Tetrazine-Ligated CRISPR sgRNAs for Efficient Genome Editing

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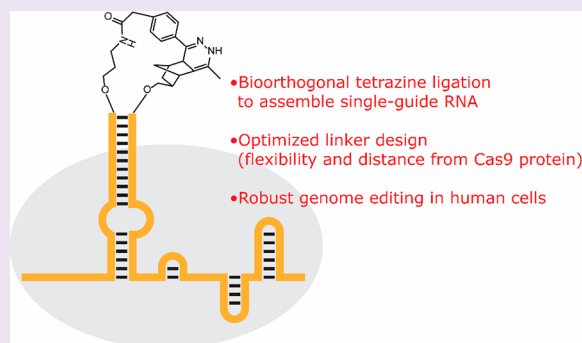
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ABSTRACT: CRISPR-Cas technology has revolutionized genome editing. Its broad and fast-growing application in biomedical research and therapeutics has led to increased demand for guide RNAs. The synthesis of chemically modified single-guide RNAs (sgRNAs) containing >100 nucleotides remains a bottleneck. Here we report the development of a tetrazine ligation method for the preparation of sgRNAs. A tetrazine moiety on the 3′-end of the crRNA and a norbornene moiety on the 5′-end of the tracrRNA enable successful ligation between crRNA and tracrRNA to form sgRNA under mild conditions. Tetrazine-ligated sgRNAs allow efficient genome editing of reporter and endogenous loci in human cells. High-efficiency editing requires structural optimization of the linker.

- Bioorthogonal tetrazine ligation to assemble single-guide RNA
- Optimized linker design (flexibility and distance from Cas9 protein)
- Robust genome editing in human cells

CRISPR-Cas genome editing has profoundly advanced biomedical research and holds promise as a therapeutic modality.[1] CRISPR-Cas systems use programmable guide RNAs that direct sequence-specific DNA cleavage by Cas nucleases.[2−5] CRISPR-mediated editing can be performed in cells or organisms by DNA-, RNA-, or ribonucleoprotein (RNP)-based delivery of the effector and guide RNA.[6] Direct chemical synthesis can be used to generate chemically modified gRNAs with improved efficiency, enhanced stability, reduced off-target editing, and improved delivery and cellular uptake relative to unmodified guides.[7−16]

Most CRISPR-Cas systems, including *Streptococcus pyogenes* Cas9 (SpyCas9), use single-guide RNAs (sgRNAs) containing >100 nucleotides.[2] Oligonucleotides this long are expensive to synthesize, and yields tend to be low. A dual-guide approach can be used, consisting of two short RNA pieces (crRNA and tracrRNA) assembled by hybridization.[2,7] However, sgRNAs are more effective than dual-guide RNAs for genome editing in many cases. For example, after lipid nanoparticle delivery of Cas9 mRNA and guide RNA to the mouse liver, sgRNA provided substantially higher in vivo editing than dual-guide RNA.[13] Therefore, efficient, scalable production of safe and effective sgRNAs remains a key challenge.

Ligation of short synthetic RNAs offers an alternative to the synthesis of a long RNA. However, enzymatic ligation is time-consuming and difficult to scale.[17] Instead, chemical ligation is relatively easy to implement and scale. Indeed, copper-catalyzed azide−alkyne cycloaddition (CuAAC) chemistry has recently been used to ligate two RNA components into a sgRNA.[18] However, the ligated sgRNAs were significantly less effective than in vitro-transcribed sgRNAs, although their efficiency can be aided by installing chemical modifications on the ligated sgRNAs.[18] Moreover, the copper catalyst is toxic,

and therefore, the products must be carefully purified before in vivo use.[19−21] We have also observed that copper can have side reactions with phosphorothioate (PS) linkages, which are desired for stability and uptake of sgRNAs in vivo. Therefore, we sought to develop a metal-free chemical ligation method to prepare sgRNAs.[22]

The tetrazine-based inverse-electron-demand Diels−Alder (IEDDA) reaction (Figure 1a) has emerged as a promising bioorthogonal ligation chemistry with rapid kinetics that does not depend on a metal catalyst.[23−27] Despite many examples of nucleic acid applications,[27−36] the tetrazine-based IEDDA reaction has not been used to produce long RNA molecules. Here we show that tetrazine-based ligation can be used to form sgRNAs that support efficient genome editing.

