduction to practice until there was an actual reduction to practice. It is this actual reduction to practice that is completely lacking in the '086 priority application and the '256 priority application, and the difficulties in extrapolating to eukaryotic cells (*see, e.g.,* discussion below), and the literature, including all the admissions by named inventors on the '772 publication, it is respectfully submitted, indicate that an actual reduction to practice is necessary to having an enabling written description—that is, an anticipating disclosure or a novelty-destroying disclosure—or even a suggestion of the presently claimed invention. As such is lacking in the '086 priority application and the '256 priority application, the '772 publication via the '086 priority application and the '256 priority application do not anticipate and could not have even suggested the instantly claimed invention.

**THE '256 PRIORITY APPLICATION AND THE '086 PRIORITY APPLICATION FAIL TO ENABLE OR DESCRIBE THE PRESENT INVENTION, AND HENCE FAIL TO TEACH THE PRESENT INVENTION; AND IN VIEW OF THE AFOREMENTIONED LITERATURE, THE PRESENT INVENTION IS SURPRISING, UNEXPECTED AND NONOBVIOUS**

4. I have read and understood all the rejections raised in the January 13, 2014 Office Action. It is my understanding that for a reference to anticipate or destroy novelty, the reference needs to fully enable and describe a claimed invention. In this regard, I understand that for there to be invention there needs to be conception and reduction to practice, and that in certain instances where there is unpredictability, for there to be conception there must be an actual reduction to practice, i.e., an actual working of the invention. That is, I understand that in certain instances there needs to be simultaneous conception and actual reduction to practice for there to be invention, and hence for there to be an enabling written description of such an invention in a patent application, there needs to be an actual reduction to practice, i.e., an actual working example. In the instance of the CRISPR-Cas system working in a eukaryotic cell as claimed in

the present application, I assert that there must be simultaneous conception and actual reduction to practice for there to be invention, and hence an actual working example in a previously-filed patent application for that patent application to have an enabling written description to anticipate or destroy novelty as to the CRISPR-Cas system working in a eukaryotic cell as claimed in the present application. In view of my education, training and experience, I submit that there is absolutely no teaching whatsoever in the '086 priority application and the '256 priority application that enables and describes the CRISPR-Cas system working in a eukaryotic cell as claimed in the present application. The lack of an actual reduction to practice in both the '086 priority application and the '256 priority application is a serious and critical deficiency in the '086 priority application and the '256 priority application. The lack of an actual reduction to practice in both the '086 priority application and the '256 priority application is a basis for how and why these applications fail to anticipate the CRISPR-Cas system working in a eukaryotic cell as claimed in the present application. Indeed, there are several critical criteria that have to be met to show that an engineered prokaryotic system can function in a novel eukaryotic environment. For example, eukaryotic cells present an entirely different cellular context than the bacterial intracellular environment of natural wild type CRISPR cascade. In order to engineer a CRISPR-Cas type II system (e.g. a CRISPR-Cas9 system) to work in the non-natural (to it) eukaryotic environment, it is important to ensure not only that the protein and RNAs are successfully expressed in the eukaryotic cell at a level necessary to achieve activity, but also that the protein and RNAs are appropriately processed and folded into the proper conformation, and that they are efficiently transported into the desired intracellular location (e.g. organelle), and are not degraded by endogenous protein recycling machineries (e.g. the ubiquitin system). Furthermore, the temperature and ion composition status of eukaryotic cells are not compatible with the intended catalytic activity. Given the inherent differences between prokaryotic cells and eukaryotic cells, e.g. bacterial cells are inherently different from human cells, it is impossible to predict whether a prokaryotic protein:RNA:DNA complex would be able to assemble, much less carry out the same function when introduced into an eukaryotic cell. Quite simply, there was no reasonable expectation of success as to the CRISPR-Cas system working in a eukaryotic cell as claimed in the present application, including as evidenced by the articles concerning the "many frustrations" Doudna experienced in attempting to get the system to work in eukaryotic cells. To provide yet another example, the Group II intron (Lambowitz, A. M. & Zimmerly, S. Group II

introns: mobile ribozymes that invade DNA. Cold Spring Harbor perspectives in biology 3, a003616 (2011). Pubmed), which utilizes a combination of protein and RNA has been successfully used for genome editing in prokaryotic cells, but has not been successfully translated into eukaryotic cells. One of the challenges described by researchers attempting to develop the Group II intron for eukaryotic cells has been related to ion concentrations, as reported in Mastroianni M et al. (2008) Group II Intron-Based Gene Targeting Reactions in Eukaryotes. PLoS ONE 3(9): e3121. doi:10.1371/journal.pone.0003121. Accordingly, I reiterate that in the '086 priority application and the '256 priority application there is also absolutely no enabling written description or suggestion of the CRISPR-Cas system working in a eukaryotic cell as claimed in the present application.

**The '086 Priority Application Especially Fails To Describe Or Suggest The Present Invention**

4.1 I understand that the Examiner alleges that at least paragraphs [0033], [0083], and [00165] of the '086 priority application discloses methods of, and compositions and CRISPR-Cas systems for interfering with a target DNA sequence in both prokaryotic and eukaryotic cells using CRISPR RNA (crRNA) and a CRISPR-associated (cas) protein/nucleic acid, where the cas protein is Cas9. I submit that in the '086 priority application there is no teaching whatsoever that enables and describes methods of, and compositions and CRISPR-Cas systems for interfering with a target DNA sequence in a prokaryotic or a eukaryotic cell.

4.2 I understand that the Examiner alleges that at least paragraphs [00157]-[00159] of the '086 priority application) discloses a method of inhibiting the function of a DNA target sequence, which will reduce the expression of a gene in cells by interfering with transcription of the sequence, which can include knock-out experiments. I submit that in the '086 priority application there is no teaching whatsoever that enables and describes a method of inhibiting the function of a DNA target sequence, which will reduce the expression of a gene in a eukaryotic cell by interfering with transcription of the sequence, which can include knock-out experiments.

4.3 I understand that the Examiner alleges that at least paragraphs [0083], [0091]-[0092], and [00132]-[00133] of the '086 priority application discloses an engineered system and vectors that comprise a regulatory sequence linked to an RNA sequence that guides the system to the target sequence by base pairing, and where the RNA sequences and Cas

sequences can be naturally-occurring or non-naturally occurring (i.e., programmable). I submit that in the '086 priority application there is no teaching whatsoever that enables and describes an engineered system and vectors that comprise a regulatory sequence linked to an RNA sequence that guides the system to the target sequence by base pairing, and where the RNA sequences and Cas sequences can be naturally-occurring or non-naturally occurring (i.e., programmable) in a cell.

4.4 I understand that the Examiner alleges that at least paragraphs [00153] and [00160] of the '086 priority application discloses that a promoter can be utilized in the system to drive expression of the system, as well as other regulatory sequences, which can be employed with both the guide RNA and the Cas9 polypeptide. I submit that in the '086 priority application there is no teaching whatsoever that enables and describes a promoter being utilized in the system to drive expression of the system, as well as other regulatory sequences, which can be employed with both the guide RNA and the Cas9 polypeptide in a eukaryotic cell.

4.5 I understand that the Examiner alleges that at least paragraphs [0077] and [00187] of the '086 priority application discloses that the system can be used to target multiple genes in a cell, and will inhibit function of those genes, or even provide for a knock-out targeted genes (i.e., expression of those genes will be decreased). I submit that in the '086 priority application there is no teaching whatsoever that enables and describes a system can be used to target multiple genes in a cell, and will inhibit function of those genes, or even provide for a knock-out targeted genes (i.e., expression of those genes will be decreased) in a eukaryotic cell.

4.6 I understand that the Examiner alleges that at least paragraph [00159] of the '086 priority application discloses that the presence of a nuclear localization signal allows for the system to provide for subcellular localization. I submit that there is no teaching in the '086 priority application whatsoever that enables and describes the use of a nuclear localization signal that allows for the system to provide for subcellular localization in a eukaryotic cell.

4.7 The Examiner alleges that at least paragraph [0083] of the '086 priority application discloses that the RNA guide sequences can be fused to tracr sequences. I submit that in the '086 priority application there is no teaching whatsoever that enables and describes RNA guide sequences that can be fused to tracr sequences in a eukaryotic cell.

4.8 The Examiner alleges that at least paragraphs [00165]-[00166] of the '086 priority application discloses that the Cas9 can be codon optimized for use in eukaryotic cells,

such as human cells. I submit that in the '086 priority application there is no teaching whatsoever that enables and describes the Cas9 being codon optimized for use in eukaryotic cells, such as human cells.

4.9 The Examiner alleges that at least [paragraphs [00165]-[00166] of the '086 priority application discloses that the CRISPR/Cas system can be used to generate target mutants in mammalian cells, including human cells. I submit that in the '086 priority application there is no teaching whatsoever that enables and describes the CRISPR/Cas system can be used to generate target mutants in mammalian cells, including human cells.

4.10 In view of the foregoing, I submit that it is clear that the '772 publication via the '086 priority application and the '256 priority application fails to anticipate the presently claimed invention. I further submit that Example 1 of the '086 priority application does not deal with the CRISPR-Cas system working in any cell but rather solely deals with *in vitro* conditions in which the recombinant DNA-targeting polypeptide was heterologously expressed in *E.coli* and further purified and then used to cleave target DNA *in vitro*. In view of the foregoing, and especially the various statements in the art, it is surprising that I was able to obtain that which is claimed in the present application. I understand obtaining that which was unexpected or surprising evinces nonobviousness. I accordingly assert that the claimed invention in the present application is novel and nonobvious in views over the '772 publication via the '086 priority application and the '256 priority application in view of this Declaration and that which is herein cited.

