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CRISPR-Cas: From the Bacterial Adaptive Immune System to a Versatile Tool for Genome Engineering

*Marion Kirchner and Sabine Schneider**

Cas9 · CRISPR · genome engineering · precision medicine

The field of biology has been revolutionized by the recent advancement of an adaptive bacterial immune system as a universal genome engineering tool. Bacteria and archaea use repetitive genomic elements termed clustered regularly interspaced short palindromic repeats (CRISPR) in combination with an RNA-guided nuclease (CRISPR-associated nuclease: Cas) to target and destroy invading DNA. By choosing the appropriate sequence of the guide RNA, this two-component system can be used to efficiently modify, target, and edit genomic loci of interest in plants, insects, fungi, mammalian cells, and whole organisms. This has opened up new frontiers in genome engineering, including the potential to treat or cure human genetic disorders. Now the potential risks as well as the ethical, social, and legal implications of this powerful new technique move into the limelight.

1. Discovery and Function of the RNA-Mediated Immunological Memory System

Following the identification and description of clustered regularly interspaced short palindromic repeats (CRISPR) in *Escherichia coli* in 1987,[1] CRISPR sequences were found in numerous bacterial and archaeal species.[2] However, their role remained elusive until the first decade of the 21st century, when these spacers were identified to be of viral and plasmid origin, thereby providing the first evidence for the existence of a bacterial adaptive immune system.[3] Indeed it was shown that these sequences are transcribed to antisense RNAs (CRISPR RNAs/crRNAs), which guide the CRISPR-associated nuclease (Cas) to the target DNA, whereupon the foreign DNA is destroyed.[4]

Three types of CRISPR-Cas systems have been identified, which can be differentiated by their molecular mechanisms: While types I and III employ a Cas–protein complex for target DNA recognition and cleavage, the type II system uses only a single protein. A prerequisite for destruction of invading DNA by the type I and II systems is an additional sequence motif 3′ adjacent to the crRNA-targeted sequence, the so-called protospacer adjacent motif (PAM). The minimalistic type II CRISPR-Cas system of *Streptococcus pyogenes* requires only a single endonuclease (Cas9), one guide RNA, and a PAM in the targeted DNA. It is this system that has been developed into an RNA-programmable genome editing tool. In the following Minireview, we will thus focus on the molecular mechanism and engineering of the *S. pyogenes* type II CRISPR-Cas system and review how it has been turned into a powerful technique that now offers the potential for therapeutic genome editing.[5]

Figure 1 illustrates the components of the bacterial type II immune system and how adaptive immunity is achieved. First, short fragments derived from invader DNA, so-called spacers, are inserted into the CRISPR array (acquisition). Then the trans-activating crRNA (tracrRNA) locus and the CRISPR-spacer array are transcribed into tracrRNA and precursor crRNA (pre-crRNA), respectively (expression). tracrRNA and pre-crRNA form a duplex via the repeat sequence (pre-crRNA) and its complementary sequence (tracrRNA). They are then subject to further processing by RNase III. The so-formed mature tracrRNA:crRNA (guide RNA) then builds a ribonucleoprotein complex with the Cas9 endonuclease. When DNA with complementary sequence to the crRNA is encountered by the complex, it is cleaved by Cas9 (interference).[6]

2. Mechanism of DNA Targeting by CRISPR-Cas9

Crystal structures and cryo-EM studies of Cas9 orthologues from *S. pyogenes* and *Actinomyces naeslundii* belonging to the type II system provide insight into the molecular

[*] M. Sc. M. Kirchner, Dr. S. Schneider
Center for Integrated Protein Science
Department of Chemistry, Technische Universität München
Lichtenbergstrasse 4, 85748 Garching (Germany)
E-mail: sabine.schneider@mytum.de

