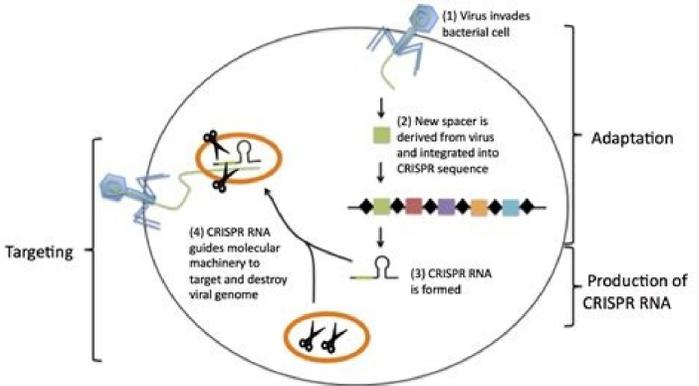
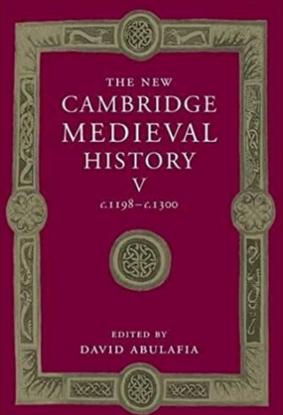
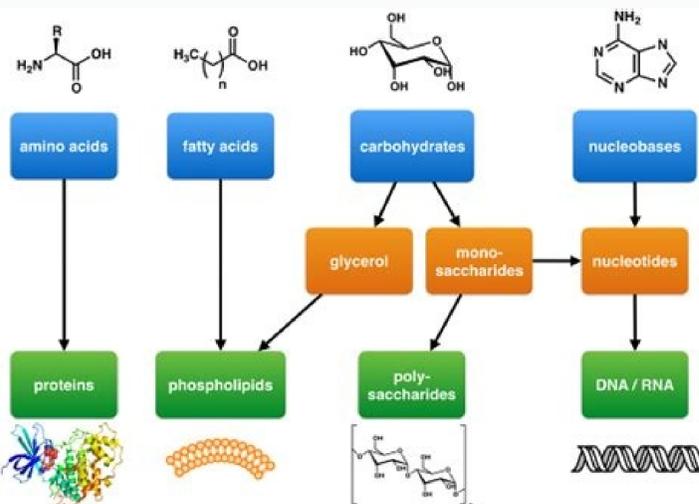


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Small guide rna.

The spacer region of the guide undergoes Watson and Crick base pairing with the complementary stand to the DNA protospacer. doi: 10.1038/nmeth.3433 PubMed Abstract | CrossRef Full Text | Google Scholar Shmakov, S., Smargon, A., Scott, D., Cox, D., Pzyzcha, N., Yan, W., et al. 17, 1097-1107. doi: 10.1126/sciadv.aaz0051 PubMed Abstract | CrossRef Full Text | Google Scholar Liu, Y., Zou, R. doi: 10.1038/nature16526 PubMed Abstract | CrossRef Full Text | Google Scholar Kocak, D. Chemically modified guide RNAs enhance CRISPR-Cas9 genome editing in human primary cells. The aforementioned work by Yin et al. Taken together, the mode of intracellular delivery of gRNA-Cas9 complexes, whether gRNA is delivered with Cas9 mRNA or protein, and the number and positions of chemical modifications are all key factors that must be considered when planning CRISPR-Cas9 genome editing experiments. demonstrated that the addition of 2'-O-Me and PS groups on the 2'-OH and phosphate backbone within synthesized gRNAs completely abolished any immune response (Schubert et al., 2018). This significantly hinders the ability to study the full spectrum of DSB formation and subsequent DNA repair dynamics. ACS Cent. 22, 169-174. L., Strezoska, Z., He, K., Vermeulen, A., and Smith, A. A later study by Kocak et al. Adding modifications only on the 3' and 5' ends of gRNAs would protect the gRNA from exonucleolytic but not endonucleolytic activity inside the cells, which also may impair the editing efficiency by reducing gRNA stability. The donor DNA is fused to the 5' end of the guide region. A codon consists of three continuous nucleotide bases that code for an amino acid or signal the end of translation. A different approach relied on simultaneously expressing engineered gRNAs containing MS2/PCP aptamers, MS2/PCP binding proteins fused to fluorescent proteins, and dCas9. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. W., and Liu, D. *Physiol. Exp.* Several studies have shown that gRNA modifications in Type V CRISPRs (Cas12a), including 3' terminal chemical modifications (Li et al., 2017; McMahon et al., 2018) and crRNA elongation (Bin Moon et al., 2018; Park et al., 2018), stabilize the complex and enhance editing efficiency. G., Cho, H. H., Camarena, J., Sifra, W., et al. E., Curry, B., Lucas, A. F., and Rahdar, M. Y., Lee, G., Lim, D. S., Li, L., Wu, X., Choi, U., Allen, C., Kozntz, S., et al. Nonetheless, caution should be exercised when introducing RNA modifications since further analysis found that over modification of the gRNA in the seed region, the ten nucleotides in the spacer region that recognize the target DNA closest to the PAM sequence, also known as the PAM-proximal portion, inhibits proper DNA-RNA hybridization and can significantly hinder efficiency (Rahdar et al., 2015; Basila et al., 2017; Yin et al., 2017). Classification and evolution of type II CRISPR-Cas systems. doi: 10.1038/nmeth.4430 PubMed Abstract | CrossRef Full Text | Google Scholar Nihongaki, Y., Yamamoto, S., Kawano, F., Suzuki, H., and Sato, M. A., Bounoutas, G. A., Fraietta, J. D., Goepfert, A. Acad. Basila et al. V., Bielias, J. P., Bak, R. H., Bratovic, M., Charpentier, E., et al. However, since primary cells are known to mount an innate immune response to the foreign DNA (Sun et al., 2013), as well as to the in vitro transcribed crRNAs (as discussed below), chemical synthesis represents a cost-effective, expeditious alternative