***As Much As Each Of The '086 Priority Application And The '256 Priority Application Shows Is Antedated.***

5. I understand that under the US Patent Law to which the present application is subject, if I can show conception and actual reduction to practice prior to the filing dates of the '086 priority application and the '256 priority application, in certain territories (particularly the United States), of at least as much as each of the '086 priority application and the '256 priority application discloses, then I have removed the '086 priority application and the '256 priority application from being prior art against the present application, and I have thus removed the '772 publication from being a reference against the present application.

5.1 Attached hereto as Exhibit 7 are redacted records of experiments that were performed by me or under my direction, supervision and/or control in ordinary course of

business of the Broad Institute in the USA, prior to May 2012, i.e., prior to the respective filing dates of each of the '086 priority application and the '256 priority application. Where work of Exhibit 7 was done under my direction, supervision and/or control, this work was also reported to me, in the USA, prior to May 2012, i.e., prior to the respective filing dates of each of the '086 priority application and the '256 priority application. I am empowered by the Broad Institute to speak as to the documents of Exhibit 7 and the work reported therein and introduce these documents into evidence, including because the work reported therein was done by me or under my direction, supervision and/or control in ordinary course of business of the Broad Institute in the USA, and because the documents of Exhibit 7 are maintained by me and the Broad Institute in the USA in ordinary course of business of the Broad Institute. With respect to all statements herein as to the documents of Exhibit 7, the foregoing statements all apply. Also attached as Exhibit 6 is the manuscript Cong et al. "CRISPR-Assisted Mammalian Genome Engineering". I submitted this manuscript to the journal Science on October 5, 2012, prior to the October 19, 2012 filing date of the '256 priority application. Exhibit 6 reports records of experiments that were performed by me or under my direction, supervision and/or control in ordinary course of business of the Broad Institute in the USA, prior to October 5, 2012, i.e., prior to the filing date of the '086 priority application. Where work of Exhibit 6 was done under my direction, supervision and/or control, this work was also reported to me, in the USA, prior to October 5, 2012, i.e., prior to the filing date of the '256 priority application. I am empowered by the Broad Institute to speak as to Exhibit 6 and the work reported therein and introduce this document into evidence, including because the work reported therein was done by me or under my direction, supervision and/or control in ordinary course of business of the Broad Institute in the USA, and because Exhibit 6 is maintained by me and the Broad Institute in the USA in ordinary course of business of the Broad Institute. With respect to all statements herein as to Exhibit 6, the foregoing statements all apply.

5.1.1 As the October 5, 2012 Manuscript (Exhibit 6) shows at least as much as (and much more than) in the '256 priority application, I respectfully assert that I have removed the '772 publication via the '256 priority application from being prior art to the present application. In this regard, Exhibit 6 shows that the CRISPR crRNA processing and interference system was reconstituted in mammalian cells and demonstrates that the Cas9 nuclease could be targeted to specific genomic loci by short crRNA guides to induce DNA double strand breaks.

The disclosure in the October 5, 2012 Manuscript (Exhibit 6) parallels that which is in US provisional application Serial No. 61/736,527, filed December 12, 2012, in the lineage of the present application. Hence the manuscript and the December 12, 2012 provisional application provides support for claims 1-20. I understand that this showing removes the '772 publication and the '256 priority application from being prior art with regard to the present application.

5.1.2 As the documents of Exhibit 7 show at least as much as (and much more than) in the '086 priority application, I respectfully assert that I have removed the '772 publication via the '086 priority application from being prior art to the present application. The documents of Exhibit 7 are redacted records, e.g., to remove dates. The documents of Exhibit 7 describe and enable that which is claimed in the present application and read on methods of, and compositions and CRISPR-Cas systems for altering expression of at least one gene product by introducing into a eukaryotic cell a CRISPR-Cas system that functions in a eukaryotic cell.

5.1.2.1 In particular, Page Z-2 of Exhibit 7 shows the setup of an experiment in which the expression of luciferase is decreased by a CRISPR-Cas system comprising a spacer (guide sequence) and a Streptococcus thermophilus Csn-1 (Cas5 or Cas9) enzyme, when vectors encoding the spacer (guide sequence), the Csn-1 (Cas5 or Cas9) enzyme and luciferase are transfected into a eukaryotic cell (human embryonic kidney cells, 293FT). The spacers (guide sequences) used in the experiment are depicted as Sp2 and Sp3 and the control is a junk spacer depicted as Jk. Note the "guide sequence" recitations of the claims.

5.1.2.2 In particular, Pages Z-3 and Z-4 of Exhibit 7 further show the selection of spacers (guide sequences) to be used in the experiment for insertion into a backbone vector and the basic flowchart to clone these spacers (guide sequences) into the backbone vector to target DNA, respectively. On page Z-3, Insert 2 and Insert 3 refer to Sp2 and Sp3 on page Z-2, respectively. Note the "guide sequence" recitations of the claims.

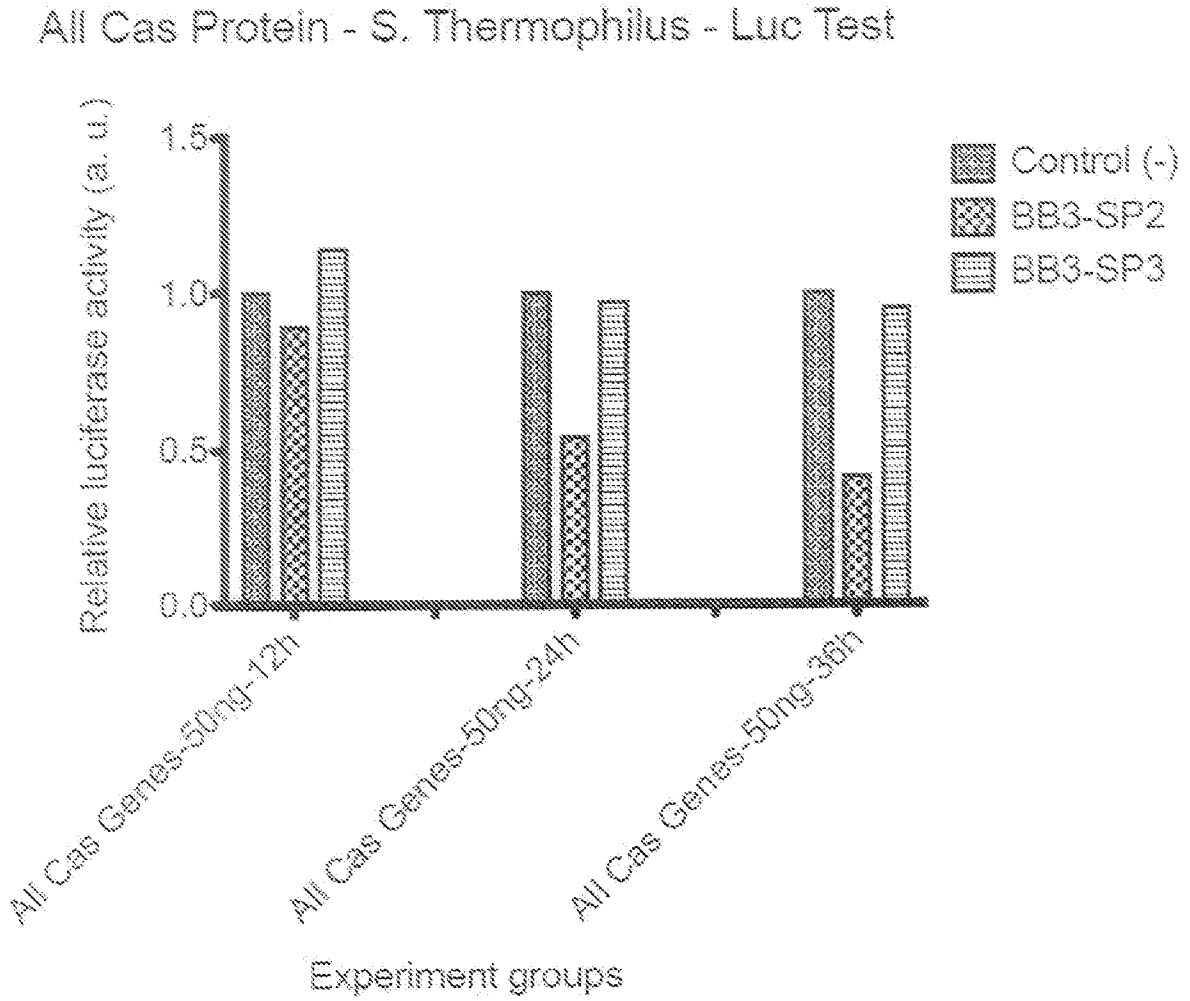
5.1.2.3 In particular, Pages Z-5 to Z-23 of Exhibit 7 show a vector with a CMV promoter and a human codon optimized Csn-1 (Cas 5 or Cas9) that was engineered and ordered from DNA2.0. A nuclear localization signal is present in the construct as indicated on page Z-8 of Exhibit 7. Note the "Cas9", "NLS" and codon optimized recitations of the claims.

5.1.2.4 In particular, Page Z-24 of Exhibit 7 shows the backbone vector into which the spacers (guide sequences) were cloned. In this regard, note the "guide sequence" recitations of the claims.

5.1.2.5 In particular, Pages Z-25 to Z-26 of Exhibit 7 shows the experimental protocol followed to transfect eukaryotic cells (human embryonic kidney cells, 293FT) with the various vectors and how to test and measure site-specificity of endonuclease activity. In this regard, note the "eukaryotic" claim recitation.