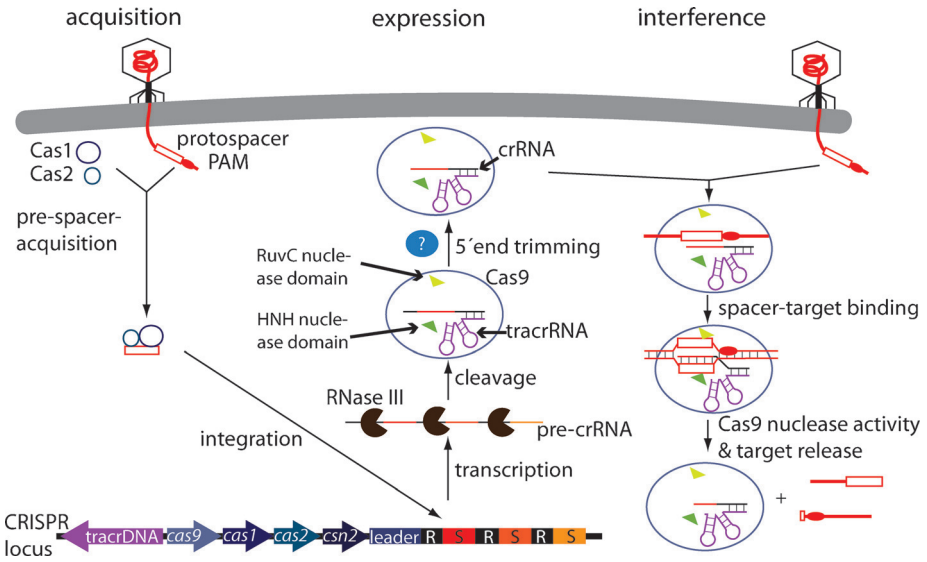

Figure 1. Illustration of the three stages of the type II CRISPR-Cas system. In the acquisition stage, invader DNA (red) enters the bacterial cell (cell wall shown in grey) and is processed by endonucleases (Cas1, Cas 2; blue circles). The fragments (protospacer) are integrated into the CRISPR locus in the bacterial chromosome (S = protospacer, R = repeat). Upon expression of the CRISPR locus and processing of pre-crRNA by RNase III, the crRNA:tracRNA complex is bound by Cas9. The Cas9-tracr:crRNA complex is now ready to cleave invading DNA containing the PAM motif and sequence complementary to the crRNA.

architecture and mechanism of CRISPR-Cas9 action. All Cas9 proteins have a conserved structural core and consist of two lobes: The nuclease (NUC) lobe contains both an HNH-like and a RuvC-like endonuclease domain.[7] The two are connected by a bridge helix and are responsible for the cleavage of target DNA complementary to the 20 nucleotide (nt) guide sequence in crRNAs (complementary strand) and the DNA 5′ adjacent to the PAM motif in the counter strand (non-complementary strand). The second lobe, the recognition (REC) lobe, has an alpha-helical and a topoisomerase-homology domain and is crucial for binding of the repeat:anti-repeat RNA duplex of the guide RNA, as well as stemloop 1 of the tracrRNA.[8] It was shown that the cr:tracrRNA can be combined into a single guide RNA (sgRNA) by connecting them through an artificial tetraloop,[6b] with the crRNA providing the 20 nt guide RNA and the 12 nt repeat sequence, whereas the tracrRNA contains the 14 nt anti-repeat sequence and the three stem-loops (Figure 2). While stem-loops 2 and 3 aid stabilization of the complex through interaction with the NUC lobe, stem-loop 1 is essential for Cas9 function.[8] Recognition and binding of the PAM motif in the target DNA occurs in the positively charged cleft at the junction of the two lobes (PAM-interaction domain: PI).

Variation of the structural organization of these domains among different Cas orthologues is responsible for the diversity in guide RNA and PAM specificities, with the REC domain being the least conserved. For instance, SpCas9 recognizes its specific PAM sequence 5′-NGG-3′ by base-specific readout of the GG motif in the major groove of the DNA duplex through two arginine residues of the PI domain.[9] In contrast, the Cas9 orthologue from *Lactobacillus buchnerie* employs two glutamine residues at the structurally equivalent position to recognize a 5′-NAAAA-3′ PAM sequence.[10] Recently, it was shown that the presence of the PAM is essential for target-sequence recognition: DNA sequences fully complementary to the guide RNA are not recognized by Cas9 in the absence of a PAM.[11] Off-target binding by Cas9 to sites bearing a PAM and only a partially

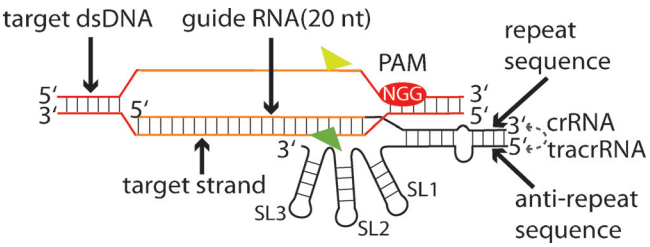

Figure 2. Schematic representation of guide and target nucleic acids. Cleavage of the target DNA requires the presence of the appropriate PAM sequence 3′ to the protospacer (here the *S. pyogenes* PAM 5′-NGG-3′), as well as complementarity between the protospacer and spacer sequence. (SL = stem-loop, green triangles = cleavage sites). The dotted arrow indicates the position of the tetraloop in the engineered sgRNA.

Sabine Schneider (b. 1976) studied at the Ludwig Maximilians Universität in Munich (Dipl.) and the University of Nottingham, UK (PhD with M. Paoli). Following post-doctoral research at the Ludwig Maximilians Universität in Munich (T. Carell), she was awarded a Liebig-Fellowship of the Fonds der Chemischen Industrie (2010) and became an independent junior group leader at the Technische Universität München.

