Structural roles of guide RNAs in the nuclease activity of Cas9 endonuclease

Youngbin Lim1, So Young Bak2, Keewon Sung2, Euihwan Jeong2, Seung Hwan Lee3, Jin-Soo Kim2,3, Sangsu Bae2,3,† & Seong Keun Kim1,2

The type II CRISPR-associated protein Cas9 recognizes and cleaves target DNA with the help of two guide RNAs (gRNAs; tracrRNA and crRNA). However, the detailed mechanisms and kinetics of these gRNAs in the Cas9 nuclease activity are unclear. Here, we investigate the structural roles of gRNAs in the CRISPR-Cas9 system by single-molecule spectroscopy and reveal a new conformation of inactive Cas9 that is thermodynamically more preferable than active apo-Cas9. We find that tracrRNA prevents Cas9 from changing into the inactive form and leads to the Cas9:gRNA complex. For the Cas9:gRNA complex, we identify sub-conformations of the RNA–DNA heteroduplex during R-loop expansion. Our single-molecule study indicates that the kinetics of the sub-conformations is controlled by the complementarity between crRNA and target DNA. We conclude that both tracrRNA and crRNA regulate the conformations and kinetics of the Cas9 complex, which are crucial in the DNA cleavage activity of the CRISPR-Cas9 system.
Most archaea and 40% of bacteria have an adaptive immune system called the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated) complex that provides protection against DNAs of invasive bacteriophages1–3. The CRISPR locus consists of a repetitive sequence interspersed by short sequences (‘spacers’) derived from fragments of invasive foreign genomes (protospacers)4. CRISPR RNA (crRNA), which contains individual spacer sequences, recognizes target DNA sites via Watson–Crick base pair binding to facilitate targeted double-strand breaks5. Among CRISPR-Cas systems, the type II CRISPR-Cas system is widely used for genome editing in cultured cells and various organisms because of its high specificity and ease of use6.

In the type II CRISPR-Cas system, only one protein, Cas9, serves as an RNA-guided endonuclease utilizing two noncoding RNAs, crRNA and trans-activating crRNA (tracrRNA)7. A hybridized duplex of crRNA-tracrRNA binds to Cas9 and induces the cleavage of target DNA sequences containing protospacer-adjacent motif (PAM) at the 5′ end of target DNA8–10.

Since Cas9 endonuclease derived from Streptococcus pyogenes was first applied to genome editing in human cells11–14, extensive conformational studies of guide RNAs (gRNAs; that is, crRNA and tracrRNA) that form Cas9gRNA protein–nucleic acid complexes have been performed. The RNA conformations of Cas9gRNA are important in RNA–DNA base pairing, facilitating the discrimination between the target sites and off-target sites of target DNA. Similar to other nucleic acid-related enzymatic processes, gRNAs lead to multiple conformationally different Cas9gRNA intermediates with different enzymatic activities. Previous biochemical assays15 and structural studies using X-ray crystallography16–18 and single-particle electron microscopy imaging19 have shown that gRNAs are involved in the expansion of the R-loop structure and initiate large-scale conformational changes of Cas9 for target DNA recognition. However, the mechanistic details of the independent roles of the two gRNAs in Cas9 nucleic acid activation are still unclear.

The goal of this study is to investigate and unravel the molecular mechanism of the two gRNAs separately on Cas9gRNA complexation and their conformational changes associated with the nucleic activity. Using single-molecule fluorescence spectroscopy, we find an inactive state of apo-Cas9, transition toward which is suppressed by the tracrRNA–Cas9 interaction. We also find conformational heterogeneity in the crRNA–DNA R-loop structure, whose distribution regulates the nucleic activity of Cas9 endonuclease. These results show that tracrRNA governs the formation of the catalytically active Cas9–gRNA complex, whereas crRNA regulates the nucleic activation of the Cas9–gRNA–DNA ternary complex.