that produces highly purified gRNA at scalable quantities. Incorporation of bridged nucleic acids into CRISPR RNAs improves Cas9 endonuclease specificity. J., Clark, J., Punjya, N., Sebastiano, V., et al. 45:e98. The crRNA sequence can be divided into a guide region and a repeat region, while the tracrRNA sequence consists of an anti-repeat region and three stem-loop (numbered 1-3) structures. conducted a genome editing screen in Cas9 expressing HEK293T cells, which revealed that in living cells, the tail region, or the PAM-distal portion of the guide sequence was more amenable to DNA replacement than the seed region. 9:313. Therefore, when translating these discoveries to the clinic, the relevant modifications must be validated in primary cells and animal models. C., and MacLachlan, I. L., 198-200. Ribosomal RNA is responsible for creating the peptide bonds between the amino acids in the polypeptide chain. A., and Gagnon, K. *Methods* 14, 891-896. N., Castro, M., and Cooper, T. These sequences are transcribed together into CRISPR RNAs (crRNAs), which are subsequently utilized to recognize and destroy the invading complementary DNA or RNA molecules by Cas nucleases (Horvath and Barrangou, 2010; Terns and Terns, 2011; Morange, 2015). Though much more work remains to be done to optimize modified gRNAs for future routine human genome-editing-based therapies, there is no denying that the future of modified gRNAs and CRISPR-based therapeutics remains exceptionally bright. This method of modifying the gRNA to facilitate light-induced Cas9 activation allows for synchronous DNA cleavage across a population of cells. doi: 10.1002/cam4.2257 PubMed Abstract | CrossRef Full Text | Google Scholar Ge, Z., Zheng, L., Zhao, Y., Jiang, J., Zhang, E. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Structure and specificity of the RNA-guided endonuclease Cas9 during DNA interrogation, target binding and cleavage. 50, 259-293. CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. 24, 1216-1224. doi: 10.1089/ol.2009.0180 CrossRef Full Text | Google Scholar Romero, Z., Lovomova, A., Said, S., Miggelbrink, A., Kuo, C. High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. In addition, BNAAC nucleotides have been shown to be less toxic than LNA nucleotides when delivered to cultured cells (Manning et al., 2017). R., Patel, M. Class 2 is distinguished by a multi-domain effector Cas nuclease and uses trans-activating CRISPR RNA (tracrRNA), in addition to crRNA, for target recognition and cleavage (Makarova et al., 2020). *Biosci.* They demonstrated that using a phosphorothioate (PS) (Figure 2) modified backbone in tandem with 2'-O-Me modifications on the terminal five nucleotides on both ends of the crRNA enhanced the editing activity, presumably by diminishing crRNA susceptibility to nucleolytic cleavage. With three types in each class and more than a dozen subtypes, the CRISPR-Cas system represents a fruitful field for developing bioengineering tools. E., Joutng, J., Abudayyeh, O. 2162, 37-48. *Science* 339, 819-823. Notably, chemically-modified gRNAs are not restricted to the genome-editing via DSBs but can be exploited for a variety of applications involving catalytically-inactive Cas9 nucleases, Cas9 nickases, base editors and prime editors. doi: 10.1126/scitranslmed.aaf9336 PubMed Abstract | CrossRef Full Text | Google Scholar Dow, L. Production of gRNAs like other types of RNA. gRNAs consist of ribonucleotides covalently bound together by phosphodiester bonds. doi: 10.7554/eLife.25312 PubMed Abstract | CrossRef Full Text | Google Scholar Lennox, K. Therefore, Adeno Associated Virus (AAV) vectors have become a method of choice to introduce donor templates (Gaj et al., 2017). Cell 155, 1479-1491. S., Dawlaty, M. G., Lee, N. RNA interference in mammalian cells by chemically-modified RNA. The popular method for CRISPR-mediated gene editing in cultured cells involves transfection with plasmid DNA that expresses both gRNA and Cas9 protein under constitutive promoters (Ran et al., 2013). (2015). The partial replacement of RNA nucleotides with DNA nucleotides in the crRNA has emerged as a potential approach to enhance CRISPR-Cas9 complex specificity by reducing off-target activity (Rueda et al., 2017; Kartje et al., 2018; Yin et al., 2018). revealed that although PS, 2'-F, and 2'-O-Me modifications are tolerated in all of the non-Cas9 interacting nucleotides to improve gRNA stability, the extent of off-target editing between unmodified and modified sgRNA was comparable in both cultured cell lines and mice liver cells (Yin et al., 2017). doi: 10.1038/nbt.2808 PubMed Abstract | CrossRef Full Text | Google Scholar Gaj, T., Staahl, B. Following the CRISPR-induced DSB, the endogenous cellular DNA repair mechanism, called non-homologous end joining (NHEJ), can repair the break, often resulting in small insertions or deletions (indels), which can lead to frameshift mutations, thereby inactivating the target gene (Yang et al., 2020). Therefore, for clinical purposes, the CRISPR-Cas9 system must possess a limited intracellular lifespan to allow for quick and efficient gene editing while minimizing off-target effects. doi: 10.1371/journal.pbio.2005840 PubMed Abstract | CrossRef Full Text | Google Scholar Wu, Y., Zeng, J., Roscoe, B. Together, these advancements in the engineering of synthetically modified gRNAs have enabled tremendous improvements in CRISPR-mediated genome editing's stability, specificity, and safety. Engl. E., Roy, S., et al. Contrary to the in vitro activity assays, in cultured cells, any hydrogen-bond-disrupting modifications on the Cas9-interacting nucleotides reduced editing activity (O'Reilly et al., 2019). (2009). On the other hand, G-rich hairpins or G4 structures at the 5' end completely abolished Cas9-mediated cleavage (Nar et al., 2018). Additionally, we thank the Israel Science Foundation (ISF) (Grant No. 203/1/19) and The Israel Cancer Research Fund (ICRF) (Grant No. 19-701-IFC) for their funding contributions. CRISPR-Cas for fungal genome editing: a new tool for the management of plant diseases U.S.A. 112, 3002-3007. Rapidly inducible Cas9 and DSB-dPCR to probe editing kinetics. Although single stranded, RNA is not always linear. R., Benjamin Cummings, 2011. doi: 10.1126/science.aax7852 PubMed Abstract | CrossRef Full Text | Google Scholar Wang, H., Yang, H., Shivalila, C. 5101. Extensive work has been done to produce inducible Cas9 systems to control nuclease activity by modifying the Cas9 protein to be activated only when induced chemically (Dow et al., 2015; Rose et al., 2017) or optically (Hemphill et al., 2015; Nihongaki et al., 2015a,b, 2017; Polstein and Gersbach, 2015; Richter et al., 2016; Zhou et al., 2018). Kartje et al. doi: 10.7554/eLife.33761.032 PubMed Abstract | CrossRef Full Text | Google Scholar Schubert, M. Rep. By taking advantage of this endogenous repair pathway, efficient gene editing and gene knock-in are possible. *Science* 351, 84-88. Photocaging with light-sensitive 6-nitropiperonyloxymethyl (NPOM) thymidine modifications on the distal portion of the guide region prevents the gRNA from binding completely to its DNA target. Funding We gratefully acknowledge the funding support from the European Research Council (ERC) under