Standard sgRNA designs comprise a crRNA fused to a tracrRNA by a four-nucleotide linker (GAAA).[2] This linker forms a tetraloop that protrudes from the nuclease in CRISPR-Cas9 structures,[37−39] suggesting that SpyCas9 can accommodate structural changes in the loop. We therefore chose the junction between the 3′-end of the crRNA and the 5′-end of the tracrRNA as the ligation point (Figure 1b). We selected norbornene as the alkene in the tetrazine ligation because it can be readily incorporated into solid-phase RNA synthesis.[28] We successfully synthesized norbornene phosphoramidites (Figure S2) and installed the norbornene moiety at the 5′-end of the tracrRNA during solid-phase synthesis. To generate a
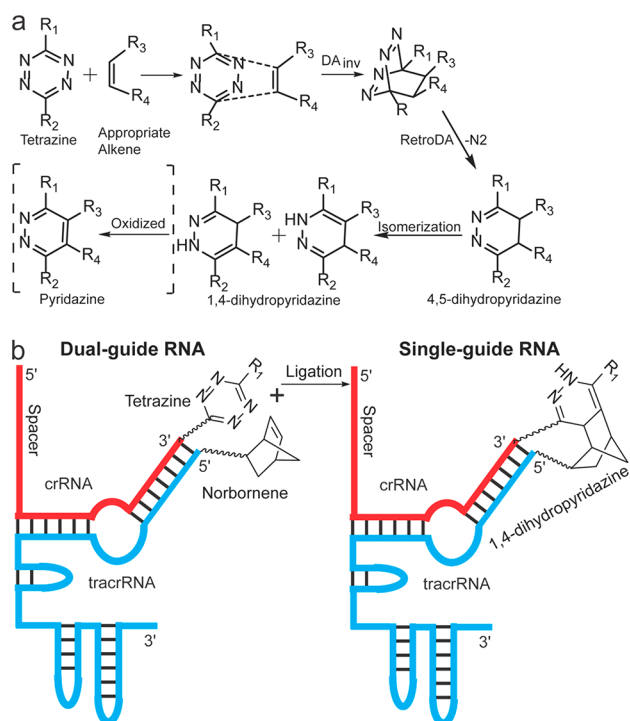
Figure 1. (a) General mechanism of tetrazine-based ligation and (b) proposed tetrazine ligation strategy for sgRNA.

tetrazine moiety on the crRNA, we synthesized the 3′-amino-modified crRNA and then installed the tetrazine moiety via postsynthetic conjugation with tetrazine *N*-hydroxysuccinimide (NHS) ester. Because the structure of the linker may affect binding of the tetrazine-ligated sgRNA to Cas9, we used two different tetrazine NHS esters to generate sgRNAs with either a short linker 1 or a long linker 2 (Figure 2a, Figure S3). Linker 2 includes an extra octaethylene glycol (PEG8) segment. To perform the tetrazine ligation, we combined the 3′-tetrazine-modified crRNA and the 5′-norbornene-modified tracrRNA and incubated the molecules under mild conditions (20 mM Tris-HCl, 200 mM NaCl, pH 7.4) for ∼20 h at room temperature (Figure S4). We verified the success of the ligation reactions by PAGE analysis (Figure S5) and confirmed the identities of the purified ligation products by HPLC-MS (Table S1 and Figures S7 and S8).

To facilitate testing in cells, we designed the ligated sgRNAs to target a traffic light reporter construct, TLR1.[40] This reporter has a validated guide RNA sequence with good editing efficiency and allows the use of flow cytometry for easy quantification. Its nature as a gain-of-function assay facilitates low background when editing is scored. In this assay, CRISPR-mediated cleavage of TLR1 is repaired by mutagenic end-joining pathways, which shifts an out-of-frame mCherry coding region into the correct reading frame in a subset of edited cells, resulting in mCherry expression. The percentage of mCherry-positive cells measured by flow cytometry therefore provides a lower-limit measure of the editing efficiency.

To explore how the tetrazine-ligated sgRNAs affect the editing efficiency, we assembled TLR1 sgRNA–SpyCas9 RNP complexes and electroporated the complexes into HEK-293T TLR1 reporter cells. We compared the functions of linker 1 and linker 2 sgRNAs at various RNP dosages with a fixed Cas9:sgRNA ratio of 1:3 (Figure 2b). We also analyzed various Cas9:sgRNA ratios with a fixed RNP dosage of 2.5 pmol
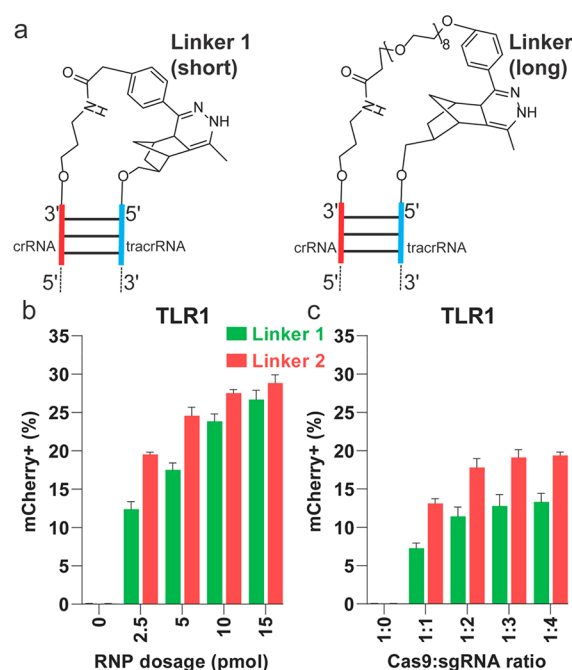

Figure 2. Initial testing of tetrazine-ligated sgRNAs with linkers 1 and 2. (a) Illustration of tetrazine-ligated sgRNAs with linkers 1 and 2. (b) Dose titration of sgRNAs with linkers 1 and 2 in the HEK-293T TLR1 assay with the Cas9:sgRNA ratio fixed at 1:3. (c) Cas9:sgRNA ratio titration of sgRNAs with linkers 1 and 2 in the HEK-293T TLR1 assay with the RNP dosage fixed at 2.5 pmol. Data are reported as mean ± SD of three independent biological replicates.