Results

Visualization of Cas9-mediated cleavage. To visualize Cas9-induced DNA cleavage activities on the immobilized target DNA, we used total internal reflection fluorescence microscopy. We designed biotinylated 48-bp double-stranded DNA (dsDNA) dual-labelled with Cy3 and Cy5 at the opposite ends of each strand and tethered to the surface via biotin–neutravidin interaction (Supplementary Fig. 1 and Supplementary Table 1). We pre-incubated 2 nM Cas9 with excess gRNAs (300 nM) for 20 min at 37°C to form Cas9gRNAs and then injected the mixture onto the immobilized DNA. Progress of DNA cleavage by Cas9gRNAs and dissociation of cleaved DNA fragments from the Cas9gRNAs were monitored by measuring the ratio of the number of Cy5 molecules to that of Cy3 molecules. Immediately on adding Cas9gRNAs to the target DNA, we found only minor changes in the number of Cy3 and Cy5 molecules, which is consistent with a previous report in which Cas9gRNAs were found to remain bound to the DNA even after DNA cleavage15.

To explicitly observe dissociation of Cas9gRNAs from the cleaved DNA, we injected 7 M urea solution while rapidly performing a wash to prevent urea-mediated denaturation of neutravidin or DNA double-helix structures. Once Cas9gRNAs were dissociated from immobilized DNA, the ratio of Cy5 to Cy3 decreased significantly; approximately 80% of target DNAs were cleaved in 5 min (Supplementary Fig. 1).

Effect of tracrRNA on Cas9 conformation. To investigate the role of gRNAs in the DNA cleavage reaction, we used a set of partially pre-incubated Cas9:gRNAs to compare the DNA cleavage efficiency. Three different processes of pre-incubation and mixing scheme were used before we injected the mixture onto the immobilized DNA (Fig. 1a): (i) apo-Cas9 and tracrRNA pre-incubated at 37°C for 20 min, and crRNA added immediately afterwards; (ii) apo-Cas9 and crRNA pre-incubated at 37°C for 20 min, and tracrRNA added later; and (iii) apo-Cas9 alone pre-incubated at 37°C for 20 min, and tracrRNA and crRNA added later. In addition, we also used apo-Cas9 pre-incubated at 37°C for 20 min with both gRNAs (full complex) as a positive control. The cleavage efficiency was then measured at 37°C after an incubation time of 5 min, the time required to achieve maximum cleavage under our experimental conditions. For Cas9 partially pre-incubated with tracrRNA ((i) in Fig. 1a,b), the efficiency of DNA cleavage (~80% DNA cleaved) was almost the same as that of the ‘full complex’ (Fig. 1b, green bar). In contrast, Cas9 pre-incubated in the absence of tracrRNA ((ii) and (iii) in Fig. 1a,b) yielded much lower cleavage efficiencies, which suggests that the tracrRNA plays a crucial role in facilitating crRNA binding and DNA cleavage by Cas9.

DNA cleavage by Cas9 pre-incubated without tracrRNA (process (ii) in Fig. 1a) also showed a strong dependence on the pre-incubation temperature, that is, the cleavage efficiency was reduced as the pre-incubation temperature was increased (Fig. 1c, yellow bars). As a control, however, the cleavage efficiency for the direct injection of the mixture with apo-Cas9, crRNA and tracrRNA (‘without pre-incubation’, Fig. 1c, grey bar) retained nearly 100% of the cleavage activity of the ‘full complex’. Because Cas9gRNAs do not undergo multiple turnover cycles15, different DNA cleavage efficiencies represent different molecular ratios of catalytically active Cas9 to inactive Cas9. Therefore, as the pre-incubation temperature increases, more active Cas9 species are converted into the inactive conformation in the absence of tracrRNA, possibly through an energy-barrier route. Temperature-dependent circular dichroism (CD) spectra suggest that the energy-consuming transformation from active Cas9 to inactive Cas9 is associated with a conformational change (Fig. 1d). As the pre-incubation temperature increased from 25°C to 37°C, a change in the ellipticity of Cas9 was observed in the characteristic band near 210–220 nm (from the green curve to the black one). However, when we annealed Cas9 by slowly cooling it from 37°C to 25°C, no reverse change was observed (the dashed red curve remaining identical to the green curve), indicating that there was no reverse conformational rearrangement to the active form. Taken together, these results suggest that the conformational structure of inactive Cas9 is thermodynamically more stable and kinetically trapped at lower temperatures owing to the large refolding barrier.