Marion Kirchner (b. 1988) studied at the Ludwig Maximilians Universität in Munich. Since 2014, she has been conducting research for her PhD with Sabine Schneider at the Technische Universität München.

complementary sequence to the sgRNA is in principle a serious problem associated with genome editing and is a limiting factor in small interfering RNA technology as well. Off-target effects by CRISPR-Cas9 have indeed been observed in chromatin immunoprecipitation high-throughput sequencing with genomic DNA from mouse embryonic stem cells (mESC)[12] and human embryonic kidney cells (HEK293).[13] However, cleavage of DNA bearing a PAM but an imperfectly complementary sequence to the guide RNA was found to be a rare event, which shows that interrogation, binding, and cleavage are decoupled.[11]

So how is Cas9 activity regulated? A number of structural studies of *S. pyogenes* Cas9 (PDB ID: 4CMP) and Cas9-sgRNA:target-DNA complexes (PDB IDs: 4OO8, 4UN3) revealed that Cas9 action is driven by conformational changes.[8–10] In its apo state, the enzyme is in a catalytically inactive form. Interaction with the guide RNA results in a large rotational movement of the NUC lobe, thereby shaping a central channel crucial for target DNA binding. Upon DNA binding and PAM-sequence recognition through hydrogen-bonding of the Arg residues with the Hoogsteen faces of the guanine residues, the DNA duplex is destabilized adjacent to the PAM motif through the interaction of a Ser and a Lys residue (phosphate lock-loop) with the +1 phosphate. This interaction provides the seed for DNA melting and heteroduplex formation through Watson–Crick base pairing with the guide RNA, and is accompanied by further tightening of the complex and scission of the DNA. The HNH domain cleaves the complementary target DNA strand 3 nt upstream of the PAM sequence. The RuvC-like domain cleaves the non-complementary counter DNA strand. (Figure 3)[6b] However, if there are mismatches between the DNA and guide RNA, particularly adjacent to the PAM region, the heteroduplex cannot form, which most likely impedes further strand separation and the essential tightening of the enzyme binding to its target, thus preventing off-target cleavage. Catalytically, the two nuclease domains use different mechanisms to nick the DNA: The HNH endonuclease domain shares structural similarity with the family of HNH endonucleases, which are characterized by a ββα-metal fold. T4 endonuclease IV (PDB ID: 1EN7) employs a catalytic triad (His-Asp-Asn) in a one-metal-ion catalytic mechanism. Here, a hydrated Mg^{2+} ion coordinated by the His and Asp residue binds the scissile phosphate in the target DNA strand, with the His acting as a base to deprotonate the attacking water nucleophile.[14] In the Cas9 HNH domain, the corresponding residues are Asp 839, His 840, and Asn 863. RuvC-like nucleases share an RNase H fold. They coordinate two Mg^{2+} ions through a conserved Asp-Asp-Glu-His motif and most likely act via a two-metal-ion mechanism.[14] In the Cas9 RuvC domain, Asp 10, Glu 762, His 983, and Asp 986 coordinate two Mg^{2+} ions and are located at positions similar to those of the catalytic residues of RuvC from *Thermus thermophilus* (PDB ID: 4LD0).[9] Asp 10, His 840, and the presence of Mg^{2+} ions are essential for Cas9 function, and mutation of both residues to alanine results in a catalytically inactive enzyme (dCas9).[6b,8–11,15]

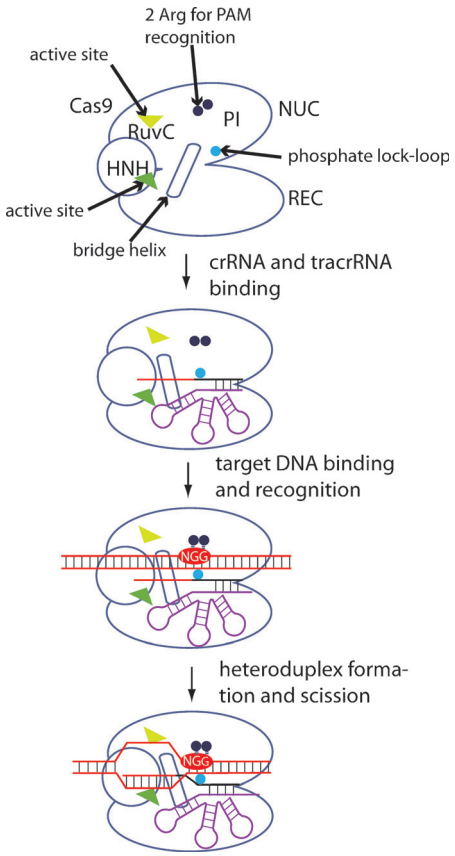

Figure 3. Overview of the type II-A SpCas9 binding and cleavage mechanism. Cas9 consists of a recognition (REC) lobe with the so-called bridge helix, and a nuclease (NUC) lobe with a PAM-interacting domain (PI) as well as the RuvC and HNH nuclease domains. The corresponding active sites are marked in green. crRNA (black and red line) and tracrRNA (lilac) binding results in conformational changes, thereby leading to the formation of a positively charged channel between the two lobes, where the guide–target and repeat–antirepeat duplexes are located. After DNA binding, the PAM motif (red oval) on the non-complementary strand is recognized by two arginine residues (dark blue) of the PI domain. DNA melting is initialized by interaction of the phosphate lock-loop (light blue) with the target strand. Subsequently, the RNA–DNA hybrid is formed and the DNA strands are cleaved.