(Figure 2c). The sgRNA with long linker 2 performed better than that with the short linker 1 under all of the conditions tested, especially at low RNP dosages. At the higher RNP dosages, the assay may be near saturation. These results indicate that tetrazine-ligated sgRNAs support CRISPR-mediated editing and confirm the importance of the linker structure in the sgRNA activity.

We next applied the linker 2 design to sgRNAs that target endogenous loci in human cells. To benchmark the activity of the tetrazine-ligated sgRNAs, we compared them to standard synthetic sgRNAs with a GAAA tetraloop linking the crRNA and tracrRNA. At each end of the sgRNAs (standard and tetrazine-ligated), the last three nucleotides were chemically modified with PS linkages and 2′-*O*-methyl (2′-OMe) groups to protect them from degradation. Tetrazine-ligated sgRNAs targeting TLR1 and four endogenous loci (*CCR5*, *HEK3*, *TRAC*, and *HPRT*) were generated, and their identities were confirmed by HPLC-MS (Table S1 and Figures S9–S13). The ability to generate these tetrazine-ligated sgRNAs indicates that the tetrazine ligation chemistry is generally compatible with chemical modifications (PS, 2′-OMe) that are important for sgRNA stability and activity in cells.

We first electroporated TLR1 RNPs into HEK-293T TLR1 reporter cells to compare tetrazine-ligated and standard sgRNAs at various RNP dosages with a fixed Cas9:sgRNA ratio of 1:3 (Figure 3a) or with various Cas9:sgRNA ratios at a fixed RNP dosage of 2.5 pmol (Figure 3b). The TLR1 tetrazine-ligated sgRNA exhibited activity comparable to that of standard sgRNA at RNP dosages of 10 pmol (27% vs 30%) and 15 pmol (28% vs 30%), but it was less active than standard sgRNA at lower RNP dosages (e.g., 17% vs 30% editing at 2.5 pmol) (Figure 3a). The reduced editing activity of tetrazine-
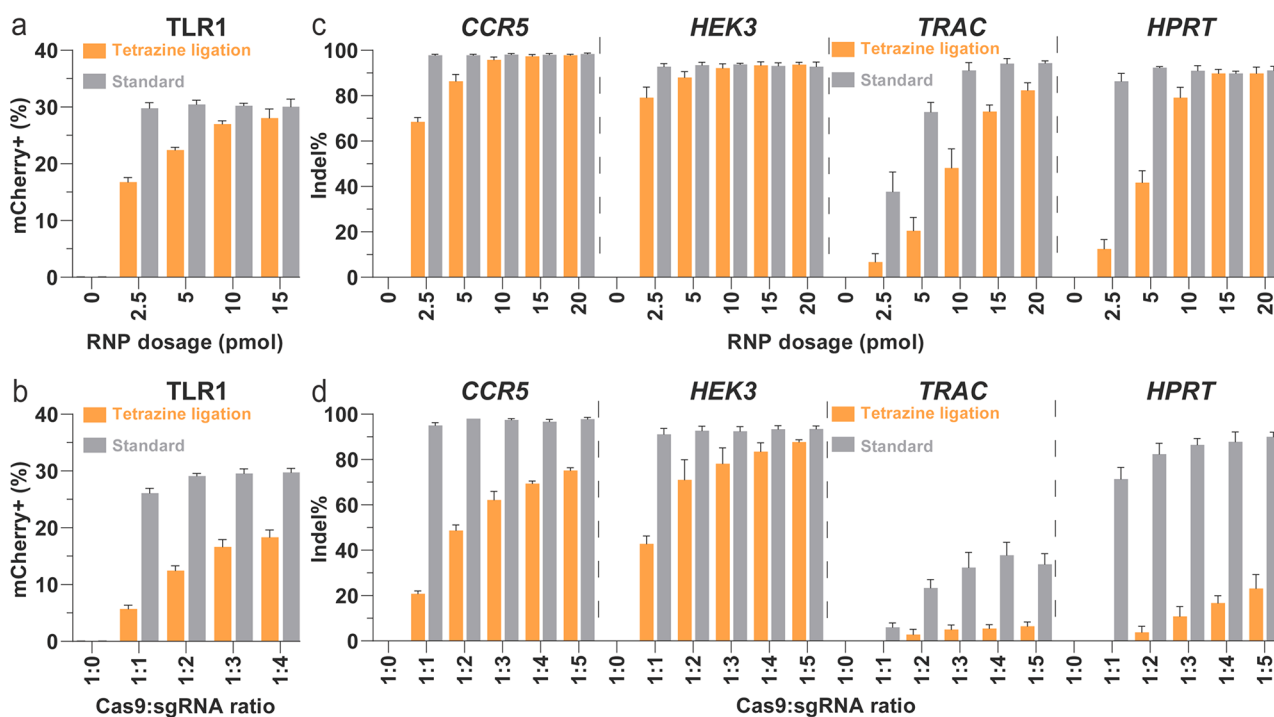
Figure 3. Comparison of the genome editing efficiencies of tetrazine-ligated (linker 2) and standard sgRNAs. (a) Titration of tetrazine-ligated and standard sgRNAs in the HEK-293T TLR1 assay with the Cas9:sgRNA ratio fixed at 1:3. (b) Cas9:sgRNA ratio titration of tetrazine-ligated and standard sgRNAs in the HEK-293T TLR1 assay with the RNP dosage fixed at 2.5 pmol. (c) Dose titration of tetrazine-ligated and standard sgRNAs for endogenous loci (*CCR5*, *HEK3*, *TRAC*, and *HPRT*) in HEK-293T cells with the Cas9:sgRNA ratio fixed at 1:3. (d) Cas9:sgRNA ratio titration of tetrazine-ligated and standard sgRNAs for endogenous loci (*CCR5*, *HEK3*, *TRAC*, and *HPRT*) in HEK-293T cells with the RNP dose fixed at 2.5 pmol. Data are reported as mean ± SD of three independent biological replicates.
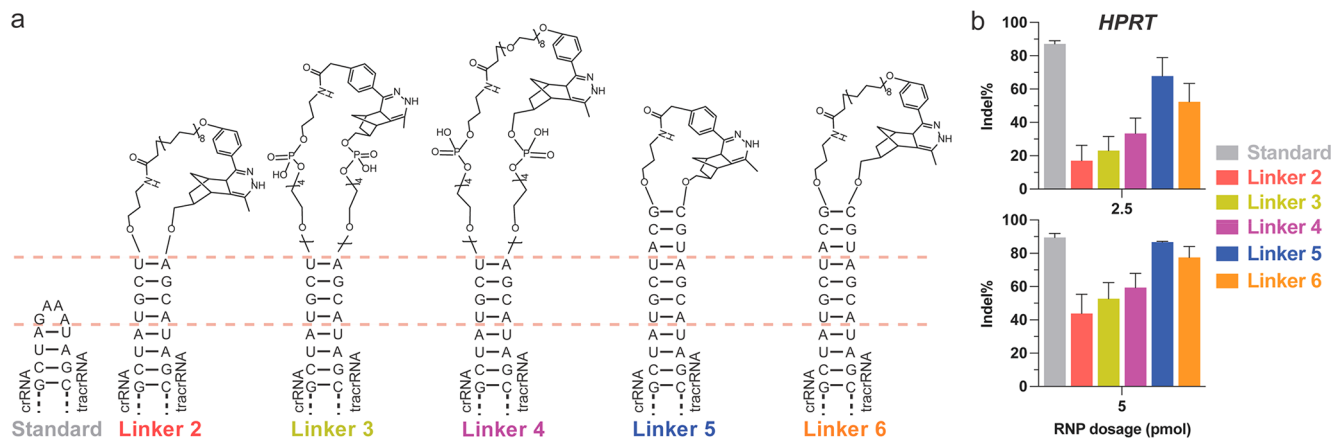