To clarify whether inactive Cas9 interacts with gRNAs to form catalytically active Cas9:gRNA, we first prepared inactive Cas9 by heating as in Fig. 1d and conducted a cleavage assay after...
Conformational heterogeneity of the RNA–DNA heteroduplex.

For enzymatic cleavage, the ternary complex comprising Cas9:gRNA and target DNA (Cas9:gRNA:DNA) must be assembled by the incorporation of target DNA onto Cas9:gRNA. To elucidate the molecular functions of gRNAs in the formation of the ternary complex, we performed a single-molecule fluorescence resonance energy transfer (FRET) experiment to probe the conformational dynamics of DNA and crRNA during Cas9:gRNA:DNA formation. As shown in Fig. 2a, we designed a FRET pair between biotinylated target DNA internally labelled with Cy3 (donor) at the distal 5' end of the protospacer positioned at the 25th nucleotide from the PAM site ( +25 ) and crRNA end-labelled with Cy5 (acceptor) at the 5' end, so that we could monitor conformational changes occurring during the RNA–DNA hybridization. We first demonstrated FRET measurement for Cas9:gRNA interacting with the fully matched target DNA (wild type), containing a proper PAM (in our measurement, 5'-GGG-3') and a protospacer with perfect complementarity to crRNA. When we injected Cy5-labelled Cas9:gRNA onto Cy3-labelled DNA while using an optical excitation scheme for Cy3, FRET signals from Cy5 that represented individual DNA binding events were observed in real time (Supplementary Fig. 3). After sufficient incubation ( >30 min) of Cas9:gRNA with the immobilized target DNA to reach chemical equilibrium, the FRET efficiency data showed two distinct bound states between Cas9:gRNA and DNA characterized by a high-FRET value (0.83) and a low-FRET value (0.27; Fig. 2b). When Cas9 was replaced with dCas9 (‘dead’ Cas9, a catalytically inactivated form of Cas9 resulting from the mutations D10A and H840A) in a control experiment, identical FRET states were also observed (Supplementary Fig. 4), suggesting that neither FRET state is related to the cleaved product. Rather, these two different values of the FRET efficiency may represent the structural flexibility associated with an intermediate state in the DNA binding and cleavage process, unrelated to the cleaved product.

Next, we characterized the structural conformation and kinetics of each FRET state. As Cas9:gRNA binds to wild-type DNA, we found that binding events in a single-molecule
and PAM (yellow), protospacer (black), crRNA (blue) and the approximate location of mismatched bases on target DNA (red cross).

The dwell-time of transient, repetitive transitions between the two FRET states does not remain in the high-FRET state, but undergoes a series of conformational transitions (termed the 'zipped conformation') and the observed FRET change from low-FRET to high-FRET may correspond to the expansion of the R-loop structure. The FRET value of the zipped conformation (0.83) amounts to a direct observation of the sub-steps of the R-loop structure kinetics (Supplementary Fig. 5). With the removal of the complementarity in the PAM-distal region (from +15 to +20) between the two DNA strands (termed 'bubble DNA'), the FRET data (Fig. 2d, bottom histogram) shows only the high-FRET population centred around a FRET value of 0.75 (a notably lower value from that of wild-type DNA, possibly expected to cause a subtle perturbation of the conformational distortion of the dsDNA by mismatched bases within the protospacer) with no more repetitive transitions. This further supports the idea that the observed FRET transitions represent the relative motions between the target strand of DNA and crRNA at the PAM-distal end. Our results amount to a direct observation of the sub-steps of the R-loop expansion in the ternary complex Cas9:gRNA:DNA, with a clear distinction of the conformational subpopulations of the RNA–DNA heteroduplex.

crRNA-regulated conformational flexibility of Cas9:gRNA:DNA.

