Switchable Cas9

Florian Richter[1,*], Ines Fonfara[2,3,*], Renate Gelfert[4], Jennifer Nack[4],

Emmanuelle Charpentier[2,3], Andreas Möglich[4]

[1]Bayer AG, Pharmaceuticals, Protein Engineering and Assays, 50829 Köln, Germany

[2]Max Planck Institute for Infection Biology, 10117 Berlin, Germany

[3]The Laboratory for Molecular Infection Medicine Sweden (MIMS), Umeå Centre for Microbial Research (UCMR), Department of Molecular Biology, Umeå University, Umeå 90187, Sweden

[4]Universität Bayreuth, Lehrstuhl für Biochemie, Bayreuth, Germany

[*]contact: florian.richter@bayer.com, fonfara@mpiib-berlin.mpg.de

Copyright Information

published as Richter *et al.* (2017) *Curr Opin Biotech* **48**, 119-126.

http://dx.doi.org/doi:10.1016/j.copbio.2017.03.025

Abstract

Ever since its discovery, Cas9 from *Streptococcus pyogenes* has revolutionized biology by enabling analysis and engineering of genomes with unprecedented precision and ease. To fine-tune on-target effects and to mitigate adverse effects caused by untimely and off-target action of Cas9, strategies have been developed to control its activity at the post-translational stage via external trigger signals. Control is either achieved by modifying the Cas9 protein itself or its programmable RNA molecules. To date, switchable Cas9 variants responding to small ligands, light or temperature have been engineered. With these variants in hand, the regulation and modification of genomes can be accomplished in graded and ever more precise manner.

Highlights

- diverse Cas9 variants have been engineered that are switchable by external signals
- suitable signals include addition of small molecules, light and temperature changes
- to achieve control, the Cas9 protein itself or its programmable RNAs are modified
- switchable Cas9 variants are engineered as split or single-chain proteins
- switchable variants mitigate effects caused by excessive and untimely Cas9 activity

Introduction

The advent of the programmable DNA endonuclease Cas9 has revolutionized analysis, control and engineering of genomes [1]. The most widely used Cas9 from *Streptococcus pyogenes* originally forms part of the type II-A CRISPR-Cas (clustered regularly interspaced short palindromic repeats-CRISPSR associated) adaptive immune system [2,3] and requires two non-coding RNA molecules, denoted crRNA (CRISPR RNA) and tracrRNA (trans-activating crRNA), for sequence-specific binding and cleavage of DNA double strands. Of key advantage, the Cas9 programmability to target a specific

DNA sequence is encoded in the crRNA of the dual tracrRNA:crRNA, rather than in proteinaceous components as in the alternative zinc finger or TALE (transcription activator-like effector) systems [4]. Routinely, the crRNA and tracrRNA of Cas9 are covalently linked by a hairpin to yield a chimeric RNA, termed single-guide RNA (sgRNA) [5•].

Biotechnological applications that harnesses the ability of the sgRNA to direct Cas9 to specific DNA target sites fall into two general areas (Fig. 1): first, sequence-specific endonucleolytic cleavage by Cas9 wild-type or variants introduces single-strand nicks or double-strand breaks (DSB) to trigger cellular repair mechanisms, principally non-homologous end-joining and homologous recombination [1]. Second, DNA cleavage is deliberately suspended in a Cas9 variant, termed dCas9, which bears two mutations in its RuvC and HNH nuclease domains, respectively [6]. dCas9 hence serves as a programmable sequence-specific DNA-binding protein. In the CRISPR interference (CRISPRi) approach, dCas9 is directed to the promoter or 5' region of target genes, thereby competes with RNA polymerase and represses transcription [7]. Alternatively, dCas9 is connected to transcriptional activators, repressors and silencers to exert transcriptional regulation [8,9], or to fluorescent reporters to mark certain genetic loci [10].