3. Development of CRISPR-Cas9 as a Genome Engineering Tool

Prior to the development of the CRISPR-Cas9 system as a targeted genome engineering tool,[16] other programmable nucleases such as zinc-finger nucleases (ZFN)[17] and transcription activator-like effector nucleases (TALENs)[18] were used for genome modification in various organisms. In ZFNs and TALENs, a DNA-binding domain of a protein is fused to a FokI-nuclease domain. However, targeting of specific genomic sequences requires evolution of the respective DNA-binding domain that delivers the nuclease function to the desired locus (reviewed in Ref. [19]). In contrast, the RNA-programmable CRISPR-Cas9 system does not require target-specific protein engineering and merely depends on adapting the 20 nt guide RNA sequence in the sgRNA molecule to the desired target sequence. By modifying the

guide RNA sequence, site-specific targeting of plasmid DNA in bacteria can be accomplished.[6b,15]

For genome editing in eukaryotes through adaptation of the CRISPR-Cas9-system, optimized expression and localization of its components in the cell is crucial. This was achieved by expressing codon-optimized Cas9 with an additional nuclear localization signal (NLS), together with its guide RNA.[16b,f,m] With these optimized proteins, site-specific genomic deletions and insertions were possible in a number of human and mouse cells lines, as well as in pluripotent stem cells. Although genomic editing was feasible with TALENS and ZFN systems, the optimized CRISPR-Cas9 system is faster.[16j,20] Essential for Cas9 activity is the presence of the PAM and a strictly target-complementary 8–12 nt "seed sequence".[16a,b,f] However the short PAM sequence occurs frequently in the genome and therefore it is likely that a suitable PAM near the site of interest can be identified. Cas9 tolerates mismatches to a certain extent, which is the reason for the aforementioned off-target effects.[21] Owing to the arrayed spacer architecture of the CRISPR loci, the targeting of multiple genomic loci in parallel (multiplexed genome engineering) by the CRISPR-Cas9 system is possible by expressing multiple sgRNAs at the same time.[16b,j] Cas9 can be converted into a "nickase" (nCas9), which cleaves only one strand of the target DNA, by inactivating one endonuclease function through mutation, thereby resulting in an increase in homology-directed repair over non-homologous end joining.[15,22] Owing to the fact that target recognition by Cas9 relies on the presence of the PAM motif next to the target sequence in the opposite strand, the natural Cas9 substrate is dsDNA. Nevertheless, it was shown recently that Cas9 function can be extended to the sequence-specific recognition and cleavage of RNA and ssDNA. Here, the addition of an appropriate short single-stranded oligonucleotide, the "PAMmer", as a separate oligonucleotide is required. This may lead to a general approach for sequence-specific RNA manipulation.[23]

4. Current Applications

One direct practical biotechnological application of the CRISPR-Cas9 system is the immunization of cultured bacteria in the dairy industry. For one, it is possible to increase their robustness against bacteriophages. On the other hand, this technology also allows one to make bacteria resistant to the uptake of plasmids encoding undesirable genes.[24] The applicability of the CRISPR-Cas9 technology, however, does not stop at immunizing bacteria for industrial purposes. In the last 3 years, more than 500 articles describing different applications of the CRISPR-Cas9 technology have been published and the system has been successfully transferred to various cell types and organisms, including yeast,[16c,25] Actinomycetes,[26] Drosophila,[27] zebra fish,[16e,28] *C. elegans*,[16d] mammals,[29] and plants,[16h,k,30] as a general genome editing tool (Reviewed in Ref. [5a,c,31]). For example, dCas9 was shown to be able to knock down gene expression by blocking target gene transcription.[32] In addition, dCas9 fusion proteins and appropriate guide RNAs can be used to recruit transcriptional regulators to specific loci in the genome (Figure 4).[16j,33] This enables the role of transcriptional regulators, as well as the function of endogenous genes through their specific up- or downregulation, to be deciphered. Nevertheless one transcriptional activator fused to dCas9 alone is often not sufficient for the upregulation of endogenous genes, and the amplitude of the regulatory effect depends on the locus of the targeted gene. To increase gene expression independent of the location in the genome, additional transcriptional activator domains can be recruited to the target genes by adding small aptamer domains to the sgRNA. This, in combination with a sgRNA library targeting 23 430 coding genes in a cancer cell line, followed by screening for upregulated genes that mediate resistance to a chemotherapeutic agent, led to the identification of previously unknown mechanisms of resistance.[33d] Moreover, CRISPR-Cas9 enables dissection of the