To further monitor the role of crRNA in Cas9:gRNA:DNA, we introduced truncated crRNA in which three nucleotides were omitted in the PAM-distal end. Loss or gain of stability in the interaction between crRNA and the target strand of DNA was expected to cause a subtle perturbation of the conformational equilibrium between the two FRET states. In agreement with our hypothesis, the FRET histogram with truncated crRNA after sufficient incubation time (>30 min) exhibited a notably different distribution, where not only the FRET values of the low and high FRET states are different (0.29 and 0.75 respectively) but also their relative populations have been reversed (Fig. 3a), indicating that the
Figures 5 and 6; mean times shorter than that of the 20-nt crRNA (222 ms R-loop sub-structures. the complementarity of crRNA to the target strand of DNA crRNA (Fig. 3b and Supplementary Fig. 6). Our data support that while the low-FRET dwell time was similar to that of the 20-nt sequences upstream from the PAM site were matched; DNA significantly more stable when at least half of the protospacer structure using off-target DNA sequences with the introduction only if a stable R-loop forms. These results reveal that the open conformation is incompatible with RNA–DNA heteroduplex are the rate-determining steps. These greater proportion of time spent in the open conformation the kinetics of the wild-type and bubble DNA showed that the binding affinity but showed only the open conformation, failed to Cas9 complex, while the mis-crRNA, which retained the level of mismatched nucleotides at variable positions (Supplementary structure using off-target DNA sequences with the introduction of mismatched nucleotides at variable positions (Supplementary Table 1). The binding of Cas9:gRNA to target DNA became significantly more stable when at least half of the protospacer sequences upstream from the PAM site were matched; DNA sequences containing a mutation in the ‘seed’ region did not exhibit significant DNA binding, as expected (Supplementary Fig. 8)23. In agreement with the enhanced cleavage of the bubble substrate, we found that the cleavage efficiency increased as the population of the high-FRET state increased (Fig. 4b). Notably, we found that the mismatch at 10–11 did not exhibit the high-FRET state, despite the complementarity of nine nucleotides at the PAM-distal end, suggesting that the formation of the zipped conformation, in addition to the complementarity of nucleotides at the PAM-distal end, requires the stability of the entire RNA–DNA heteroduplex. These results show that the FRET transition between the two states involves both R-loop expansion as well as the enzymatic activation of Cas9 complex. Therefore, the conformational changes between crRNA and its target strand of DNA serve as a key step leading to activation of the Cas9:gRNA:DNA structure for DNA cleavage.

Discussion
Our results demonstrate the roles of both of tracrRNA and crRNA in the regulation of Cas9 complex structures associated with Cas9 nuclease activity. We found that tracrRNA is required to maintain the active conformation of apo-Cas9 for target DNA binding, whereas crRNA regulates the R-loop structure according to the stability of the RNA–DNA heteroduplex (Fig. 5). Several structural studies of type II CRISPR-Cas9 have suggested that Cas9 undergoes conformational changes during gRNA binding and target DNA recognition, but we discovered conformational change taking place in apo-Cas9 itself without nucleic acid interactions although its significance in vivo remains to be addressed by further studies. In the absence of tracrRNA, apo-Cas9 becomes inactive, suggesting that tracrRNA (possibly the tracrRNA moiety in the partial tracrRNA:crRNA duplex) is recognized by structurally active apo-Cas9 to form Cas9:gRNA. Consistent with our model, previous biochemical data showed that Cas9–tracrRNA did not exhibit nonspecific binding; nonspecific binding was observed only with Cas9 alone or Cas9–crRNA10. These results indicate that the tracrRNA–Cas9 interaction affects the conformation of Cas9 for target DNA recognition.

A previous in vivo study showed that Cas9 is also involved in the maturation of the hybridized pre-gRNAs (pre-crRNA and pre-tracrRNA) by RNase III (ref. 5). Our in vitro study sheds some light on the role of Cas9 in this process, through which pre-tracrRNA may be recognized by Cas9 and recruited to pre-crRNA with the help of Cas9, similar to the Cas9–gRNA complexation process for target DNA cleavage.