Figure 4. Comparison of standard and different linker-ligated sgRNAs. (a) Structures of the standard and modified linkers. (b) Comparison of *HPRT* sgRNA genome editing efficiencies of standard and modified linkers at RNP dosages of 2.5 pmol (top) and 5 pmol (bottom) in HEK-293T cells. Data are reported as mean ± SD of three independent biological replicates.

ligated TLR1 sgRNA was exacerbated at lower sgRNA:Cas9 ratios (Figure 3b).

To compare the editing activities of tetrazine-ligated and standard sgRNAs targeting endogenous loci, we sequenced the intended editing regions and calculated the editing efficiencies using the ICE algorithm.[41] Consistent with the TLR assay, tetrazine-ligated and standard sgRNAs targeting endogenous loci were similarly active at high RNP dosages, but at low RNP dosages, the tetrazine-ligated sgRNAs were less active than the standard sgRNAs (Figure 3c). The editing efficiency of tetrazine-ligated sgRNA was further reduced when the Cas9:sgRNA ratio decreased (Figure 3d). Overall, the

tetrazine-ligated sgRNA with linker 2 consistently performed well at high RNP dosages but weakly at low RNP dosages.

The results of our experiments shown in Figure 3 made it clear that linker 2 is not optimal. We considered several hypotheses: (i) Linker 2 is still too short or inflexible to allow optimal conformation of the RNP; (ii) the PEG8 segment in linker 2 may not be optimally configured; or (iii) the pyridazine-based linkage in linker 2 may have certain unwanted effects with Cas9 or the rest of the guide. To test and address these possibilities, we designed several new linkers (Figure 4a). Linker 3 has the same length as linker 2, but instead of a single PEG8 segment, we incorporated a PEG4 segment on each side
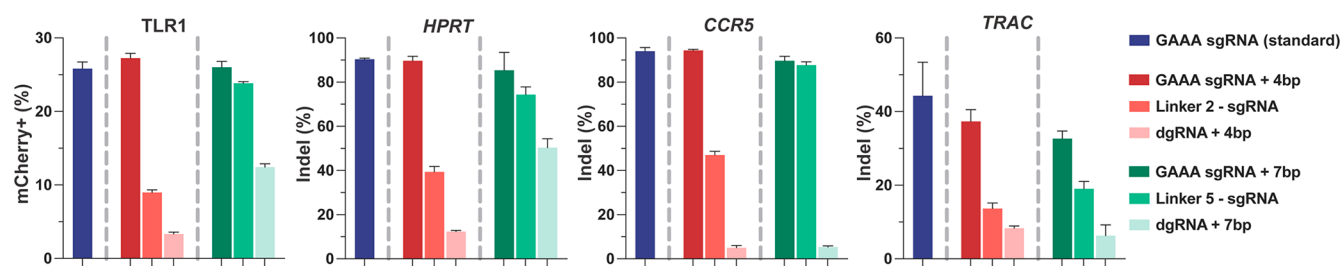
Figure 5. Comparison of linker 2- and linker 5-ligated sgRNAs with stem-length-matched sgRNA and dgRNA controls at TLR1, *HPRT*, *CCR5*, and *TRAC* loci in HEK-293T cells. Data are reported as mean ± SD of three biological replicates.