5.1.2.6 In particular, Page Z-27 of Exhibit 7 shows the quantification of data after the experiment was performed. A reduction of luciferase expression was seen at each subsequent time point from 12 hr, 24 hr and 36 hr at which the relative luciferase activity was measured for Sp2 at a concentration of 50ng (as seen represented in the following panel provided for summary purposes of that which is shown in Exhibit 7).

5.1.3 The following panel represents my understanding of the data of Exhibit 7 that I had prior to May 2012, when I received that data, prior to May 2012.




5.1.4 It is clearly seen from the data of Exhibit 7 (also summarized in the panel set forth above) that there is a reduction in luciferase activity for the experiment conducted with Sp2 as compared to the control and Sp3. I submit that this provides support for methods of, and compositions and CRISPR-Cas systems for altering expression of at least one gene product by introducing into a eukaryotic cell a CRISPR-Cas system that functions in a eukaryotic cell as claimed in the present invention. In this regard, note the claim recitations, "at least one guide RNA that targets and hybridizes to a target sequence of a DNA molecule in a eukaryotic cell, wherein the DNA molecule encodes and the eukaryotic cell expresses at least one gene product and the Cas9 protein cleaves the DNA molecule, whereby expression of the at least one gene product is altered."

5.1.5 Indeed, I submit that Exhibit 7 shows that prior to May 2012, I conceived and reduced to practice the presently claimed subject matter, including:

A method of altering expression of at least one gene product comprising introducing into a eukaryotic cell containing and expressing a DNA molecule having a target sequence and encoding the gene product an engineered, non-naturally occurring vector system comprising one or more vectors comprising:
a) a first regulatory element operable in a eukaryotic cell operably linked to at least one Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR associated (Cas) system guide RNA that hybridizes with the target sequence,
b) a second regulatory element operable in a eukaryotic cell operably linked to a Type-II Cas9 protein,
wherein components (a) and (b) are located on same or different vectors of the system,
whereby the guide RNA targets the target sequence and the Cas9 protein cleaves the DNA molecule, whereby expression of the at least one gene product is altered; and, wherein the Cas9 protein and the guide RNA do not naturally occur together

and

An engineered, non-naturally occurring vector system comprising one or more vectors comprising:
a) a first regulatory element operable in a eukaryotic cell operably linked to at least one CRISPR-Cas system guide RNA that hybridizes with a target sequence of a DNA molecule in a eukaryotic cell that contains the DNA molecule, wherein the DNA molecule encodes and the eukaryotic cell expresses at least one gene product,
b) a second regulatory element operable in a eukaryotic cell operably linked to a Type-II Cas9 protein,
wherein components (a) and (b) are located on same or different vectors of the system,
whereby the guide RNA targets and hybridizes with the target sequence and the Cas9 protein cleaves the DNA molecule, whereby expression of the at least one

gene product is altered; and, wherein the Cas9 protein and the guide RNA do not naturally occur together

and

An engineered, programmable, non-naturally occurring Type II CRISPR-Cas system comprising a Cas9 protein and at least one guide RNA that targets and hybridizes to a target sequence of a DNA molecule in a eukaryotic cell, wherein the DNA molecule encodes and the eukaryotic cell expresses at least one gene product and the Cas9 protein cleaves the DNA molecule, whereby expression of the at least one gene product is altered; and, wherein the Cas9 protein and the guide RNA do not naturally occur together.

I also submit that Exhibit 6 shows that prior to October 5, 2012 I conceived and reduced to practice the presently claimed subject matter.

6. In view of the foregoing and the documents herewith, I respectfully assert that I have shown that the '772 publication fails to teach or suggest the presently claimed invention and that the '772 publication is removed from being prior to the present application. I respectfully request that the rejection based on the '772 publication be reconsidered and withdrawn and that the present application be allowed.

7. I declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001.

Date: Jan 30, 2014

Feng Zhang

Feng ZHANG

# EXHIBIT 1

| Feng Zhang, Ph.D. | POSITION TITLE<br>Core Faculty Member, Broad Inst of MIT and Harvard<br>Investigator, McGovern Institute<br>W.M. Keck Career Development Professor in Biomedical Engineering, MIT |
|---|---|

EDUCATION/TRAINING

| INSTITUTION AND LOCATION | DEGREE | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| Harvard College, Cambridge, MA, USA | A.B. | 09/00-06/04 | Chemistry and Physics |
| Stanford University, Stanford, CA, USA | Ph.D. | 09/04-06/09 | Chemistry |

**A. Positions and Honors**

**Positions**

1/11- Core Member, Broad Institute of MIT and Harvard
Investigator, McGovern Institute for Brain Research, MIT
W.M. Keck Career Development Professor in Biomedical Engineering, MIT
Assistant Professor of Brain and Cognitive Sciences, MIT
Assistant Professor of Biological Engineering, MIT
8/09-12/10 Junior Fellow, Society of Fellows, Harvard
9/04-6/09 Graduate Student (Stanford) with Karl Deisseroth M.D. Ph.D.
5/02-8/04 Research Assistant (Harvard) with Xiaowei Zhuang Ph.D.
10/00-11/01 Research Assistant (Harvard) with Don Wiley, Ph.D.
10/97-6/99 Research Assistant (Human Gene Therapy Research Inst, Des Moines, IA) with John Levy Ph.D.

**Selected Academic and Professional Honors**

2013 MIT Technology Review Innovators Under 35 Award
2013 Popular Science Brilliant 10 Award
2013 Vallee Foundation Young Investigator Award
2012 Merkin Institute Fellow
2012 National Academy of Sciences Kavli Fellow
2012 NIH Director's Pioneer Award
2012 W. M. Keck Foundation Medical Research Award
2012 Klingenstein Fellowship
2012 Searle Scholar
2012 Perl/UNC Neuroscience Prize (for optogenetics)
2012 Australian Neuroscience Society Overseas Keynote
2011 Damon Runyon Cancer Research Foundation Technology Innovation Award
2011 McKnight Foundation Technological Innovations in Neuroscience Award
2010 Nature Medicine/Roche Junior Award for Translational Neuroscience
2009 Junior Fellowship, Society of Fellows, Harvard University
2008 Lightspeed Venture Partners Startup Seed Funding
2008 Larry M. Katz Memorial Lecture at Cold Spring Harbor Laboratories for Best Graduate Thesis
2007 BMEidea National Biomedical Engineering Competition Second Place
2007 Ruth L. Kirschstein National Research Service Award
2007 Global Life Science Innovation Competition, Finalist
2006 Stanford Graduate School of Business Fellow
2000-04 Highest Honors in Chemistry and Physics (Harvard)
2001 Gold Award, ThinkQuest International Internet Challenge, for *Cellupedia* ($20,000 scholarship)
2000 First Team Member, USAToday All-USA High School Academic Team
2000 Third Place, Intel Science and Talent Search ($50,000 scholarship)
1999 Pinnacle Award, Intel International Science and Engineering Fair ($40,000 scholarship)

**B. Publications (* denotes equal contribution or co-correspondence, in reverse chronological order)**

1. Shalem O, Sanjana NE, Hartenian E, Shi X, Scott DA, Mikkelson T, Heckl D, Ebert BL, Root DE, Doench JG, and **Zhang F**. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 2013

2. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, and **Zhang F**. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*. 2013 Nov;8(11):2281-308.
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# EXHIBIT 2

**AMENDMENT**

Kindly amend the present application, without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents, as follows.

**In the Claims:**

1. (Currently amended) A method of altering expression of at least one ~~or more~~ gene product[[s]] comprising introducing into a eukaryotic cell containing and expressing a DNA molecule[[s]] having a target sequence and encoding the ~~one or more~~ gene product[[s]] an engineered, non-naturally occurring vector system comprising one or more vectors comprising:
a) a first regulatory element operably linked to at least one ~~or more~~ Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)- CRISPR associated (Cas) system guide [[RNAs]] RNA that hybridizes with the target sequence[[s]] ~~in genomic loci of the DNA molecules encoding the one or more gene products,~~
b) a second regulatory element operably linked to a Type-II Cas9 protein,
wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide [[RNAs]] RNA targets the target sequence ~~the genomic loci of the DNA molecules encoding the one or more gene products~~ and the Cas9 protein cleaves ~~the genomic loci of~~ the DNA molecules ~~encoding the one or more gene products~~, whereby expression of the at least one ~~or more~~ gene product[[s]] is altered; and, wherein the Cas9 protein and the guide [[RNAs]] RNA do not naturally occur together..

2. (Original) The method of claim 1, wherein the expression of two or more gene products is altered.

3. (Original) The method of claim 1, wherein the vectors of the system further comprise one or more nuclear localization signal(s) (NLS(s)).

4. (Previously presented) The method of claim 1, wherein the guide RNAs comprise a guide sequence fused to a trans-activating cr (tracr) sequence.

5. (Original) The method of claim 1, wherein the Cas9 protein is codon optimized for expression in the eukaryotic cell.

6. (Original) The method of claim 1, wherein the eukaryotic cell is a mammalian or human cell.

7. (Original) The method of claim 1, wherein the expression of one or more gene products is decreased.