the Horizon 2020 Research and Innovation Program (Grant Agreement No. 755758). During translation, the anticodon region of tRNA recognizes a specific area on messenger RNA (mRNA) called a codon. Truncated gRNAs (tru-gRNAs), as short as seventeen nucleotides, have been shown to destabilize the cleavage complex formation and reduce the time spent in the zipped conformation, allowing for more specific editing (Fu et al., 2014). E., et al. conducted an extensive, high-throughput analysis of Cas9 cleavage specificity both in vitro and in cultured cells, combined with mechanistic studies to identify the precise stage of the Cas9-cleavage reaction that was affected by the BNAAC and LNA substitutions (Cromwell et al., 2018). doi: 10.1021/ja51264v PubMed Abstract | CrossRef Full Text | Google Scholar Hendel, A., Bak, R. Lastly, this research was supported by the Ministry of Science, Technology & Space (Grant No. 3-14679). This can be achieved by chemically modifying the gRNA ends to reduce degradation by exonucleases, thus improving the guide's stability (Hendel et al., 2015a). The study focused solely on modifying crRNA while being mindful of the impact on the RNA's helix conformation. GFP and BFP were shown solely as examples since CRISPR/Cas9 covers the full spectrum of combinations. doi: 10.1038/s41467-019-11962-8 PubMed Abstract | CrossRef Full Text | Google Scholar Goodwin, M., Lee, E., Lakshmanan, U., Shipp, S., Frossi, L., Barzagli, F., et al. Both ribosomal subunits travel along the mRNA molecule translating the codons on mRNA into a polypeptide chain as they go. Although there is a wide consensus regarding the profile of chemical modifications that improve the intracellular and intra-serum stability of guide RNAs, the proper design of the chemical gRNA modifications to improve the specificity of CRISPR-mediated genome editing is still to be determined. S., Charpentier, E., and Koonin, E. This highlighted the necessity for proper hydrogen bonding for Cas9-gRNA complexes in cultured cells. 9:60. (2015a), as well as on the internal residues in the crRNA and tracrRNA regions, resulted in more efficient in vivo genome editing compared to the unmodified sgRNA or sgRNA with only terminal modifications (Finn et al., 2018). S., He, S., Nihongaki, Y., Li, X., Ravasi, S., et al. 9:2641. P., Liu, P., Yao, Q., Lazzarotto, C. 26, 2431-2442. A light-inducible CRISPR-Cas9 system for control of endogenous gene activation. However, a relatively simple and cost-effective method that allows optically-induced genome editing was recently demonstrated by adding photocaged light-sensitive 6-nitropiperonyloxymethyl (NPOM) thymidine modifications on the distal portion of the gRNA (Lu et al., 2020; Moroz-Omori et al., 2020). M., and Jounng, J. Likewise, a pioneering study by Hendel et al. *Nature* 529, 490-495. This creates a potential major pitfall for CRISPR-based therapies due to the well-understood correlation between increased DSBs to increased cellular toxicity and elevated immune response (Obe et al., 1992; Lips and Gains, 2001; Nakado and Schumacher, 2016; Bednarski and Sleckman, 2019). B., Kim, D. Therapeutic potential of CRISPR/Cas9 gene editing in engineered T-cell therapy. P., Pattanayak, V., Prew, M. 8, 4254-4264. Modifying gRNA to increase HDR efficiency CRISPR-mediated DSBs can be repaired via the HDR pathway to allow for precise editing of DNA sequences, to correct genetic mutations, or to introduce novel genetic fragments. Table 2. Hence, measuring the extent of indels on the site of interest, following CRISPR-mediated editing, is considered a gold standard for assessing the CRISPR activity