of the loop. Linker 4 includes the PEG8 segment from linker 2 and both PEG4 segments from linker 3, adding length and flexibility relative to linker 2. Finally, linkers 5 and 6 are analogues of linkers 1 and 2 in which the stem formed by the crRNA and tracrRNA is extended by three base pairs, on the basis of the hypothesis that the extended (rigid) duplex structure might minimize any unwanted effects of the pyridazine-based linkage with Cas9 that might occur.

To test our new linkers (3−6) under the most demanding conditions, we applied them to sgRNAs targeting *HPRT*, for which the editing efficiency difference between the tetrazine-ligated (linker 2) sgRNA and standard sgRNA was greatest (Figure 3c,d). The tetrazine-ligated sgRNAs with linkers 3−6 were generated and confirmed by HPLC-MS (Table S1 and Figures S14−S17) and assembled into RNPs. We then compared the editing efficiencies of the tetrazine-ligated (linkers 2−6) and standard GAAA-linked sgRNAs at RNP dosages of 2.5 and 5 pmol, which showed the largest differences in activity between the linker 2- and GAAA-linked sgRNA. As shown in Figure 4b, we observed that sgRNAs with linkers 3−6 performed better at both RNP dosages than did the sgRNA with linker 2. Linker 5 sgRNA was the most effective: at the RNP dosage of 2.5 pmol, the linker 5 sgRNA (68%) was ∼4-fold more effective than linker 2 sgRNA (17%) and was only 22% less active than standard sgRNA (87%). Importantly, at the RNP dosage of 5 pmol, the activity of linker 5 sgRNA (87%) was comparable to that of standard sgRNA (90%). The linker 6 sgRNA was the next most effective tetrazine-ligated sgRNA. The improved performance of linkers 5 and 6 suggests that extending the crRNA:tracrRNA stem structure improves the activity of tetrazine-ligated sgRNAs.

We considered the possibility that the increment of editing efficiency from linker 2 to linker 5 might simply be caused by the extension of the stem itself, unrelated to the linker structure. Previous literature has shown that stem extension can improve the activity of RNP complexes, possibly as a result of increases in gRNA stability and gRNA−Cas9 assembly.[2,3,42−45] To better characterize this, we expanded our experiments and analysis with additional controls. Because linker 2 sgRNA has four more base pairs than standard tetraloop-linked sgRNA in the upper stem region, we included a GAAA-linked sgRNA control and a dual-guide (dgRNA) control with the same four additional base pairs. Similarly, for linker 5 sgRNA, we added the GAAA-linked sgRNA and dgRNA controls with seven more base pairs in the upper stem region. We compared these seven guide designs across four loci: TLR1, *HPRT*, *CCR5*, and *TRAC*.

Linker 5 sgRNAs provided higher editing efficiencies than linker 2 sgRNAs at all four loci, though the magnitude of the improvement varied (Figure 5). For instance, linker 5 sgRNAs

were almost as good as GAAA-linked sgRNAs at the TLR1, *HPRT*, and *CCR5* loci. Moreover, both linker 2 and linker 5 sgRNAs were more active than their corresponding dgRNA controls at all four loci. This confirms the importance of sgRNAs for efficient genome editing. Finally, the observation that the editing efficiencies of GAAA sgRNA controls with different stem lengths were similar suggests that the improvement in going from linker 2 to linker 5 is not simply due to the longer stem of linker 5.

It is intriguing that the effect of the linker design on the sgRNA activity depends on the context of the crRNA:tracrRNA stem length. With a shorter stem, the long linker 2 sgRNA was more active than the short linker 1 sgRNA (Figure 2b-c), but conversely, when the crRNA:tracrRNA stem was extended by three base pairs, the short linker 5 sgRNA was more active than the long linker 6 sgRNA (Figure 4).

One possible explanation for our results is that pyridazine-based linkages, particularly in the context of short stem lengths, may impair optimal interactions with Cas9. We thus performed an electrophoretic mobility shift assay (EMSA) for the seven TLR1 gRNAs used in Figure 5. The results (Figure S6) suggested that tetrazine-ligated sgRNAs had somewhat lower affinities for Cas9 than the corresponding GAAA-linked sgRNAs. Future studies should address the magnitude of this decrease more quantitatively and develop guides and linkages that increase rather than decrease Cas9 affinity.