8. (Currently amended) An engineered, non-naturally occurring vector system comprising one or more vectors comprising:

a) a first regulatory element operably linked to at least one [[or more]] CRISPR-Cas system guide RNA[[s]] that hybridizes with a target sequence[[s]] ~~in genomic loci~~ of a DNA molecule[[s]] in a eukaryotic cell that contains the DNA molecule, wherein the DNA molecule encodes and the eukaryotic cell expresses at least ~~a encoding~~ one ~~or more~~ gene product[[s]],

b) a second regulatory element operably linked to a Type-II Cas9 protein,

wherein components (a) and (b) are located on same or different vectors of the system, whereby the guide RNA[[s]] targets and hybridizes with the target sequence ~~the genomic loci of the DNA molecules encoding the one or more gene products in a eukaryotic cell~~ and the Cas9 protein cleaves ~~the genomic loci of~~ the DNA molecule~~s encoding the one or more gene products~~, whereby expression of the at least one ~~or more~~ gene product[[s]] is altered; and, wherein the Cas9 protein and the guide RNA[[s]] do not naturally occur together.

9. (Original) The vector system of claim 8, wherein the expression of two or more gene products is altered.

10. (Original) The vector system of claim 8, wherein the vectors of the system further comprise one or more NLS(s).

11. (Original) The vector system of claim 8, wherein the guide RNAs comprise a guide sequence fused to a tracr sequence.

12. (Original) The vector system of claim 8, wherein the Cas9 protein is codon optimized for expression in the eukaryotic cell.

13. (Original) The vector system of claim 8, wherein the eukaryotic cell is a mammalian or human cell.

14. (Original) The vector system of claim 8, wherein the expression of one or more gene products is decreased.

15. (Currently Amended) An engineered, programmable, non-naturally occurring Type II CRISPR-Cas system comprising a Cas9 protein and at least one ~~or more~~ guide RNA[[s]] that targets and hybridizes to [[the]] ~~genomic loci of~~ a target sequence of a DNA molecule[[s]] in a eukaryotic cell, wherein the DNA molecule encodes and the eukaryotic cell expresses at least

~~encoding~~ one ~~or more~~ gene products ~~in a eukaryotic cell~~ and the Cas9 protein cleaves ~~the genomic loci of~~ the DNA molecules ~~encoding the one or more gene products~~, whereby expression of the at least one ~~or more~~ gene product[[s]] is altered; and, wherein the Cas9 protein and the guide RNA[[s]] do not naturally occur together.

16. (Original) The CRISPR-Cas system of claim 15, wherein the expression of two or more gene products is altered.

17. (Original) The CRISPR-Cas system of claim 15, wherein the CRISPR-Cas system further comprises one or more NLS(s).

18. (Original) The CRISPR-Cas system of claim 15, wherein the guide RNAs comprise a guide sequence fused to a tracr sequence.

19. (Original) The CRISPR-Cas system of claim 15, wherein the Cas9 protein is codon optimized for expression in the eukaryotic cell.

20. (Original) The CRISPR-Cas system of claim 15, wherein the eukaryotic cell is a mammalian or human cell.

# EXHIBIT 3

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RESEARCH ARTICLE

# RNA-programmed genome editing in human cells

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**Abstract** Type II CRISPR immune systems in bacteria use a dual RNA-guided DNA endonuclease, Cas9, to cleave foreign DNA at specific sites. We show here that Cas9 assembles with hybrid guide RNAs in human cells and can induce the formation of double-strand DNA breaks (DSBs) at a site complementary to the guide RNA sequence in genomic DNA. This cleavage activity requires both Cas9 and the complementary binding of the guide RNA. Experiments using extracts from transfected cells show that RNA expression and/or assembly into Cas9 is the limiting factor for Cas9-mediated DNA cleavage. In addition, we find that extension of the RNA sequence at the 3′ end enhances DNA targeting activity in vivo. These results show that RNA-programmed genome editing is a facile strategy for introducing site-specific genetic changes in human cells.
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## Introduction

Methods for introducing site-specific double-strand DNA (dsDNA) breaks (DSBs) in genomic DNA have transformed our ability to engineer eukaryotic organisms by initiating DNA repair pathways that lead to targeted genetic re-programming. Zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have proved effective for such genomic manipulation but their use has been limited by the need to engineer a specific protein for each dsDNA target site and by off-target activity (*Urnov et al., 2010*; *Bogdanove and Voytas, 2011*). Thus, alternative strategies for triggering site-specific DNA cleavage in eukaryotic cells are of great interest.

Research into genome defense mechanisms in bacteria showed that CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) loci encode RNA-guided adaptive immune systems that can destroy foreign DNA (*Bhaya et al., 2011*; *Terns and Terns, 2011*; *Wiedenheft et al., 2012*). The Type II CRISPR/Cas systems require a single protein, Cas9, to catalyze DNA cleavage (*Sapranauskas et al., 2011*). Cas9 generates blunt DSBs at sites defined by a 20-nucleotide guide sequence contained within an associated CRISPR RNA (crRNA) transcript (*Gasiunas et al., 2012*; *Jinek et al., 2012*). Cas9 requires both the guide crRNA and a trans-activating crRNA (tracrRNA) that is partially complementary to the crRNA for site-specific DNA recognition and cleavage (*Deltcheva et al., 2011*; *Jinek et al., 2012*). Recent experiments showed that the crRNA:tracrRNA complex can be redesigned as a single transcript (single-guide RNA or sgRNA) encompassing the features required for both Cas9 binding and DNA target siterecognition (*Jinek et al., 2012*). Using sgRNA, Cas9 can be programmed to cleave double-stranded DNA at any site defined by the guide RNA sequence and including a GG protospacer-adjacent (PAM) motif (*Sapranauskas et al., 2011*; *Jinek et al., 2012*). 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

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**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 sequence. The bacterial protein, Cas9, has the potential to enable a simpler approach to genome engineering because it is a DNA-cleaving enzyme that can be programmed with short RNA molecules to recognize specific DNA sequences, thus dispensing with the need to engineer a new protein for each new DNA target sequence.

Now Jinek et al. demonstrate the capability of RNA-programmed Cas9 to introduce targeted double-strand breaks into human chromosomal DNA, thereby inducing site-specific genome editing reactions. Cas9 assembles with engineered single-guide RNAs in human cells and the resulting Cas9-RNA complex can induce the formation of double-strand breaks in genomic DNA at a site complementary to the guide RNA sequence. Experiments using extracts from transfected cells show that RNA expression and/or assembly into Cas9 is the limiting factor for the DNA cleavage, and that extension of the RNA sequence at the 3′ end enhances DNA targeting activity in vivo.

These results show that RNA-programmed genome editing is a straightforward strategy for introducing site-specific genetic changes in human cells, and the ease with which it can programmed means that it is likely to become competitive with existing approaches based on zinc finger nucleases and transcription activator-like effector nucleases, and could lead to a new generation of experiments in the field of genome engineering for humans and other species with complex genomes.

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genome editing. However, it was not known whether such a bacterial system would function in eukaryotic cells.