Our real-time single-molecule data demonstrated that the formation of the R-loop structure inside Cas9:gRNA:DNA was a multistep process and exhibited a dynamic motion at the PAM-distal end of the protospacer. The observed conformational transitions (Fig. 3a) are consistent with the previous crystal
activity after the R-loop formation. On the other hand, the rate of the observed transitions (Fig. 2c) was two orders of magnitude faster than the target cleavage rate (Fig. 1e), which suggests that the nuclease activity of Cas9 is associated with the movement, our results showed that the stability and kinetics of the R-loop structure based on the crRNA–DNA heteroduplex serve as key conformational regulators of the enzymatic behaviour of Cas9. Furthermore, this newly unveiled role of the kinetics and dynamics of the R-loop structure of Cas9:gRNA:DNA could allow prediction of off-target sites quantitatively based on the calculation of the stability of the crRNA–DNA interface, thereby enabling more efficient target design in genome editing.

**Methods**

**Cas9 and nucleic acids.** Recombinant Cas9 and dCas9 from S. pyogenes were overexpressed from *Escherichia coli* or purchased from New England Biolabs. In case of expressed proteins, Cas9 gene was subcloned into pET28-b (+). Recombinant Cas9 protein containing a nuclear localization signal, the HA epitope, and the His-tag at the amino (N) terminus was expressed in BL21(DE3) strain, purified using Ni-NTA agarose beads (Qiagen), and dialysed against 20 mM HEPES pH 7.5, 150 mM KCl, 1 mM DTT and 10% glycerol. The purified Cas9 protein was concentrated using Ultracel 100 K cellulose column (Millipore). The purity and concentration of Cas9 protein were analysed by SDS–polyacrylamide gel electrophoresis. We also confirmed that the catalytic activities and DNA binding affinities of the purified Cas9 were comparable to those of the purchased Cas9 upon our experimental conditions.

All DNA oligonucleotides were purchased from Integrated DNA Technologies. Biotin was conjugated at the 5′ end of nontarget strand. Internal amino modification for dye labelling was incorporated in each DNA strand, and both Cy3 and Cy5 were conjugated via a nucleophilic substitution reaction between amino-modified thymine and NHS ester-linked fluorescent dyes.

**Structures of Cas9, which show conformational flexibility** and with recent biophysical studies reporting that Cas9, in contrast to the type I CRISPR Cascade, does not exhibit a ‘locking’ mode in which Cas9:gRNA:DNA is locked for nuclease activity after the R-loop formation. On the other hand, the rate of the observed transitions (Fig. 2c) was two orders of magnitude faster than the target cleavage rate (Fig. 1e), which suggests that the nuclease activity of Cas9 is associated with the movement of the HNH nuclease domain that actually cleaves the target strand, it is possible that Cas9 undergoes a conformational change into the zipped conformation that may trigger structural rearrangement of the nuclease domain.

Our experiments with variable off-target sequences demonstrated that the stability of the crRNA–DNA interface was crucial for the conformational distribution and kinetics of Cas9:gRNA:DNA. Off-target sequences that failed to overcome the energetic barrier for the zipped conformation exhibited only PAM-proximal bound structures and were not cleaved (Fig. 4 and Supplementary Fig. 7). Although further experiments are necessary to elucidate the details of DNA cleavage mechanism of Cas9:gRNA:DNA such as synchronous single-molecule measurement for R-loop completion and HNH domain movement, our results showed that the stability and kinetics of the R-loop structure based on the crRNA–DNA heteroduplex serve as key conformational regulators of the enzymatic behaviour of Cas9. Furthermore, this newly unveiled role of the kinetics and dynamics of the R-loop structure of Cas9:gRNA:DNA could allow prediction of off-target sites quantitatively based on the calculation of the stability of the crRNA–DNA interface, thereby enabling more efficient target design in genome editing.