In summary, we have developed tetrazine-linked sgRNAs that support efficient editing activity in human cells, rivaling that of standard sgRNA. Moreover, our tetrazine ligation strategy is easy to implement and scale because the long, invariant tracrRNA can be made in bulk quantity, ready to be ligated to the shorter, sequence-variable crRNA according to the desired target. Therefore, tetrazine ligation provides a promising route to CRISPR sgRNAs for efficient genome editing. We expect that this approach will also be useful for the production of guide RNAs for other Cas nucleases and other editing platforms that require even longer guide RNAs,[46] thus expanding the utility of CRISPR genome editing in biomedical research and therapeutics development.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.2c00116.

Experimental methods and additional information related to synthesis, characterization, and genome editing experiments (PDF)

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Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Urnov, F. D. Genome Editing B.C. (Before CRISPR): Lasting Lessons from the "Old Testament. *CRISPR Journal* **2018**, *1* (1), 34−46.

(2) Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J. A.; Charpentier, E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **2012**, *337* (6096), 816−821.

(3) Jinek, M.; East, A.; Cheng, A.; Lin, S.; Ma, E.; Doudna, J. RNA-Programmed Genome Editing in Human Cells. *eLife* **2013**, *2*, No. e00471.

(4) Cong, L.; Ran, F. A.; Cox, D.; Lin, S.; Barretto, R.; Habib, N.; Hsu, P. D.; Wu, X.; Jiang, W.; Marraffini, L. A.; Zhang, F. Multiplex Genome Engineering Using CRISPR/Cas Systems. *Science* **2013**, *339* (6121), 819−823.

(5) Mali, P.; Yang, L.; Esvelt, K. M.; Aach, J.; Guell, M.; DiCarlo, J. E.; Norville, J. E.; Church, G. M. RNA-Guided Human Genome Engineering via Cas9. *Science* **2013**, *339* (6121), 823−826.

(6) Glass, Z.; Lee, M.; Li, Y.; Xu, Q. Engineering the Delivery System for CRISPR-Based Genome Editing. *Trends Biotechnol.* **2018**, *36* (2), 173−185.

(7) Mir, A.; Alterman, J. F.; Hassler, M. R.; Debacker, A. J.; Hudgens, E.; Echeverria, D.; Brodsky, M. H.; Khvorova, A.; Watts, J. K.; Sontheimer, E. J. Heavily and Fully Modified RNAs Guide Efficient SpyCas9-Mediated Genome Editing. *Nat. Commun.* **2018**, *9* (1), 2641.

(8) Yin, H.; Song, C.-Q.; Suresh, S.; Wu, Q.; Walsh, S.; Rhym, L. H.; Mintzer, E.; Bolukbasi, M. F.; Zhu, L. J.; Kauffman, K.; Mou, H.; Oberholzer, A.; Ding, J.; Kwan, S.-Y.; Bogorad, R. L.; Zatsepin, T.; Koteliansky, V.; Wolfe, S. A.; Xue, W.; Langer, R.; Anderson, D. G. Structure-Guided Chemical Modification of Guide RNA Enables Potent Non-Viral in Vivo Genome Editing. *Nat. Biotechnol.* **2017**, *35* (12), 1179−1187.

(9) Rahdar, M.; McMahon, M. A.; Prakash, T. P.; Swayze, E. E.; Bennett, C. F.; Cleveland, D. W. Synthetic CRISPR RNA-Cas9-Guided Genome Editing in Human Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (51), E7110−E7117.

(10) Cromwell, C. R.; Sung, K.; Park, J.; Krysler, A. R.; Jovel, J.; Kim, S. K.; Hubbard, B. P. Incorporation of Bridged Nucleic Acids into CRISPR RNAs Improves Cas9 Endonuclease Specificity. *Nat. Commun.* **2018**, *9* (1), 1448.

(11) Yin, H.; Song, C.-Q.; Suresh, S.; Kwan, S.-Y.; Wu, Q.; Walsh, S.; Ding, J.; Bogorad, R. L.; Zhu, L. J.; Wolfe, S. A.; Koteliansky, V.; Xue, W.; Langer, R.; Anderson, D. G. Partial DNA-Guided Cas9 Enables Genome Editing with Reduced off-Target Activity. *Nat. Chem. Biol.* **2018**, *14* (3), 311−316.

(12) Rueda, F. O.; Bista, M.; Newton, M. D.; Goeppert, A. U.; Cuomo, M. E.; Gordon, E.; Kröner, F.; Read, J. A.; Wrigley, J. D.; Rueda, D.; Taylor, B. J. M. Mapping the Sugar Dependency for Rational Generation of a DNA-RNA Hybrid-Guided Cas9 Endonuclease. *Nat. Commun.* **2017**, *8* (1), 1610.

(13) Finn, J. D.; Smith, A. R.; Patel, M. C.; Shaw, L.; Youniss, M. R.; van Heteren, J.; Dirstine, T.; Ciullo, C.; Lescarbeau, R.; Seitzer, J.; Shah, R. R.; Shah, A.; Ling, D.; Growe, J.; Pink, M.; Rohde, E.; Wood, K. M.; Salomon, W. E.; Harrington, W. F.; Dombrowski, C.; Strapps, W. R.; Chang, Y.; Morrissey, D. V. A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. *Cell Rep.* **2018**, *22* (9), 2227−2235.