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

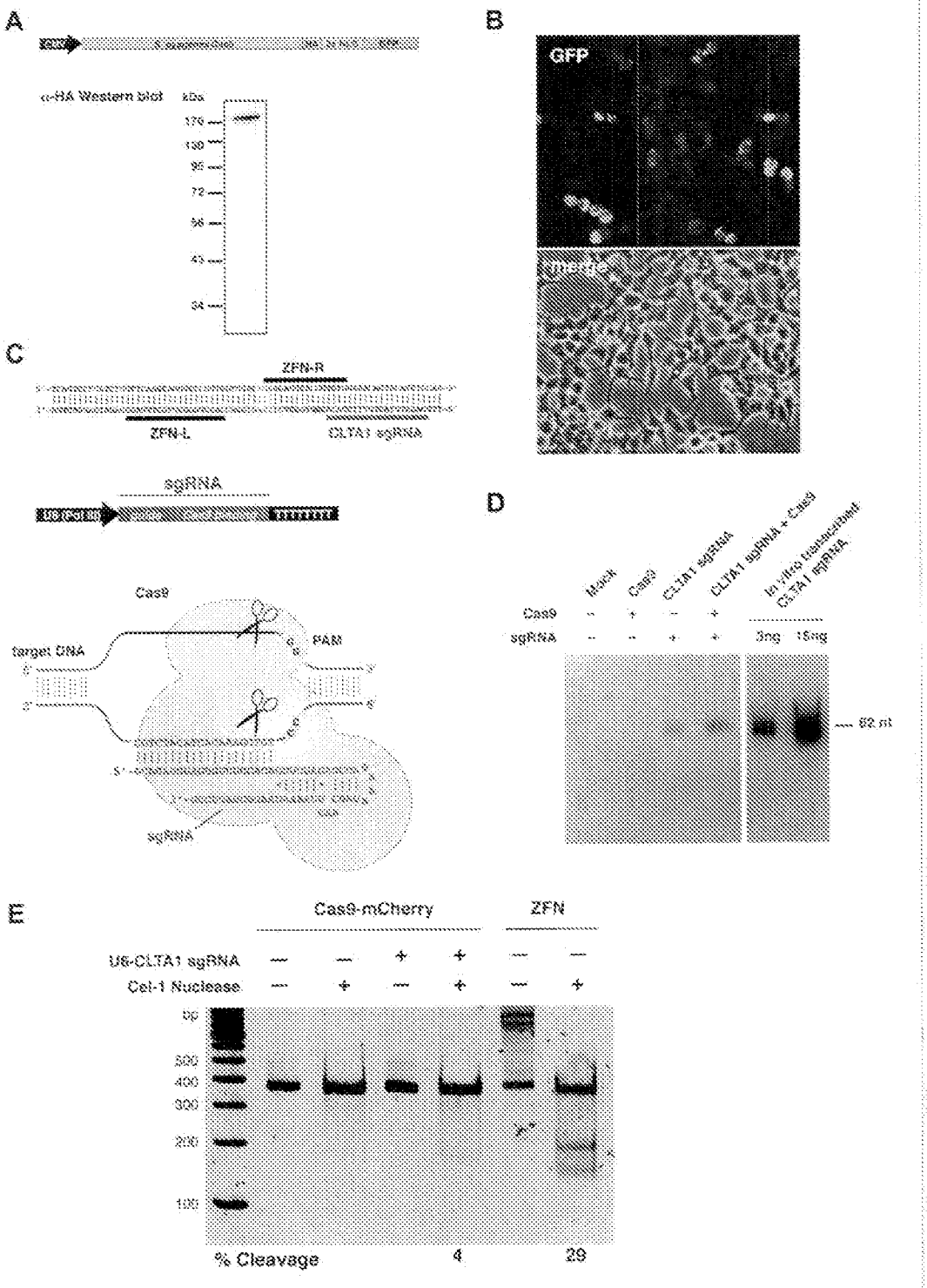


**Figure 1.** *Co-expression of Cas9 and guide RNA in human cells generates double-strand DNA breaks at the target locus.* (**A**) *Top*: schematic diagram of the Cas9-HA-NLS-GFP expression construct. *Bottom*: lysate from HEK293T cells transfected with the Cas9 expression plasmid was analyzed by Western blotting using an anti-HA antibody. (**B**) *Fluorescence microscopy of HEK293T cells expressing Cas9-HA-NLS-GFP.* (**C**) *Blue line* indicates the sequence used for the guide segment of CLTA1 sgRNA. *Top*: schematic diagram of the sgRNA target site in exon 7 of the human CLTA gene. The target sequence that hybridizes to the guide segment of CLTA1 sgRNA is indicated by the *blue line*. The GG nucleotide protospacer adjacent motif (PAM) is highlighted in *yellow*. *Black lines* denote the DNA binding regions.

*Figure 1. Continued on next page*

*Figure 1. Continued*

of the control ZFN protein. The translation stop codon of the CLTA open reading frame is highlighted in red for reference. Middle: schematic diagram of the sgRNA expression construct. The RNA is expressed under the control of the U6 Pol III promoter and a poly(T) tract that serves as a Pol III transcriptional terminator signal. Bottom: sgRNA-guided cleavage of target DNA by Cas9. The sgRNA consists of a 20-nt 5′-terminal guide segment (blue) followed by a 42-nt stem-loop structure required for Cas9 binding (red). Cas9-mediated cleavage of the two target DNA strands occurs upon unwinding of the target DNA and formation of a duplex between the guide segment of the sgRNA and the target DNA. This is dependent on the presence of a GG dinucleotide PAM downstream of the target sequence in the target DNA. Note that the target sequence is inverted relative to the upper diagram. (**D**) Northern blot analysis of sgRNA expression in HEK293T cells. (**E**) Surveyor nuclease assay of genomic DNA isolated from HEK293T cells expressing Cas9 and/or CLTA sgRNA. A ZFN construct previously used to target the CLTA locus (**Doyon et al., 2011**) was used as a positive control for detecting DSB-induced DNA repair by non-homologous end joining.

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The following figure supplements are available for figure 1:

**Figure supplement 1.** Mutated alleles of the CLTA gene in HEK293T cells as a result of Cas9-induced NHEJ.

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assay (**Qiu et al., 2004**). The region of genomic DNA targeted by Cas9:sgRNA is amplified by PCR and the resulting products are denatured and reannealed. The rehybridized PCR products are incubated with the mismatch recognition endonuclease Cel-1 and resolved on an acrylamide gel to identify Cel-1 cleavage bands. As DNA repair by NHEJ is typically induced by a DSB, a positive signal in the Surveyor assay indicates that genomic DNA cleavage has occurred. Using this assay, we detected cleavage of the CLTA locus at a position targeted by the CLTA1 sgRNA (*Figure 1E*). A pair of ZFNs that target a neighboring site in the CLTA locus provided a positive control in these experiments (**Doyon et al., 2011**).

To determine if either Cas9 or sgRNA expression is a limiting factor in the observed genome editing reactions, lysates prepared from the transfected cells were incubated with plasmid DNA harboring a fragment of the CLTA gene targeted by the CLTA1 sgRNA. Plasmid DNA cleavage was not observed upon incubation with lysate prepared from cells transfected with the Cas9-HA-NLS-GFP expression vector alone, consistent with the Surveyor assay results. However, robust plasmid cleavage was detected when the lysate was supplemented with in vitro transcribed CLTA1 sgRNA (*Figure 2A*). Furthermore, lysate prepared from cells transfected with both Cas9 and sgRNA expression vectors supported plasmid cleavage, while lysates from cells transfected with the sgRNA-encoding vector alone did not (*Figure 2A*). These results suggest that a limiting factor for Cas9 function in human cells could be assembly with the sgRNA. We tested this possibility directly by analyzing plasmid cleavage in lysates from cells transfected as before in the presence and absence of added exogenous sgRNA. Notably, when exogenous sgRNA was added to lysate from cells transfected with both the Cas9 and sgRNA expression vectors, a substantial increase in DNA cleavage activity was observed (*Figure 2B*). This result indicates that the limiting factor for Cas9 function in HEK293T cells is the expression of the sgRNA or its loading into Cas9.

As a means of enhancing the Cas9:sgRNA assembly in vivo, we next tested the effect of extending the presumed Cas9-binding region of the guide RNA. Two new versions of the CLTA1 sgRNA were designed to include an additional 4 or 10 base pairs in the helix that mimics the base-pairing interactions between the crRNA and tracrRNA (*Figure 3A*). Additionally, the 3′-end of the guide RNA was extended by five nucleotides based on the native sequence of the *S. pyogenes* tracrRNA (**Deltcheva et al., 2011**). Vectors encoding these 3′ extended sgRNAs under the control of either the U6 or H1 Pol III promoters were transfected into cells along with the Cas9-HA-NLS-GFP expression vector and site-specific genome cleavage was tested using the Surveyor assay (*Figure 3B*). These results suggest that the 3′-extended sgRNAs support more efficient Cas9 function in vivo, although more quantitative comparisons will be necessary to confirm this conclusion.

## Discussion

Besides serving as an invaluable research tool, targeted genome engineering in cells and organisms could potentially provide the path to revolutionary applications in human therapies, agricultural biotechnology and microbial engineering. Methods of modifying the genome exploit endogenous DNA repair pathways that are initiated by the introduction of site-specific dsDNA cleavages. The results presented here provide a straightforward system of RNA-guided site-specific dsDNA cleavage using

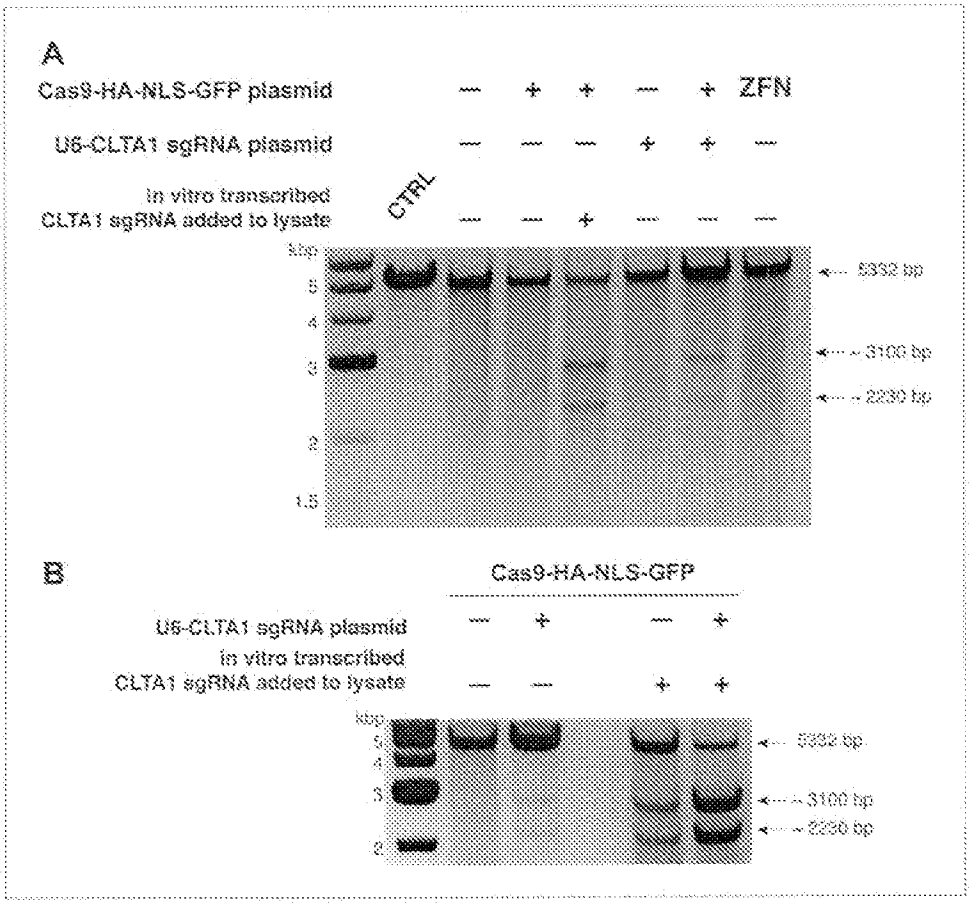


**Figure 2**. Cell lysates contain active Cas9:sgRNA and support site-specific DNA cleavage. (**A**) Lysates from cells transfected with the plasmid(s) indicated at left were incubated with plasmid DNA containing a PAM and the target sequence complementary to the CLTA1 sgRNA, where indicated, the reaction was supplemented with 10 pmol of in vitro transcribed CLTA1 sgRNA; secondary cleavage with XhoI generated fragments of ~2230 and ~3100 bp fragments indicative of Cas9-mediated cleavage. A control reaction using lysate from cells transfected with a ZFN expression construct shows fragments of slightly different size reflecting the offset of the ZFN target site relative to the CLTA1 target site. (**B**) Lysates from cells transfected with Cas9-HA-NLS-GFP expression plasmid and, where indicated, the CLTA1 sgRNA expression plasmid, were incubated with target plasmid DNA as in (**A**) in the absence or presence of in vitro-transcribed CLTA1 sgRNA.