Both areas benefit from means of precisely controlling (d)Cas9 activity, preferably with spatial and temporal resolution, and in reversible manner. In particular, off-target cleavage caused by elevated Cas9 levels can be mitigated by adjusting overall activity and by turning off Cas9 at desired times [11]. In principle, control can be achieved at the expression level, leading to changes in the production of (d)Cas9, or at the post-translational level, leading to changes in (d)Cas9 activity, availability or stability. The latter of the two offers the advantages of faster response and of being compatible with approaches where pre-assembled (d)Cas9 is injected into target cells as opposed to being expressed *in situ*. Natural mechanisms that directly affect the activity of Cas9 [12•,13•] and related CRISPR proteins [14] have only been discovered very recently and are so far limited to inhibitory proteins. Against this backdrop, the past two years have witnessed the vigorous

development of synthetic strategies for controlling (d)Cas9 activity [11,15]. Here, we review recent advances in the engineering of (d)Cas9 variants that can be modulated in their cellular availability and activity by external stimuli.

Leverage Points for Interfering with Cas9 Activity

As illustrated by the high-resolution structure of the *S. pyogenes* holo enzyme [6], Cas9 is an elaborate molecular machine that precisely mediates several reaction steps with several ligands (Fig. 1). Although the mechanistic details await full elucidation, it is evident that for proper function Cas9 needs to bind the dual tracrRNA:crRNA (or, the sgRNA) to locate the specific target site within double-stranded DNA, to cleave both DNA strands and to eventually release the reaction products [5•]. Potentially, each of these steps offers toeholds for interfering with Cas9 activity; if interference proceeds in signal-dependent manner, the desired regulation of Cas9 is accomplished. Present approaches for signal-dependent regulation of Cas9 activity rely on impeding assembly with its programmable RNA and/or correct binding to the DNA target site, as opposed to affecting nucleolytic cleavage *per se*. As a corollary, the regulatory approaches developed for the cleavage-competent Cas9 also hold for the cleavage-incompetent dCas9. The opposite is not necessarily true as dCas9 is often used as an inert, RNA-guided, sequence-specific DNA-binding protein, to which accessory effector modules are recruited that exert the desired biological activity. Regulation of this activity can be achieved via signal-modulated recruitment of accessory effector units to dCas9.

Split Variants to Regulate Cas9 Activity

Precisely orchestrated interactions between proteins and nucleic acids underpin diverse natural processes and are often subject to regulation by signals, e.g., small-molecule compounds. The moieties mediating such signal-dependent interactions have long been coopted in the engineering

of split proteins that regain function when dimerized, e.g., [16]. Several of these strategies have also been applied in order to subject split Cas9 under the control of external triggers.

Cas9 has been divided into two fragments and linked to the two halves of a split intein [17,18•]; fragment combination allowed seamless *trans* splicing and yielded native, fully functional protein. However, intein-based approaches suffer from being irreversible as reversible activation would require non-covalent fragment assembly.

The dissection of Cas9 into recognition and nuclease lobes, which are inactive on their own but regain activity when associating upon sgRNA binding, demonstrated the principal validity of the split approach for Cas9 [19]. The combination of split Cas9 parts with the FKBP protein and the rapamycin-binding domain of mTOR, respectively, yielded a system in which fragment assembly and concomitant recovery of Cas9 activity are under control of rapalogs (i.e. rapamycin or analogs) [20•]. To overcome the challenge posed by undesirable residual dimerization in the absence of rapalogs, one fragment was directed to the nucleus and the other to the cytosol via appendage of suitable localization signal peptides. Nguyen *et al.* later added an additional layer of control by linking both Cas9 fragments with the ligand-binding domain (LBD) of the estrogen receptor ERT [21•]. This domain interacts with the cytosolic chaperone Hsp90 and is thus sequestered from the nucleus. Addition of the ligand 4-hydroxytamoxifen (4-HT) disrupts the Hsp90:ERT interaction and thereby promotes nuclear translocation of ERT and the connected split fragments. Assembly in the nucleus and resultant reconstitution of Cas9 activity either occurs spontaneously or, in enhanced fashion, by rapalog addition.