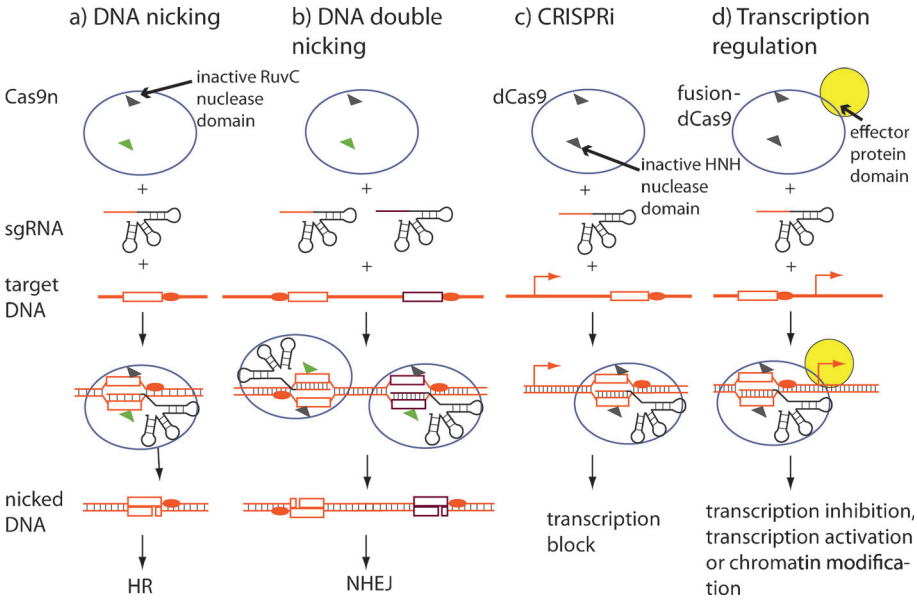

Figure 4. Applications of the type II CRISPR-Cas system. The sgRNA (black) contains a "spacer"-like part homologous to the target sequence (orange and red). a) In order to increase the rate of homology-directed repair (HDR) over non-homologous end-joining (NHEJ), a Cas9 variant (Cas9n) with an inactivated RuvC nuclease domain (grey triangle) is used. Thus only one strand of the target DNA is cleaved by the HNH nuclease domain.[22] b) To increase the genome-editing specificity, the DNA can be cleaved at two sites by using two different sgRNAs, which is followed by repair through NHEJ.[16b] c) Through targeting promoter regions with an endonuclease-inactive Cas9 variant (dCas9), transcription can be blocked (CRISPRi).[32b] d) Activation or inhibition of endogenous gene expression can be accomplished by fusing effector protein domains (yellow) to dCas9 and targeting promoter regions. In addition, the function of the effector domains can be studied.[33a,c,d]

impact of epigenetic markers such as DNA methylation, which play an essential role during embryo development. Targets for DNA methyltransferases were mapped on a single-base level through the disruption of different DNA methyltransferase genes in ES cells, in combination with whole-genome bisulfite sequencing.[34] Other applications include Cas9-GFP fusions for live-cell imaging, which permits the sequence-specific investigation of the dynamics and spatiotemporal organization of genomic elements related to genome function, such as telomeres during mitosis.[35] In addition, the CRISPR-Cas9 technology has proven to be an efficient tool for generating organisms with specific gene modifications. These can function as valuable models for studying gene function, disease, and development. Before the development of the CRISPR-Cas9 technology, their generation was complex and time consuming. Simple injection of Cas9 mRNA and sgRNA into the zygotes of mice and monkeys enables the creation of mutations in multiple genes in just one step.[16l,29] In addition, Cas9 can be used to target viral DNA, such as the human immunodeficiency virus (HIV) or the human papilloma virus (HPV), thereby resulting in protection of the cells against HIV infection[36] or the induction of apoptosis in HPV-transformed cells.[37] Recently, it was shown that the system can be extended to whole animals to study cancer development and progression. To this end, mice were implanted with lung cancer cells expressing Cas9. Through the expression of an sgRNA library targeting protein-encoding and microRNA-encoding sequences, genes could be identified for which inactivation promotes tumor progression and metastasis.[38] In addition, chromosomal translocations and point mutations can be introduced into adult mice by delivering a CRISPR-Cas9 sgRNA expression plasmids to target cells, thereby resulting in the rapid development of cancer models and functional genomics.[39]

5. Future Directions and New Frontiers

Advances in DNA sequencing technologies in combination with genome-wide association studies have provided detailed insight into disease-underlying genetics down to the single-cell level. The CRISPR-Cas9 system, with its power and apparent ease of use, has the potential to bridge the gap between this genetic information and the treatment of diseases. It promises personalized medicine with unprecedented precision. It has already been shown that a mutated gene that causes liver-based metabolic disease in a mouse model can be corrected in the whole animal.[40] In addition, the single-gene heredity defect in the CFTR gene has been repaired in stem cells derived from cystic fibrosis patients.[41] The potential future therapeutic use of Cas9 is not limited to the repair of single-gene heredity defects such as cystic fibrosis: it might also be used to prevent infections by targeting the genomes of pathogenic bacteria and viruses, to control inflammation and autoimmunity, and to repress or activate the expression of oncogenes and tumor suppressors. Moreover sequence changes in pluripotent embryonic stem cells may enable tissue engineering. While CRISPR-Cas9 offers unparalleled potential for biotechnological and medical applications, questions about its risks and ethical implications have arisen.[42] Safety issues such as off-target effects and on-target effects with unintended consequences need to be investigated. It is also possible that Cas9 exhibits toxicity or immunogenicity. This has to be thoroughly investigated prior to any therapeutic use of Cas9 in humans. The interdependency of a patient's individual genetic and disease background and environmental factors is still largely not clear. Prediction of the impact of any therapeutic genomic alteration is consequently not possible. Genome modifications of fertilized eggs or embryos are passed on to every single cell in an organism, including the germ line cells, which may have unwanted consequences. A group of researchers and interested stakeholders have already called for a restriction to use the technology for human germ-line modifications and they ask for an open discussion in a forum including scientists, clinicians, social scientists, and the general public.[42,43] One of their key recommendations on how to deal with this powerful technology prior to clinical application is to form a global representative group of developers and users of genome engineering technologies; experts in genetics, law, and bioethics; the scientific community; the public; and relevant government agencies. Clearly transparent research is now necessary to evaluate the full potential and risks of this technology.