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the Cas9 protein from a Type II bacterial CRISPR system to promote genome editing in human cells. Our data show that a codon-optimized version of Cas9, when programmed by an appropriate sgRNA, successfully assembles into Cas9 targeting complexes to trigger site-specific DNA cleavage and repair by NHEJ. The efficiency of NHEJ-induced mutagenesis at the CLTA locus investigated here is consistently in the range of 6–8%. This frequency is lower than that found for a ZFN pair that recognizes a nearby target sequence, but is within the range of frequencies observed more generally with ZFNs and TALENs (*Bogdanove and Voytas, 2011*). Our data suggest that sgRNA expression and/or its assembly into Cas9, rather than Cas9 expression, localization or folding, presently limits Cas9 function in human cells. Higher efficiencies of Cas9-mediated genome targeting could be achieved by optimization of the sgRNA construct design, its expression levels or its subcellular localization. We note that the sgRNAs used in this study are not thought to be 5′-capped or 3′-polyadenylated, which may have reduced their stability in vivo. This and other 5′ and 3′ end modifications might provide alternative approaches to enhancing Cas9:sgRNA assembly and activity in cells. Nonetheless, the levels of targeting observed in this study have been obtained with a minimal system that relies on simple base pairing to a guide RNA, in contrast to the ZFN and TALEN proteins, which require a new protein to be

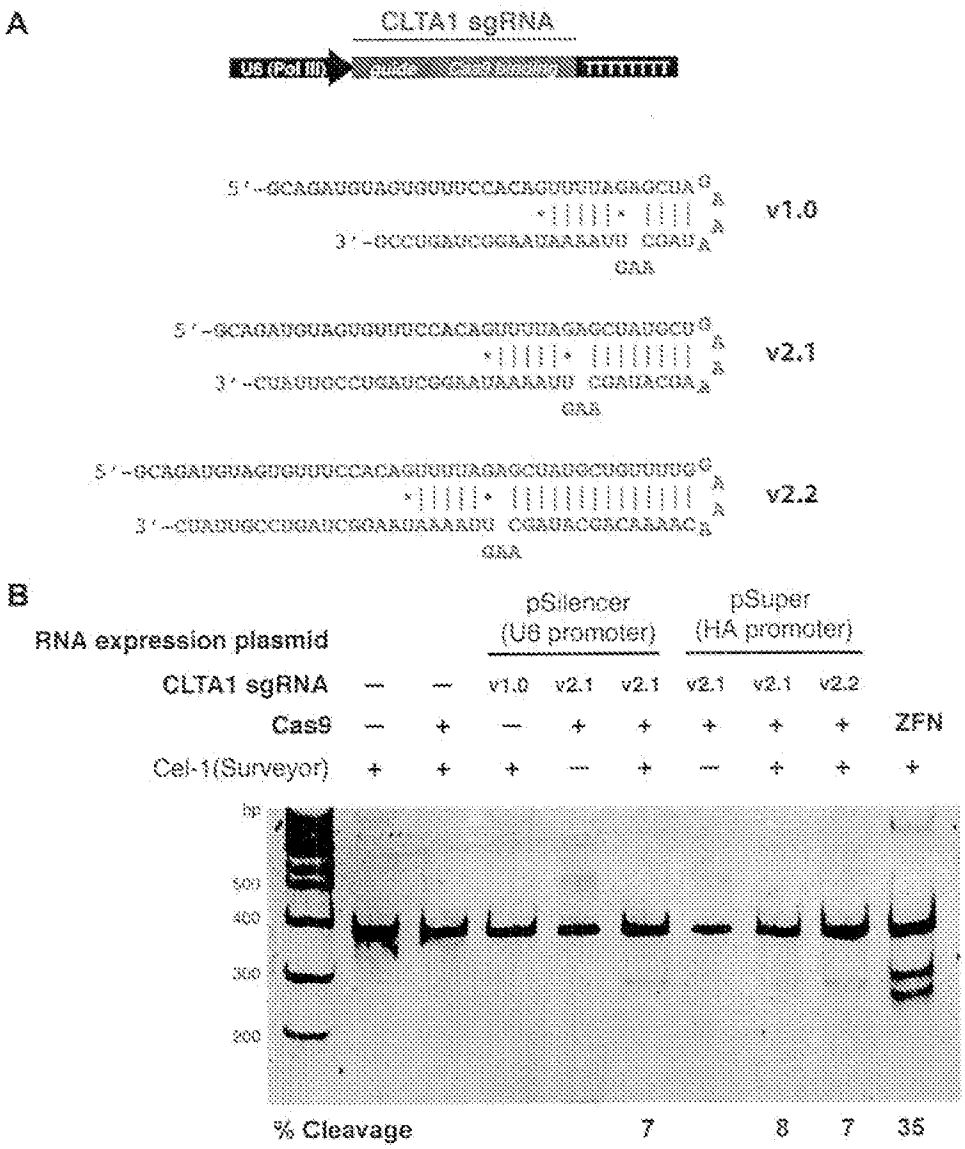


**Figure 3**. 3′ extension of sgRNA constructs enhances site-specific NHEJ-mediated mutagenesis. (**A**) The construct for CLTA1 sgRNA expression (top) was designed to generate transcripts containing the original Cas9-binding sequence v1.0 (***Jinek et al., 2012***), or sequences extended by 4 base pairs (v2.1) or 10 base pairs (v2.2). (**B**) Surveyor nuclease assay of genomic DNA isolated from HEK293T cells expressing Cas9 and/or CLTA sgRNA v1.0, v2.1 or v2.2. A ZFN construct previously used to target the CLTA locus (***Doyon et al., 2011***) was used as a positive control for detecting DSB-induced DNA repair by non-homologous end joining.

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engineered for each new cleavage site. RNA-guided genome editing would thus offer distinct advantages due to the simplicity of the sgRNA design.

Our results thus provide the framework for implementing Cas9 as a facile molecular tool for diverse genome editing applications. Although not tested explicitly in this study, a powerful feature of this system is the potential to program Cas9 with multiple sgRNAs in the same cell, either to increase the efficiency of targeting at a single locus, or as a means of targeting several loci simultaneously. Such strategies would find broad application in genome-wide experiments and large-scale research efforts such as the development of multigenic disease models. As an inexpensive and rapid mechanism for triggering site-specific genome modification, the programmable Cas9:sgRNA system could potentially transform next-generation genome-scale studies.

# Materials and methods

## Plasmid design and construction

The sequence encoding *Streptococcus pyogenes* Cas9 (residues 1–1368) fused to an HA epitope (amino acid sequence DAYPYDVPDYASL), a nuclear localization signal (amino acid sequence PKKKRKVEDPKKKRKVD) was codon optimized for human expression and synthesized by GeneArt (Regensburg, Germany; DNA and protein sequences shown in *supplementary file 1*). Ligation-independent cloning (LIC) was used to insert this sequence into a pcDNA3.1-derived GFP and mCherry LIC vectors (vectors 6D and 6B, respectively, obtained from the UC Berkeley MacroLab), resulting in a Cas9-HA-NLS-GFP and Cas9-HA-NLS-mCherry fusions expressed under the control of the CMV promoter. Guide sgRNAs were expressed using expression vector pSilencer 2.1-U6 puro (Life Technologies, Carlsbad, CA) and pSuper (Oligoengine, Seattle, WA). RNA expression constructs were generated by annealing complementary oligonucleotides to form the RNA-coding DNA sequence and ligating the annealed DNA fragment between the BamHI and HindIII sites in pSilencer 2.1-U6 puro and BglII and HindIII sites in pSuper.

## Cell culture conditions and DNA transfections

HEK293T cells were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 37°C humidified incubator with 5% $CO_2$. Cells were transiently transfected with plasmid DNA using either X-tremeGENE DNA Transfection Reagent (Roche Applied Science, Indianapolis, IN) or Turbofect Transfection Reagent (Thermo Scientific, Waltham, MA) with recommended protocols. Briefly, HEK293T cells were transfected at 60–80% confluency in 6-well plates using 0.5 μg of the Cas9 expression plasmid and 2.0 μg of the RNA expression plasmid. The transfection efficiencies were estimated to be 30–50% for Turbofect (*Figures 1E and 2A,B*) and 80–90% for X-tremegene (*Figure 3B*), based on the fraction of GFP-positive cells observed by fluorescence microscopy. 48 hr post transfection, cells were washed with phosphate buffered saline (PBS) and lysed by applying 250 μl lysis buffer (20 mM Hepes pH 7.5, 100 mM potassium chloride [KCl], 5 mM magnesium chloride [$MgCl_2$], 1 mM dithiothreitol [DTT], 5% glycerol, 0.1% Triton X-100, supplemented with Roche Protease Inhibitor cocktail) and then rocked for 10 min at 4°C. The resulting cell lysate was divided into aliquots for further analysis. Genomic DNA was isolated from 200 μl cell lysate using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

## Western blot analysis of Cas9 expression

HEK293T, transfected with the Cas9-HA-NLS-GFP expression plasmid, were harvested and lysed 48 hr post transfection as above. 5 μl of lysate were electrophoresed on a 10% SDS polyacrylamide gel, blotter onto a PVDF membrane and probed with HRP-conjugated anti-HA antibody (1:1000 dilution in 1× PBS; Sigma, St. Louis, MO).