(14) Hendel, A.; Bak, R. O.; Clark, J. T.; Kennedy, A. B.; Ryan, D. E.; Roy, S.; Steinfeld, I.; Lunstad, B. D.; Kaiser, R. J.; Wilkens, A. B.; Bacchetta, R.; Tsalenko, A.; Dellinger, D.; Bruhn, L.; Porteus, M. H. Chemically Modified Guide RNAs Enhance CRISPR-Cas Genome Editing in Human Primary Cells. *Nat. Biotechnol.* **2015**, *33* (9), 985−989.

(15) Ryan, D. E.; Taussig, D.; Steinfeld, I.; Phadnis, S. M.; Lunstad, B. D.; Singh, M.; Vuong, X.; Okochi, K. D.; McCaffrey, R.; Olesiak, M.; Roy, S.; Yung, C. W.; Curry, B.; Sampson, J. R.; Bruhn, L.; Dellinger, D. J. Improving CRISPR-Cas Specificity with Chemical Modifications in Single-Guide RNAs. *Nucleic Acids Res.* **2018**, *46* (2), 792−803.

(16) Basila, M.; Kelley, M. L.; Smith, A. v. B. Minimal 2′-O-Methyl Phosphorothioate Linkage Modification Pattern of Synthetic Guide RNAs for Increased Stability and Efficient CRISPR-Cas9 Gene Editing Avoiding Cellular Toxicity. *PLoS One* **2017**, *12* (11), No. e0188593.

(17) El-Sagheer, A. H.; Brown, T. New Strategy for the Synthesis of Chemically Modified RNA Constructs Exemplified by Hairpin and Hammerhead Ribozymes. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107* (35), 15329−15334.

(18) Taemaitree, L.; Shivalingam, A.; El-Sagheer, A. H.; Brown, T. An Artificial Triazole Backbone Linkage Provides a Split-and-Click Strategy to Bioactive Chemically Modified CRISPR sgRNA. *Nat. Commun.* **2019**, *10* (1), 1610.

(19) Gaetke, L. Copper Toxicity, Oxidative Stress, and Antioxidant Nutrients. *Toxicology* **2003**, *189* (1−2), 147−163.

(20) Jewett, J. C.; Bertozzi, C. R. Cu-Free Click Cycloaddition Reactions in Chemical Biology. *Chem. Soc. Rev.* **2010**, *39* (4), 1272.

(21) Neumann, S.; Biewend, M.; Rana, S.; Binder, W. H. The CuAAC: Principles, Homogeneous and Heterogeneous Catalysts, and Novel Developments and Applications. *Macromol. Rapid Commun.* **2020**, *41* (1), 1900359.

(22) Taemaitree et al.[18] also demonstrated that a copper-free SPAAC reaction could ligate sgRNAs that were functional for in vitro DNA cleavage, but they did not present cellular data.

(23) Blackman, M. L.; Royzen, M.; Fox, J. M. Tetrazine Ligation: Fast Bioconjugation Based on Inverse-Electron-Demand Diels-Alder Reactivity. *J. Am. Chem. Soc.* **2008**, *130* (41), 13518−13519.

(24) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. Tetrazine-Based Cycloadditions: Application to Pretargeted Live Cell Imaging. *Bioconjugate Chem.* **2008**, *19* (12), 2297−2299.

(25) Devaraj, N. K.; Upadhyay, R.; Haun, J. B.; Hilderbrand, S. A.; Weissleder, R. Fast and Sensitive Pretargeted Labeling of Cancer Cells through a Tetrazine/*trans*-Cyclooctene Cycloaddition. *Angew. Chem., Int. Ed.* **2009**, *48* (38), 7013−7016.

(26) Knall, A.-C.; Slugovc, C. Inverse Electron Demand Diels-Alder (IEDDA)-Initiated Conjugation: A (High) Potential Click Chemistry Scheme. *Chem. Soc. Rev.* **2013**, *42* (12), 5131.

(27) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse Electron Demand Diels-Alder Reactions in Chemical Biology. *Chem. Soc. Rev.* **2017**, *46* (16), 4895−4950.

(28) Schoch, J.; Wiessler, M.; Jäschke, A. Post-Synthetic Modification of DNA by Inverse-Electron-Demand Diels-Alder Reaction. *J. Am. Chem. Soc.* **2010**, *132* (26), 8846−8847.

(29) Schoch, J.; Ameta, S.; Jäschke, A. Inverse Electron-Demand Diels-Alder Reactions for the Selective and Efficient Labeling of RNA. *Chem. Commun.* **2011**, *47* (46), 12536−12537.

(30) Schoch, J.; Staudt, M.; Samanta, A.; Wiessler, M.; Jäschke, A. Site-Specific One-Pot Dual Labeling of DNA by Orthogonal Cycloaddition Chemistry. *Bioconjugate Chem.* **2012**, *23* (7), 1382−1386.

(31) Šečkutė, J.; Yang, J.; Devaraj, N. K. Rapid Oligonucleotide-Templated Fluorogenic Tetrazine Ligations. *Nucleic Acids Res.* **2013**, *41* (15), No. e148.