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[1] Y. Ishino, H. Shinagawa, K. Makino, M. Amemura, A. Nakata, *J. Bacteriol.* **1987**, *169*, 5429–5433.

[2] a) R. Jansen, J. D. Embden, W. Gaastra, L. M. Schouls, *Mol. Microbiol.* **2002**, *43*, 1565–1575; b) A. Bolotin, B. Quinquis, A. Sorokin, S. D. Ehrlich, *Microbiology* **2005**, *151*, 2551–2561; c) F. J. Mojica, C. Diez-Villasenor, J. Garcia-Martinez, E. Soria, *J. Mol. Evol.* **2005**, *60*, 174–182; d) C. Pourcel, G. Salvignol, G. Vergnaud, *Microbiology* **2005**, *151*, 653–663.

[3] a) K. Makarova, A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N. Pavlova, V. Karamychev, N. Polouchine, et al., *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 15611–15616; b) R. Barrangou, C. Fremaux, H. Deveau, M. Richards, P. Boyaval, S. Moineau, D. A. Romero, P. Horvath, *Science* **2007**, *315*, 1709–1712; c) S. J. Brouns, M. M. Jore, M. Lundgren, E. R. Westra, R. J. Slijkhuis, A. P. Snijders, M. J. Dickman, K. S. Makarova, E. V. Koonin, J. van der Oost, *Science* **2008**, *321*, 960–964; d) H. Deveau, R. Barrangou, J. E. Garneau, J. Labonte, C. Fremaux, P. Boyaval, D. A. Romero, P. Horvath, S. Moineau, *J. Bacteriol.* **2008**, *190*, 1390–1400; e) L. A. Marraffini, E. J. Sontheimer, *Science* **2008**, *322*, 1843–1845; f) C. R. Hale, P. Zhao, S. Olson,

M. O. Duff, B. R. Graveley, L. Wells, R. M. Terns, M. P. Terns, *Cell* **2009**, *139*, 945 – 956.

[4] a) J. E. Garneau, M. E. Dupuis, M. Villion, D. A. Romero, R. Barrangou, P. Boyaval, C. Fremaux, P. Horvath, A. H. Magadan, S. Moineau, *Nature* **2010**, *468*, 67 – 71; b) R. Sapranauskas, G. Gasiunas, C. Fremaux, R. Barrangou, P. Horvath, V. Siksnys, *Nucleic Acids Res.* **2011**, *39*, 9275 – 9282; c) T. R. Sampson, S. D. Saroj, A. C. Llewellyn, Y. L. Tzeng, D. S. Weiss, *Nature* **2013**, *497*, 254 – 257.

[5] a) P. Mali, K. M. Esvelt, G. M. Church, *Nat. Methods* **2013**, *10*, 957 – 963; b) R. Barrangou, *Science* **2014**, *344*, 707 – 708; c) J. A. Doudna, E. Charpentier, *Science* **2014**, *346*, 1258096.

[6] a) E. Deltcheva, K. Chylinski, C. M. Sharma, K. Gonzales, Y. Chao, Z. A. Pirzada, M. R. Eckert, J. Vogel, E. Charpentier, *Nature* **2011**, *471*, 602 – 607; b) M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J. A. Doudna, E. Charpentier, *Science* **2012**, *337*, 816 – 821.

[7] K. S. Makarova, D. H. Haft, R. Barrangou, S. J. Brouns, E. Charpentier, P. Horvath, S. Moineau, F. J. Mojica, Y. I. Wolf, A. F. Yakunin, et al., *Nat Rev Microbiol* **2011**, *9*, 467 – 477.

[8] H. Nishimasu, F. A. Ran, P. D. Hsu, S. Konermann, S. I. Shehata, N. Dohmae, R. Ishitani, F. Zhang, O. Nureki, *Cell* **2014**, *156*, 935 – 949.

[9] M. Jinek, F. Jiang, D. W. Taylor, S. H. Sternberg, E. Kaya, E. Ma, C. Anders, M. Hauer, K. Zhou, S. Lin, et al., *Science* **2014**, *343*, 1247997.

[10] C. Anders, O. Niewoehner, A. Duerst, M. Jinek, *Nature* **2014**, *513*, 569 – 573.

[11] S. H. Sternberg, S. Redding, M. Jinek, E. C. Greene, J. A. Doudna, *Nature* **2014**, *507*, 62 – 67.

[12] X. Wu, D. A. Scott, A. J. Kriz, A. C. Chiu, P. D. Hsu, D. B. Dadon, A. W. Cheng, A. E. Trevino, S. Konermann, S. Chen, et al., *Nat. Biotechnol.* **2014**, *32*, 670 – 676.