**Figure 4** Cas9 cleavage activity with various mutated target DNA. (a) The relative target binding (grey bars) versus the fraction of cleaved product (red bars) for DNA sequences containing various positions of mismatched bases. (mean ± s.e.m., n ≥ 5). (b) Histograms of FRET upon binding with each mismatched mutant with Gaussian fits (black line). The ratio of high-FRET states to all DNA-bound states calculated from the FRET histograms (white bars) versus the DNA cleavage efficiency (red bars) for different DNA sequences containing various positions of mismatched bases. The base positions are labelled by numbering from the 5′ end of PAM as shown on top panel.
Guide RNAs were in vitro transcribed through run-off reactions by T7 RNA polymerase using the MEGAshortscript T7 kit (Ambion) according to the manufacturer’s instructions. Transcribed RNA was purified by phenol-chloroform extraction, chloroform extraction and ethanol precipitation. Purified RNA was quantified by spectrometry. We also used guide RNAs based on solid-phase synthesis (purchased from ST Synthesizes) in single-molecule FRET measurements. Cy3-labelled eRNA was purchased and HPLC-purified from ST Pharm. All RNA-related solutions were prepared with nuclease-free water. Devices and lab space were treated with 70% ethanol, and all disposable tips and microcentrifuge tubes were certified as RNase-free.

DNA/RNA sequences and the positions of biotin and fluorophores are provided in Supplementary Table 1.

**Single-molecule measurement.** Coverslips and quartz glasses were passivated by polyethylene glycol to prevent samples from nonspecific binding on the glass surface26. All imaging and cleavage reaction were performed at 37 °C (unless otherwise specified) with the following buffer composition: 100 mM NaCl, 50 mM Tris-HCl pH 7.9, 10 mM MgCl2, 1 mM DTT and 0.1 mg ml−1 BSA. For cleavage and binding measurements and smFRET experiments for FRET reporter on Cy3 and Cy5 were collected by a microscope objective lens (UPlanApo 60x, Nikon) along with the green laser were used to excite Cy3 and Cy5 molecules independently. The emitting photons from Cy3 and Cy5 were collected by a green laser (645dcxr, Chroma Technology Corp.) and detected by back-illuminated water-immersion objective lens (UPlanApo 60x, Nikon) with an appropriate threshold. In this work, we used hidden Markov modelling based on variational Bayesian expectation maximization (VBayesEM) to account for the drop in single-molecule experiments. CD spectra were obtained using 1 mg ml−1 of Cy3 protein in Cas9 storage buffer (20 mM HEPES pH 7.5, 150 mM KC1, 1 mM NaCl, and 10% glycerol). Each spectrum was obtained from an average of three scans and the result was presented as mean residue ellipticity (deg cm2 dmol−1) at each wavelength.

**CD measurement.** CD spectra of Cas9 protein were measured over the range of 190 to 260 nm with Chirascan plus (Applied Photophysics) at various temperatures. Wave scans were acquired by sampling data at 1 nm intervals between 190 and 260 nm. Temperature was controlled by Peltier-type temperature controller in the range of 25 to 37 °C. CD spectra were obtained using 1 mg ml−1 of Cas9 protein in Cas9 storage buffer (20 mM HEPES pH 7.5, 150 mM KC1, 1 mM NaCl, and 10% glycerol). Each spectrum was obtained from an average of three scans and the result was presented as mean residue ellipticity (deg cm2 dmol−1) at each wavelength.

**Data availability.** The data that support the findings of this study are available from the corresponding author on request.