## Surveyor assay

The Surveyor assay was performed as previously described (*Qiu et al., 2004*; *Miller et al., 2007*; *Dayon et al., 2011*). Briefly, the human clathrin light chain A (CLTA) locus was PCR amplified from 200 ng of genomic DNA using a high fidelity polymerase, Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA) and forward primer 5′-GCAGCAGAAGAAGCCTTTGT-3′ and reverse primer 5′-TTCCTCCTCTCCCTCCTCTC-3′. 300 ng of the 360 bp amplicon was then denatured by heating to 95°C and slowly reannealed using a heat block to randomly rehybridize wild type and mutant DNA strands. Samples were then incubated with Cel-1 nuclease (Surveyor Kit; Transgenomic, Omaha, NE) for 1 hr at 42°C. Cel-1 recognizes and cleaves DNA helices containing mismatches (wild type:mutant hybridization). Cel-1 nuclease digestion products were separated on a 10% acrylamide gel and visualized by staining with SYBR Safe (Life Technologies). Quantification of cleavage bands was performed using ImageLab software (Bio-Rad, Hercules, CA). The percent cleavage was determined by dividing the average intensity of cleavage products (160–200 bps) by the sum of the intensities of the uncleaved PCR product (360 bp) and the cleavage product. Mutations (indels) resulting from Cas9-induced DNA repair reactions were identified by Sanger sequencing of cloned amplicons. Briefly, the 360-bp PCR amplicon was treated with Taq DNA polymerase (NEB) and dATP to attach an 'A' base to the 3′ end of the DNA fragment. The DNA was then ligated into pGEM-T Easy vector (Promega, Madison, WI) according to the manufacturer's protocol. Transformants were grown on Luria Broth agar plates containing 100 μg/ml of ampicillin, 70 μg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside and 100 μM

isopropyl β-D-1-thiogalactopyranoside. Random white colonies were selected for Sanger sequencing (Quintara Biosciences, Albany, CA). Representative DNA sequences are shown in *Figure 1—figure supplement 1*.

### In vitro transcription

Guide RNA was in vitro transcribed using recombinant T7 RNA polymerase and a DNA template generated by annealing complementary synthetic oligonucleotides as previously described (*Sternberg et al., 2013*). RNAs were purified by electrophoresis on 7 M urea denaturing acrylamide gel, ethanol precipitated, and dissolved in DEPC-treated water.

### Northern blot analysis

RNA was purified from HEK293T cells using the mirVana small-RNA isolation kit (Ambion). For each sample, 800 ng of RNA were separated on a 10% urea-PAGE gel after denaturation for 10 min at 70°C in RNA loading buffer (0.5× TBE [pH 7.5], 0.5 mg/ml bromophenol blue, 0.5 mg xylene cyanol and 47% formamide). After electrophoresis at 10 W in 0.5× TBE buffer until the bromophenol blue dye reached the bottom of the gel, samples were electroblotted onto a Nytran membrane at 20 V for 1.5 hr in 0.5× TBE. The transferred RNAs were cross-linked onto the Nytran membrane in UV-Crosslinker (Stratagene) and were pre-hybridized at 45°C for 3 hr in a buffer containing 40% formamide, 5× SSC, 3× Denhardt's solution (0.1% each of ficoll, polyvinylpyrollidone, and BSA) and 200 μg/ml Salmon sperm DNA. The pre-hybridized membranes were incubated overnight in the prehybridization buffer supplemented with 5′-$^{32}$P-labeled antisense DNA oligo probe at 1 million cpm/ml. After several washes in SSC buffer (final wash in 0.2× SCC), the membranes were imaged phosphorimaging.

### In vitro cleavage assay

Cell lysates were prepared as described above and incubated with CLTA-RFP donor plasmid (*Dayon et al., 2011*). Cleavage reactions were carried out in a total volume of 20 μl and contained 10 μl lysate, 2 μl of 5× cleavage buffer (100 mM HEPES pH 7.5, 500 mM KCl, 25 mM $MgCl_2$, 5 mM DTT, 25% glycerol) and 300 ng plasmid. Where indicated, reactions were supplemented with 10 pmol of in vitro transcribed CLTA1 sgRNA. Reactions were incubated at 37°C for 1 hr and subsequently digested with 10 U of XhoI (NEB) for an additional 30 min at 37°C. The reactions were stopped by the addition of Proteinase K (Thermo Scientific) and incubated at 37°C for 15 min. Cleavage products were analyzed by electrophoresis on a 1% agarose gel and stained with SYBR Safe. The presence of ~2230 and ~3100 bp fragments is indicative of Cas9-mediated cleavage.

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## Additional information

### Competing interests

MJ, Founder of Caribou Biosciences and member of its scientific advisory board. Has filed a provisional patent related to this work. JD, Founder of Caribou Biosciences and member of its scientific advisory board. Has filed a provisional patent related to this work. The other authors declare that no competing interests exist

### Funding


| Funder | Grant reference number | Author |
|---|---|---|
| Howard Hughes Medical Institute | | Jennifer Doudna |
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The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication

Author contributions

MJ, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; AE, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; AC, Conception and design, Drafting or revising the article; SL, Acquisition of data, Analysis and interpretation of data; EM, Acquisition of data, Drafting or revising the article; JD, Conception and design, Analysis and interpretation of data, Drafting or revising the article

## Additional files

Supplementary files

- Supplementary file 1. DNA and protein sequences relating to plasmid design and construction.

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# EXHIBIT 4

# Right on target: New era of fast genetic engineering

28 January 2014 by Colin Barras
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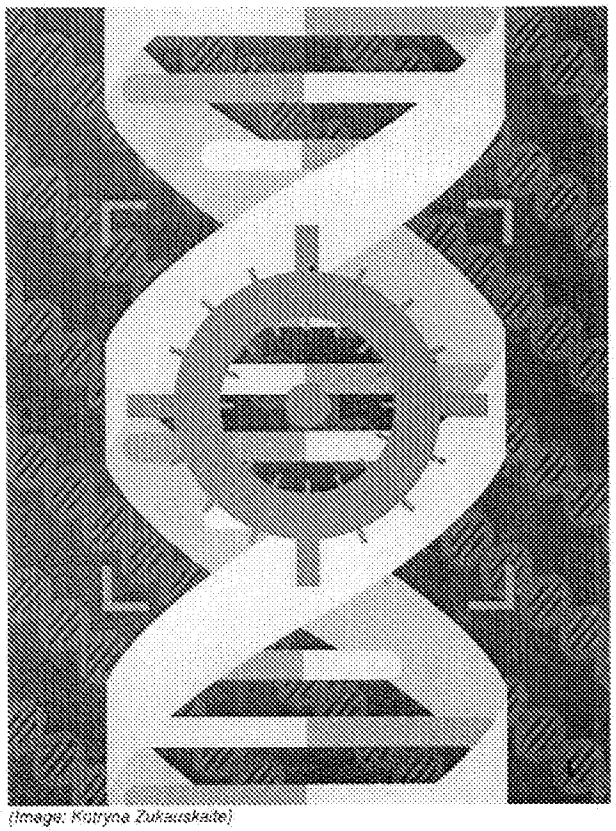


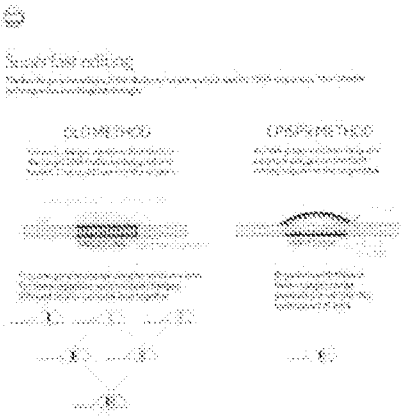
(Image: Kotryna Zukauskaite)

*A simple, very powerful method is making genome editing much easier and faster – prepare for a revolution in biology and medicine*

SEQUENCING genomes has become easy. Understanding them remains incredibly hard. While the trickle of sequence information has turned into a raging torrent, our knowledge isn't keeping up. We still have very little understanding of what, if anything, all our DNA does.

This is not a problem that can be solved by computers. Ultimately, there is only one way to be sure what a particular bit of DNA does – you have to alter it in real, living cells to see what happens. But genetic engineering is very difficult and expensive.

At least, it used to be. Last month, two groups announced that they had performed a mind-boggling feat. They targeted and disabled nearly every one of our genes in cells growing in a dish. They didn't knock out all the genes in each cell at once, of course, but one gene at a time. That is, they individually



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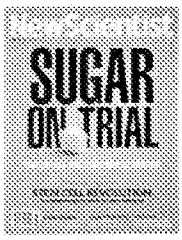


01 February 2014

modified a staggering 20,000 genes. "It's truly remarkable," says Eric Lander, director of the Broad Institute of MIT and Harvard, who led one of the studies. "This is transformative."

To put it into perspective, in 2007 an international project was launched to target and "knock out" each of the 20,000 genes a mouse possesses. It took the collective effort of numerous labs around the world more than five years to complete, and it cost $100 million. Now two small teams have each done something similar in a fraction of the time and cost. The secret: a simple and powerful new way of editing genomes. The term breakthrough is overused, but this undoubtedly is one. "It's a game-changer," says Feng Zhang, also at the Broad Institute, who led the other study.