[13] C. Kuscu, S. Arslan, R. Singh, J. Thorpe, M. Adli, *Nat. Biotechnol.* **2014**, *32*, 677 – 683.

[14] W. Yang, *Nat. Struct. Mol. Biol.* **2008**, *15*, 1228 – 1231.

[15] G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, *Proc. Natl. Acad. Sci. USA* **2012**, *109*, E2579 – 2586.

[16] a) S. W. Cho, S. Kim, J. M. Kim, J. S. Kim, *Nat. Biotechnol.* **2013**, *31*, 230 – 232; b) L. Cong, F. A. Ran, D. Cox, S. Lin, R. Barretto, N. Habib, P. D. Hsu, X. Wu, W. Jiang, L. A. Marraffini, et al., *Science* **2013**, *339*, 819 – 823; c) J. E. DiCarlo, J. E. Norville, P. Mali, X. Rios, J. Aach, G. M. Church, *Nucleic Acids Res.* **2013**, *41*, 4336 – 4343; d) A. E. Friedland, Y. B. Tzur, K. M. Esvelt, M. P. Colaiacovo, G. M. Church, J. A. Calarco, *Nat. Methods* **2013**, *10*, 741 – 743; e) W. Y. Hwang, Y. Fu, D. Reyon, M. L. Maeder, S. Q. Tsai, J. D. Sander, R. T. Peterson, J. R. Yeh, J. K. Joung, *Nat. Biotechnol.* **2013**, *31*, 227 – 229; f) M. Jinek, A. East, A. Cheng, S. Lin, E. Ma, J. Doudna, *eLife* **2013**, *2*, e00471; g) D. Li, Z. Qiu, Y. Shao, Y. Chen, Y. Guan, M. Liu, Y. Li, N. Gao, L. Wang, X. Lu, et al., *Nat. Biotechnol.* **2013**, *31*, 681 – 683; h) J. F. Li, J. E. Norville, J. Aach, M. McCormack, D. Zhang, J. Bush, G. M. Church, J. Sheen, *Nat. Biotechnol.* **2013**, *31*, 688 – 691; i) W. Li, F. Teng, T. Li, Q. Zhou, *Nat. Biotechnol.* **2013**, *31*, 684 – 686; j) P. Mali, J. Aach, P. B. Stranges, K. M. Esvelt, M. Moosburner, S. Kosuri, L. Yang, G. M. Church, *Nat. Biotechnol.* **2013**, *31*, 833 – 838; k) V. Nekrasov, B. Staskawicz, D. Weigel, J. D. Jones, S. Kamoun, *Nat. Biotechnol.* **2013**, *31*, 691 – 693; l) H. Wang, H. Yang, C. S. Shivalila, M. M. Dawlaty, A. W. Cheng, F. Zhang, R. Jaenisch, *Cell* **2013**, *153*, 910 – 918; m) P. Mali, L. Yang, K. M. Esvelt, J. Aach, M. Guell, J. E. DiCarlo, J. E. Norville, G. M. Church, *Science* **2013**, *339*, 823 – 826.

[17] M. L. Maeder, S. Thibodeau-Beganny, J. D. Sander, D. F. Voytas, J. K. Joung, *Nat. Protoc.* **2009**, *4*, 1471 – 1501.

[18] V. M. Bedell, Y. Wang, J. M. Campbell, T. L. Poshusta, C. G. Starker, R. G. Krug 2nd, W. Tan, S. G. Penheiter, A. C. Ma, A. Y. Leung, et al., *Nature* **2012**, *491*, 114 – 118.

[19] J. A. Doudna, E. J. Sontheimer, *Methods Enzymol.* **2014**, *546*, xix – xx.

[20] Q. Ding, S. N. Regan, Y. Xia, L. A. Oostrom, C. A. Cowan, K. Musunuru, *Cell Stem Cell* **2013**, *12*, 393 – 394.

[21] V. Pattanayak, S. Lin, J. P. Guilinger, E. Ma, J. A. Doudna, D. R. Liu, *Nat. Biotechnol.* **2013**, *31*, 839 – 843.

[22] F. A. Ran, P. D. Hsu, C. Y. Lin, J. S. Gootenberg, S. Konermann, A. E. Trevino, D. A. Scott, A. Inoue, S. Matoba, Y. Zhang, et al., *Cell* **2013**, *154*, 1380 – 1389.

[23] M. R. O'Connell, B. L. Oakes, S. H. Sternberg, A. East-Seletsky, M. Kaplan, J. A. Doudna, *Nature* **2014**, *516*, 263 – 266.

[24] R. Barrangou, P. Horvath, *Annu. Rev. Food. Sci. Technol.* **2012**, *3*, 143 – 162.

[25] J. Z. Jacobs, K. M. Ciccaglione, V. Tournier, M. Zaratiegui, *Nat. Commun.* **2014**, *5*, 5344.

[26] Y. Tong, P. Charusanti, L. Zhang, T. Weber, S. Y. Lee, *ACS Synth. Biol.* **2015**, DOI: 10.1021/acssynbio.1025b00038.

[27] F. Port, H. M. Chen, T. Lee, S. L. Bullock, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, E2967 – 2976.