**References**

1. Jansen, R., van Embden, J. D. A., Gaastra, W. & Schouls, L. M. Identification of genes that are associated with DNA repeats in prokaryotes. Mol. Microbiol. 43, 1657–1675 (2002).
2. Bolotin, A., Quinquis, B., Sorkin, A. & Ehrlich, S. D. Clustered regularly interspaced short palindromic repeats (CRISPRs) have spacers of extrachromosomal origin. Microbiology 151, 2551–2561 (2005).
3. Marraffini, L. A. & Sontheimer, E. J. CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. Nat. Rev. Genet. 11, 181–190 (2010).
4. Barragou, R. et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709–1712 (2007).
5. Delcheva, E. et al. CRISPR RNA maturation by trans-encoded small RNA and host factor Rnas II. Nature 471, 602–607 (2011).
6. Kim, M. & Kim, J. S. A guide to genome engineering with programmable nucleases. Nat. Rev. Genet. 15, 321–334 (2014).
7. Makarova, K. S. et al. Evolution and classification of the CRISPR-Cas systems. Nat. Rev. Microbiol. 9, 467–477 (2011).
8. Mojica, F. J. M., Diez-Villasenor, C., Garcia-Martinez, J. & Almendros, C. Short motif sequences determine the targets of the prokaryotic CRISPR defence system. Microbiology 155, 749–759 (2009).
9. Deveau, H. et al. Phage response to CRISPR-encoded resistance in Streptococcus thermophilus. J. Bacteriol. 190, 1390–1400 (2008).
10. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816–821 (2012).
11. Cong, L. et al. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 818–823 (2013).
12. Jinek, M. et al. RNA-programmed genome editing in human cells. Elife 2, e00471 (2013).
13. Cho, S. W., Kim, S., Kim, J. M. & Kim, J. S. Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31, 230–232 (2013).
14. Mali, P. et al. RNA-guided human genome engineering via Cas9. Science 339, 823–826 (2013).
15. Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C. & Doudna, J. A. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature 507, 62–67 (2014).
16. Anders, C., Niewoehner, O., Duerst, A. & Jinek, M. Structural basis of PAM-dependent target DNA recognition by the Cas9 endonuclease. Nature 513, 569–573 (2014).
17. Nishimasu, H. et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 156, 935–949 (2014).
18. Jiang, F., Zhou, K., Ma, L., Gressel, S. & Doudna, J. A. Structural biology of Cas9-guide RNA complex reprogrammed for target DNA recognition. Science 348, 1477–1481 (2015).
19. Jinek, M. et al. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. Science 343, 1247997 (2014).
20. Jiang, F. et al. Structures of a CRISPR-Cas9-R-loop complex primed for DNA cleavage. Science 351, 867–871 (2016).
21. Fu, Y. F., Sander, J. D., Reyon, D., Casico, V. M. & Joung, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nat. Biotechnol. 32, 279–284 (2014).
22. Slaymaker, I. M. et al. Rationally engineered Cas9 nucleases with improved specificity. Science 351, 84–88 (2016).
23. Wu, X. B. et al. Genome-wide binding of the CRISPR endonuclease Cas9 in mammalian cells. Nat. Biotechnol. 32, 670–676 (2014).
24. Szczelkun, M. D. et al. Direct observation of R-loop formation by single RNA-guided Cas9 and Cascade effector complexes. Proc. Natl Acad. Sci. USA 111, 9798–9803 (2014).
25. Sternberg, S. H., LaFrance, B., Kaplan, M. & Doudna, J. A. Conformational control of DNA target cleavage by CRISPR-Cas9. Nature 527, 110–113 (2015).
26. Roy, R., Hohng, S. & Ha, T. A practical guide to single-molecule FRET. Nat. Methods 5, 507–516 (2008).
27. Swoboda, M. et al. Enzymatic oxygen scavenging for photostability without pH drop in single-molecule experiments. ACS Nano 6, 6364–6369 (2012).
28. Robinson, J. E., Fei, J. Y., Hofman, J. M., Gonzalez, R. L. & Wiggins, C. H. Learning rates and states from biophysical time series: a Bayesian approach to model selection and single-molecule FRET data. Biophys. J. 97, 3196–3205 (2009).
Acknowledgements
This work was supported by the National Research Foundation of Korea through the Global Frontier Program for Bioconvergence (NRF-2014M3A6A4075063). Y.L. acknowledges the support from the Fostering Core Leaders of the Future Basic Science Program (NRF-2011-0002378). This work was also supported by the Institute for Basic Science (IBS-R021-D1) for J.-S.K. and the Plant Molecular Breeding Center of Next Generation BioGreen 21 Program (PJ01119201) grant to S.B. We also acknowledge the BK21 Plus Program and SNU Brain Fusion grant.

Author contributions
Y.L., S.B., J.-S.K. and S.K.K. designed the research. Y.L., S.Y.B. and K.S. performed the experiments. Y.L., K.S. and S.B. analysed the data. E.J. and S.H.L. prepared the biological samples. S.B. and S.K.K. supervised the research.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/

How to cite this article: Lim, Y. et al. Structural roles of guide RNAs in the nuclease activity of Cas9 endonuclease. Nat. Commun. 7, 13350 doi: 10.1038/ncomms13350 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.