The technique, unveiled just a year ago, is generating tremendous excitement as its potential becomes clear. It is already starting to accelerate the pace of research – Lander and Zhang used it to find out which genes help cancer cells resist a drug, for instance. In years to come, it is likely to be used in gene therapy, and to create a new generation of genetically engineered organisms with extensive but precise changes to their genomes. And if we ever do decide to genetically modify people, this is the tool to do it with.

While genetic engineers have done some amazing things, their first tools were very crude. They bombarded cells with extra DNA – sometimes literally – in the hope that it might occasionally get added to a cell's genome. But there was no way to control where in the genome it went, and if added DNA ends up in the wrong place it can cause havoc. Also, this approach does not allow for any tinkering with existing genes, which is the key to finding out what they and their variants do.

So in the past couple of decades the focus has switched to genome editing. To visualise how it works, imagine the genome as a collection of cookbooks written on long scrolls of paper and cared for by blind librarians. The librarians try to repair any damage but because they can't read they are easily tricked.

If you cut a scroll in two in the middle of a recipe, the librarians will join the pieces together again but in the process they often wreck the recipe. In other words, you can disable, or "knock out", a gene by cutting it.

What's more, if you add an extra piece of paper and then cut a scroll in two, the librarians will often assume the piece was cut from the scroll and add it in where the cut was made. In this way, segments of DNA can be added exactly where you want.

So the secret of genome editing is to cut DNA at just the right spot, and let the cell's DNA repair mechanisms do the rest for you. In practice, this means finding a molecule that, if added to a cell, will bind to a specific DNA sequence and cut the DNA at that point. There are natural proteins that do exactly this, but the chances of finding an existing protein that happens to target the one site in the entire genome that you are interested in are vanishingly small.

Instead, artificial proteins that bind to a specific DNA sequence have to be designed, made and tested for each edit you want to make. That can and is being done in many research labs around the world. Indeed, this kind of gene editing could soon be used in gene therapies to treat everything from sickle cell anaemia to HIV. Yet although there are now various tricks for speeding up the process of creating a designer DNA-binding protein, it is still far from easy. It can still take months or years of work to do yourself, or cost tens of thousands of dollars to have it done for you. To complicate matters further, much of the underlying technology has been patented.

Now, though, there is an alternative that is much faster, cheaper and – so far – freely available to all. The story of how it came about begins in the late 1980s. While studying the genome of the *E. coli* bacterium, a group in Japan noticed a peculiar series of repeating sequences, separated by what appeared to be random bits of DNA that were later called "spacers". These characteristic sets of repeats and spacers are now known as clustered regularly interspaced short palindromic repeats, or CRISPR (pronounced "crisper").

CRISPRs have turned out to be extremely common. They are present in half of all bacteria and 90 per cent of archaea. This means they must do something incredibly useful. But what? Their purpose remained elusive until 2005, when three groups reported that the apparently random pieces of spacer DNA actually matched parts of the DNA of viruses that attack simple cells.

To biologists such as Eugene Koonin of the National Institutes of Health in Bethesda, Maryland, this immediately suggested a purpose: perhaps these

human cloners. We shouldn't let them set the agenda

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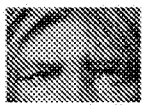

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spacers are the equivalent of a series of "wanted" posters, allowing cells to recognise and destroy viral DNA. In other words, an immune system.

By 2009, further studies had confirmed this suspicion and also revealed how the system works. The process begins with an RNA copy being made of a spacer and its flanking repeats. This RNA then joins up with a CRISPR-associated, or Cas, protein. The RNA provides the brain, and the Cas protein the brawn: the RNA will bind to any matching viral DNA in the cell, and the Cas protein will slice it up.

What's more, the cut-up pieces of viral DNA are sometimes spliced into the CRISPR region to form new spacers. In this way the wanted posters can be kept up to date even as viruses change their appearance. Learning how to fight off specific viruses is something that animal immune systems can do, but no one had imagined simple cells could do it too. "To my knowledge, CRISPR-Cas is the first and only adaptive immune system in archaea and bacteria," says Emmanuelle Charpentier of Umeå University in Sweden.

In fact, this system is better than ours in one way, because immunity can be passed down the generations. As Koonin pointed out in 2006, the CRISPR system can be seen as a form of Lamarckian evolution – an adaptation acquired during an individual's lifetime is passed on to its offspring.

But that's another story. What got a few geneticists very excited was the fact that the DNA-targeting process involves RNA rather than a protein. "What is attractive about CRISPR-Cas is it relies on a different way to recognise DNA," says Jennifer Doudna at the University of California, Berkeley. Could this simple system be used to edit genes?

## Extremely powerful

Initial work proved disappointing, because the DNA-cutting Cas protein turned out to be an intimate complex made of several different proteins, which would be tricky to work with. Then, in 2011, Charpentier's team made a key discovery. They were studying the CRISPR-Cas system in a bacterium that often infects people, called *Streptococcus pyogenes*. They discovered it was quite different to other systems. "Instead of a complex of proteins it used only one," says Charpentier.

It was more complicated in another way – it required not one but two pieces of RNA to guide the DNA-cutting protein, now called Cas9, to the target site. However, in 2012 Charpentier and Doudna together showed that those two RNAs can be combined into a single artificial RNA molecule that guides the Cas9 protein just as effectively.

What they had created was a potentially very simple yet extremely powerful genome-editing tool. Because an RNA molecule does the targeting, there is no need to design proteins to do this. All researchers need to do is make an RNA that contains a 20-base-pair sequence complementary to the target DNA, as well as the usual sequences needed for the guide RNA to hook up with the Cas9 protein. "It's a much simpler way to do site-specific targeting," says Doudna.

Crucially, making custom pieces of RNA is far quicker and cheaper than designing proteins. Automated machines can churn out short DNAs or RNAs with any desired sequence in hours, at a cost of just a few dollars.

But Charpentier and Doudna's study involved targeting bits of DNA floating loose in a tube. Would the bacterial system work inside the complex cells of animals and plants? Doudna's team set out to test this but they were beaten to it by Zhang's team and that of George Church at Harvard University, both of which reported in the first week of 2013 that the CRISPR-Cas9 system worked beautifully in human cells (*Science*, vol 339, p 819 and p 823). This was a vital demonstration says Doudna, whose own study appeared just three weeks later.

After that, everybody wanted to try CRISPR-Cas9. "It's exploded, and it's continuing to move very quickly," says Charles Gersbach at Duke University in Durham, North Carolina. "That's testament to how easy it is to use, and how robust the tool is."

Already, the CRISPR-Cas9 system has been shown to work in a wide variety of organisms, including mice, zebra fish and fruit flies. It also works in plants, including rice and wheat, the most widely grown crops. No one has yet done like-for-like studies comparing the CRISPR system with other genome-editing methods, so it is not yet clear which is more accurate in terms of hitting only the

target DNA, and more efficient in terms of the percentage of cells successfully modified. But for sheer speed and ease of use, CRISPR wins hands down.

And those are not the only advantages: because the system evolved to target multiple viruses, it can be used to modify more than one gene in a cell at a time. "In bacteria, the CRISPR system is already multiplex, so when I first learned about it, multiplex genome engineering was the first thing that came to mind," says Zhang.

He has shown that it is possible to edit at least five genes at once (*Cell*, vol 153, p 910). "Privately people have told me they've modified many more genes simultaneously," he says. With other techniques, making multiple changes is much more difficult. Church has developed an "evolution machine" potentially capable of making thousands of changes to cells (*New Scientist*, 27 June 2011, p 34), but it is complicated and works only with bacteria for now.

Why modify so many genes at once? "Look at the mutations associated with disease," Zhang says. "There's never a single mutation responsible for diabetes, or heart problems – it's always a combination." To study their effects, you have to create cell lines or animals possessing these particular combinations.

That used to mean engineering several strains of mice each with one modification then spending years crossbreeding them to create a strain with all of the modifications (see diagram). Now it can be done in one step, by using the CRISPR system to make multiple changes to a mouse embryo while it is still at the single-cell stage.

What used to take two years or more can now be done in six weeks, says Zhang. "That's a big difference." For those who have spent years trying to make just one or two specific changes to plants or animals, this is revolutionary.

## Make your own drugs

And as if this were not enough, this is not all that the CRISPR system can do. The activity of genes is controlled by proteins known as transcription factors. These proteins recognise and bind to specific DNA sequences near a gene and, by boosting or blocking the activity of the enzymes that transcribe DNA into messenger RNA, either increase that gene's activity or switch it off. Design your own transcription factors, and you can control gene activity – an ability that has all kinds of uses in research and medicine. But most attempts so far have involved designing new proteins, which as we have seen is not easy.

Gersbach and a few others realised that the Cas9 protein can be modified so that instead of cutting DNA it acts a transcription factor. In July, his team proved this works in human cells (*Nature Methods*, vol 10, p 973).

That opens up all kinds of possibilities. For instance, the approach could be used to treat autoimmune disorders such as arthritis by getting cells within a person's body to make more of a specific anti-inflammatory protein. "Rather than providing the protein as a drug, we could induce cells to make it themselves," says Gersbach.

Still, many hurdles remain to be overcome before the CRISPR system can be used for treating people. One major issue is delivery. "How do you put this into the right cells, at the right time, in the body?" asks Zhang.

And where CRISPR is used to cut DNA, the risk of it cutting it in the wrong place needs to be established. This happens to some extent with all methods – biology is a messy business – but CRISPR may be particularly prone to it because the system evolved to target continually changing viruses.

But confidence abounds in the field. "You have to remember we're still in the first year of development for use in human cells," says Gersbach. "It's clear there will be lots of advances in the coming years. We're still working with CRISPR 1.0."

*This article appeared in print under the headline "Right on target"*

*Colin Barras is a freelance writer based in Ann Arbor, Michigan*

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