[28] W. Y. Hwang, Y. Fu, D. Reyon, M. L. Maeder, P. Kaini, J. D. Sander, J. K. Joung, R. T. Peterson, J. R. Yeh, *PLoS One* **2013**, *8*, e68708.

[29] a) Y. Niu, B. Shen, Y. Cui, Y. Chen, J. Wang, L. Wang, Y. Kang, X. Zhao, W. Si, W. Li, et al., *Cell* **2014**, *156*, 836 – 843; b) H. Yang, H. Wang, C. S. Shivalila, A. W. Cheng, L. Shi, R. Jaenisch, *Cell* **2013**, *154*, 1370 – 1379.

[30] Q. Shan, Y. Wang, J. Li, Y. Zhang, K. Chen, Z. Liang, K. Zhang, J. Liu, J. J. Xi, J. L. Qiu, et al., *Nat. Biotechnol.* **2013**, *31*, 686 – 688.

[31] P. D. Hsu, E. S. Lander, F. Zhang, *Cell* **2014**, *157*, 1262 – 1278.

[32] a) M. H. Larson, L. A. Gilbert, X. Wang, W. A. Lim, J. S. Weissman, L. S. Qi, *Nat. Protoc.* **2013**, *8*, 2180 – 2196; b) L. S. Qi, M. H. Larson, L. A. Gilbert, J. A. Doudna, J. S. Weissman, A. P. Arkin, W. A. Lim, *Cell* **2013**, *152*, 1173 – 1183.

[33] a) L. A. Gilbert, M. H. Larson, L. Morsut, Z. Liu, G. A. Brar, S. E. Torres, N. Stern-Ginossar, O. Brandman, E. H. Whitehead, J. A. Doudna, et al., *Cell* **2013**, *154*, 442 – 451; b) M. L. Maeder, S. J. Linder, V. M. Cascio, Y. Fu, Q. H. Ho, J. K. Joung, *Nat. Methods* **2013**, *10*, 977 – 979; c) P. Perez-Pinera, D. D. Kocak, C. M. Vockley, A. F. Adler, A. M. Kabadi, L. R. Polstein, P. I. Thakore, K. A. Glass, D. G. Ousterout, K. W. Leong, et al., *Nat. Methods* **2013**, *10*, 973 – 976; d) S. Konermann, M. D. Brigham, A. E. Trevino, J. Joung, O. O. Abudayyeh, C. Barcena, P. D. Hsu, N. Habib, J. S. Gootenberg, H. Nishimasu, et al., *Nature* **2015**, *517*, 583 – 588.

[34] J. Liao, R. Karnik, H. Gu, M. J. Ziller, K. Clement, A. M. Tsankov, V. Akopian, C. A. Gifford, J. Donaghey, C. Galonska, et al., *Nat. Genet.* **2015**, *47*, 469 – 478.

[35] C. Chen, L. A. Fenk, M. de Bono, *Nucleic Acids Res.* **2013**, *41*, e193.

[36] W. Zhu, R. Lei, Y. Le Duff, J. Li, F. Guo, M. A. Wainberg, C. Liang, *Retrovirology* **2015**, *12*, 1 – 7.

[37] S. Zhen, L. Hua, Y. H. Liu, L. C. Gao, J. Fu, D. Y. Wan, L. H. Dong, H. F. Song, X. Gao, *Gene Ther.* **2015**, *22*, 404 – 412.

[38] S. Chen, N. E. Sanjana, K. Zheng, O. Shalem, K. Lee, X. Shi, D. A. Scott, J. Song, J. Q. Pan, R. Weissleder, et al., *Cell* **2015**, *160*, 1246 – 1260.

[39] a) D. Maddalo, E. Manchado, C. P. Concepcion, C. Bonetti, J. A. Vidigal, Y. C. Han, P. Ogrodowski, A. Crippa, N. Rekhtman, E. de Stanchina, et al., *Nature* **2014**, *516*, 423 – 427; b) W. Xue, S. Chen, H. Yin, T. Tammela, T. Papagiannakopoulos, N. S. Joshi, W. Cai, G. Yang, R. Bronson, D. G. Crowley, et al., *Nature* **2014**, *514*, 380 – 384.

[40] H. Yin, W. Xue, S. Chen, R. L. Bogorad, E. Benedetti, M. Grompe, V. Koteliansky, P. A. Sharp, T. Jacks, D. G. Anderson, *Nat. Biotechnol.* **2014**, *32*, 551 – 553.

[41] G. Schwank, B. K. Koo, V. Sasselli, J. F. Dekkers, I. Heo, T. Demircan, N. Sasaki, S. Boymans, E. Cuppen, C. K. van der Ent, et al., *Cell Stem Cell* **2013**, *13*, 653 – 658.

[42] D. Baltimore, P. Berg, M. Botchan, D. Carroll, R. A. Charo, G. Church, J. E. Corn, G. Q. Daley, J. A. Doudna, M. Fenner, et al., *Science* **2015**, *348*, 36 – 38.

[43] E. Lanphier, F. Urnov, S. E. Haecker, M. Werner, J. Smolenski, *Nature* **2015**, *519*, 410 – 411.

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