in cultured cells and in vivo. R., van Heteren, J., et al. Since it was first reported in 2013 that the CRISPR system could be repurposed into a reliable and straightforward genome editing technique in mammalian cells (Cong et al., 2013; Mali et al., 2013; Hsu et al., 2014), the CRISPR-Cas system has championed the field of gene editing. Global transcriptional response to CRISPR/Cas9-AAV6-based genome editing in CD34(+) hematopoietic stem and progenitor cells. doi: 10.1038/s41467-020-17626-6 PubMed Abstract | CrossRef Full Text | Google Scholar Ran, F. Hypothesized that maximal 2'-modified ribose rings and modified backbone phosphate groups inside the crRNA and tracrRNA should generate the required gRNA formulation for clinical studies; albeit, all of the work in the study was conducted on HEK293 cells, without in vivo validation. Cell Biosci. R., Debacker, A., et al. 10.1016/j.jmth.2018.06.002 PubMed Abstract | CrossRef Full Text | Google Scholar Cromwell, C. R., Sung, K., Park, J., Kryslar, A. 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Disorders that can be treated by this method include  $\beta$ -globin-associated diseases such as sickle-cell anemia and  $\beta$ -thalassaemia (Dever et al., 2016; DeWitt et al., 2016; Park et al., 2019; Romero et al., 2019; Wu et al., 2019), as well as Severe Combined Immunodeficiency (SCID) (Pavel-Dinu et al., 2019), Polyendocrinopathy Enteropathy X-linked Syndrome (PHEX) (Goodwin et al., 2020), Wiskott-Aldrich Syndrome (Rai et al., 2020), X-linked chronic granulomatous disease (De Ravin et al., 2017), and Mucopolysaccharidosis Type I (Gomez-Ospina et al., 2019). Editing the sickle cell disease mutation in human hematopoietic stem cells: comparison of endonucleases and homologous donor templates. Additionally, in Cas12a, modifications in the seed region or on the 5' handle were not well-tolerated (Safari et al., 2019). 8:360a1314. In this procedure, hematopoietic stem and progenitor cells (HSPCs) or T lymphocytes are isolated from the patient's blood, undergo the desired gene correction ex vivo, and are then transfused back to the patient's bloodstream. R-loop formation is depicted with Watson and Crick base pairing of the RNA:DNA heteroduplex. doi: 10.1126/science.1231143 PubMed Abstract | CrossRef Full Text | Google Scholar Cromer, M. doi: 10.1038/s41467-018-06129-w PubMed Abstract | CrossRef Full Text | Google Scholar Braasch, D. Throughout evolution, bacteria and archaea acquired the ability to store copies of portions of invading foreign genetic material such as plasmids, phage genomes, or RNA, as segments between clustered repetitive sequences in the genome. *Microbiol. A., Macherer, T., Ragone, F. M., et al. J., Baral, R., Barrera, L., 10:135. M., Gao, L., Zetsche, B., Scott, D. Acknowledgments We thank Dr. Adi Tovim-Recht for her useful support. Natl. Cytokine Biol. 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Nov 04, 2021 - The U5 and U6 small nuclear RNAs as active site components of the spliceosome. Science, Dec. 24, 1993;262(5142):1989-96, cited by applicant. Sousa, R. et al., T7 RNA polymerase. *Prog Nucleic Acid Res Mol Biol.* 2003;73:1-41. cited by applicant. Sousa, R. Use of T7 RNA polymerase and its mutants for incorporation of nucleoside analogs into RNA. Concepts of Biology is designed for the introductory biology course for nonmajors taught at most two- and four-year colleges. The scope, sequence, and level of the program are designed to match typical course syllabi in the market. Concepts of Biology includes interesting applications, features a rich art program, and conveys the major themes of biology.