(32) Asare-Okai, P. N.; Agustin, E.; Fabris, D.; Royzen, M. Site-Specific Fluorescence Labelling of RNA Using Bio-Orthogonal Reaction of Trans-Cyclooctene and Tetrazine. *Chem. Commun.* **2014**, *50* (58), 7844−7847.

(33) Pyka, A. M.; Domnick, C.; Braun, F.; Kath-Schorr, S. Diels-Alder Cycloadditions on Synthetic RNA in Mammalian Cells. *Bioconjugate Chem.* **2014**, *25* (8), 1438−1443.

(34) Domnick, C.; Eggert, F.; Kath-Schorr, S. Site-Specific Enzymatic Introduction of a Norbornene Modified Unnatural Base into RNA and Application in Post-Transcriptional Labeling. *Chem. Commun.* **2015**, *51* (39), 8253−8256.

(35) Holstein, J. M.; Rentmeister, A. Current Covalent Modification Methods for Detecting RNA in Fixed and Living Cells. *Methods* **2016**, *98*, 18−25.

(36) Winz, M.-L.; Linder, E. C.; Becker, J.; Jäschke, A. Site-Specific One-Pot Triple Click Labeling for DNA and RNA. *Chem. Commun.* **2018**, *54* (83), 11781−11784.

(37) Nishimasu, H.; Ran, F. A.; Hsu, P. D.; Konermann, S.; Shehata, S. I.; Dohmae, N.; Ishitani, R.; Zhang, F.; Nureki, O. Crystal Structure of Cas9 in Complex with Guide RNA and Target DNA. *Cell* **2014**, *156* (5), 935−949.

(38) Jiang, F.; Doudna, J. A. CRISPR-Cas9 Structures and Mechanisms. *Annu. Rev. Biophys.* **2017**, *46* (1), 505−529.

(39) Jinek, M.; Jiang, F.; Taylor, D. W.; Sternberg, S. H.; Kaya, E.; Ma, E.; Anders, C.; Hauer, M.; Zhou, K.; Lin, S.; Kaplan, M.; Iavarone, A. T.; Charpentier, E.; Nogales, E.; Doudna, J. A. Structures of Cas9 Endonucleases Reveal RNA-Mediated Conformational Activation. *Science* **2014**, *343* (6176), 1247997.

(40) Certo, M. T.; Ryu, B. Y.; Annis, J. E.; Garibov, M.; Jarjour, J.; Rawlings, D. J.; Scharenberg, A. M. Tracking Genome Engineering Outcome at Individual DNA Breakpoints. *Nat. Methods* **2011**, *8* (8), 671−676.

(41) Conant, D.; Hsiau, T.; Rossi, N.; Oki, J.; Maures, T.; Waite, K.; Yang, J.; Joshi, S.; Kelso, R.; Holden, K.; Enzmann, B. L.; Stoner, R. Inference of CRISPR Edits from Sanger Trace Data. *CRISPR J* **2022**, *5*, 123−130.

(42) Gasiunas, G.; Barrangou, R.; Horvath, P.; Siksnys, V. Cas9-CrRNA Ribonucleoprotein Complex Mediates Specific DNA Cleavage for Adaptive Immunity in Bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (39), E2579−E2586.

(43) Briner, A. E.; Donohoue, P. D.; Gomaa, A. A.; Selle, K.; Slorach, E. M.; Nye, C. H.; Haurwitz, R. E.; Beisel, C. L.; May, A. P.; Barrangou, R. Guide RNA Functional Modules Direct Cas9 Activity and Orthogonality. *Mol. Cell* **2014**, *56* (2), 333−339.

(44) Chen, B.; Gilbert, L. A.; Cimini, B. A.; Schnitzbauer, J.; Zhang, W.; Li, G.-W.; Park, J.; Blackburn, E. H.; Weissman, J. S.; Qi, L. S.; Huang, B. Dynamic Imaging of Genomic Loci in Living Human Cells by an Optimized CRISPR/Cas System. *Cell* **2013**, *155* (7), 1479−1491.

(45) Grevet, J. D.; Lan, X.; Hamagami, N.; Edwards, C. R.; Sankaranarayanan, L.; Ji, X.; Bhardwaj, S. K.; Face, C. J.; Posocco, D. F.; Abdulmalik, O.; Keller, C. A.; Giardine, B.; Sidoli, S.; Garcia, B. A.; Chou, S. T.; Liebhaber, S. A.; Hardison, R. C.; Shi, J.; Blobel, G. A. Domain-Focused CRISPR Screen Identifies HRI as a Fetal Hemoglobin Regulator in Human Erythroid Cells. *Science* **2018**, *361* (6399), 285−290.

(46) Edraki, A.; Mir, A.; Ibraheim, R.; Gainetdinov, I.; Yoon, Y.; Song, C.-Q.; Cao, Y.; Gallant, J.; Xue, W.; Rivera-Pérez, J. A.; Sontheimer, E. J. A Compact, High-Accuracy Cas9 with a Dinucleotide PAM for In Vivo Genome Editing. *Mol. Cell* **2019**, *73* (4), 714−726.