technology in filamentous fungi: progress and perspective. The second type of gRNA that can complex with Cas is a synthetic sgRNA (~100 nt) where the bridged portion between the crRNA and the tracrRNA is covalently linked by an artificial tetraloop (Jinek et al., 2012) (Figure 1B). Recently, Tammitree and colleagues presented a simplified method for producing sgRNAs via synthesis of the variable guide sequence (20 nt) and subsequently ligating the product to the remaining constant region (79 nt) by a triazole linkage (Tammitree et al., 2019). doi: 10.1093/nar/gkx119 PubMed Abstract | CrossRef Full Text | Google Scholar Safar, F., Zare, K., Negahdaripour, M., Barezaki-Mowahed, M., and Ghasemi, Y. D., Smith, A. D., Singh, M., et al. Recently, a thoroughly optimized protocol for using end-modified sgRNA in human primary HSPCs was evaluated, demonstrating high editing efficiency and specificity through the delivery of the CRISPR system as an RNP complex (Shapiro et al., 2020, 2021). 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The many years of progress in enhancing small RNA-based technologies, such as antisense RNA and RNA interference (RNAi) (Levin, 2019), includes improving RNA stability by incorporating chemical modifications onto the small RNAs (Braasch et al., 2003; Chiu and Rana, 2003; Behlke, 2008; Bennett and Swaney, 2010; Delevay and Damha, 2012; Lennox and Behlke, 2020). K., Doudna, J. CRISPR-Cas9-based photoactivatable transcription systems to induce neuronal differentiation. However, in this study, the tracrRNA remained unmodified, and the potential of using these chemical modifications in vivo was not explored. Optical control of CRISPR/Cas9 gene editing. E., Welch, M. Another approach that has been shown to increase HDR efficiency without the need to cotarget the endogenous DNA utilizes RNA aptamers. doi: 10.1062/nla.201901054. 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B. demonstrated that introducing G-quadruplex (G4) structure at the 3' end of the sgRNA resulted in increased in vitro serum stability and higher editing efficiency in the zebrafish embryos, compared to the unmodified sgRNA (Nahar et al., 2018). 37, 657–666. doi: 10.1016/j.tibtech.2019.011.009 PubMed Abstract | CrossRef Full Text | Google Scholar Moroz-Omori, E. Science 337, 816–821. Cancer Med. V., Satyapartewi, D., Rameil, M., 43, 3389–3404, demonstrated that in vitro cleavage of DNA duplexes by Cas9 could be facilitated by chimeric crRNA-DNA crRNAs. Contrary to expectations, they showed that DNA substitutions inside the crRNA end, but not within the guide sequence, resulted in the Cas9-mediated cleavage being less tolerant of mismatches in the target sequence (Kartje et al., 2018). 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CRISPR-Cas9/Cas12a biotechnology and application in bacteria. doi: 10.1146/annurev-pharmtox.010909.105654 PubMed Abstract | CrossRef Full Text | Google Scholar Bin Moon, S., Lee, J. S., et al. doi: 10.3390/jms12186461 PubMed Abstract | CrossRef Full Text | Google Scholar Yao, R., Liu, D., Jia, X., Zheng, Y., Liu, W., and Xiao, Y. It has the ability to fold into complex three-dimensional shapes and form hairpin loops. When a termination codon is reached on the mRNA molecule, the translation process ends. (B) Synthetic sgRNA formulation where the crRNA and tracrRNA are covalently fused by a tetraloop. doi: 10.1101/gr.162339.113 PubMed Abstract | CrossRef Full Text | Google Scholar Chylinski, K., Makarova, K. H., Lee, C. doi: 10.1126/science.aad5227 PubMed Abstract | CrossRef Full Text | Google Scholar Song, R., Zhai, Q., Sun, L., Huang, E., Zhang, Y., Zhu, Y., et al. Q., Suresh, S., Kwan, S. Mol. Commun. In nature, rRNA is found as a two-part molecule consisting of crRNA (~36–42 nt), which contains the DNA-binding spacer sequence, and the tracrRNA (~67–89 nt) (Jinek et al., 2012). In addition, the researchers achieved higher specificity using the engineered hairpin structures than with the tr-gRNA analog when tested side by side. CRISPR Cpfl proteins: structure, function and implications for genome editing. 8:1711. In addition to engineering the Cas9 protein (Aird et al., 2018; Savic et al., 2018; Ling et al., 2020) or the DNA donor (Renaud et al., 2016) to improve HDR efficiency, modifications on the gRNA itself have great potential to enhance HDR efficiency in a non-viral manner to increase the relevance of the CRISPR-Cas9 gene-editing tool for many biotechnological applications. To that end, a combination of the truncated or hairpin-modified sgRNAs in tandem with the previously discussed terminal chemical modifications could prevent hairpin removal by intrinsic intracellular nuclease activity, thus maximizing the editing capabilities of engineered sgRNAs. It is important to note that hairpin structures' design must be stringent to ensure thermodynamic stability since below a specific free energy cut-off, the nuclease activity is severely impacted. Improving the efficiency that in vitro cleavage of DNA duplexes by Cas9 could be facilitated by chimeric crRNA-DNA crRNAs. Contrary to expectations, they showed that DNA substitutions inside the crRNA end, but not within the guide sequence, resulted in the Cas9-mediated cleavage being less tolerant of mismatches in the target sequence (Kartje et al., 2018). 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