Rather than by chemical means, split Cas9 fragments can also be functionally reconstituted via light-mediated dimerization. To this end, Cas9 fragments were linked to derivatives of the *Neurospora crassa* light-oxygen-voltage (LOV) photoreceptor Vivid, dubbed Magnets [22], that assemble into a heterodimer upon blue-light-exposure [23••]. In contrast to the above approaches, this light-regulated system benefits from higher spatiotemporal resolution and reversible activation.

At least some of the above approaches achieve external control of Cas9 activity with high dynamic range, but they all require the use of two separate polypeptides. Although the two-component nature can be advantageous, e.g., enabling regulatory strategies and making for smaller gene constructs (e.g., beneficial for viral transfection) [18•], at least under certain circumstances it may also impart disadvantages, in particular a strong concentration dependence of the response [24] and sensitivity to different expression levels of the individual compounds. Cas9 derivatives that can be triggered by external signals yet are engineered as a single polypeptide thus represent viable alternatives to the split systems.

Single-chain Variants to Regulate Cas9 Activity

Modification of a lysine residue nearby the crRNA binding site of Cas9 with a photolabile caging group put RNA binding and catalytic activity under control of UV light [25•]. Notably, the photocaging group was incorporated site-specifically as an unnatural amino acid (UAA) via *amber* suppression, and in its photocaged form, Cas9 showed negligible activity. However, after UV-induced deprotection unmodified, fully functional enzyme was obtained. Modification via *amber* suppression of the same lysine with a different protecting group rendered uncaging and restoration of Cas9 activity dependent on addition of a small-molecule compound [26•]. The requirement for the heterologous machinery for UAA incorporation, and, in case of [25•], for irreversible activation by UV light, may limit the wider uptake of these systems.

In an approach similar to that of Nguyen *et al.* [21•], Liu *et al.* [27] sequestered Cas9 in the cytosol by flanking it N- and C-terminally with two copies each of the ERT LBD. Addition of the ligand 4-HT triggered nuclear localization and increase of Cas9 activity. (Interestingly, the underlying concept of this approach had already been demonstrated within the iGEM competition [28].) Conceptually

similar, optogenetic control of Cas9 activity might be achieved by resorting to systems for blue-light-mediated nuclear import and export [29,30].

Control over Cas9 activity has also been achieved by insertion of functional domains, in particular inteins [31], LBDs [32••] and LOV photosensors [33•]. An engineered version of the RecA intein from *Mycobacterium tuberculosis* whose splicing activity is regulated by 4-HT was inserted into different surface-exposed sites of Cas9 and cleavage activity thus abrogated [31]. Addition of the ligand 4-HT promoted splicing out of the intein and restored Cas9 activity, albeit in irreversible manner. In another study, an inert PDZ domain was randomly placed throughout the Cas9 protein by transposon mutagenesis to determine suitable insertion sites; ensuing high-throughput screening identified permissive sites that still support Cas9 activity [32••]. Replacement of the PDZ domain by the ERT LBD generated a Cas9 variant, called arCas9, that is allosterically regulated by 4-HT. The dynamic range of arCas9 could be further enhanced by combining allosteric activation with nuclear localization induced by 4-HT, cf. above. In another approach, we inserted the dimeric LOV photoreceptor *Rs*LOV from *Rhodobacter sphaeroides* [34] which undergoes blue-light-dependent dissociation at candidate, surface-exposed sites of Cas9. We reasoned that Cas9 activity might thus be repressed by forcing the enzyme into an unproductive dimeric complex and by restricting access to its active site [33•]. High-throughput screening identified the variant paRC9 which was modestly activated by blue light. In an unexpected twist, we discovered that insertion of *Rs*LOV bestowed temperature sensitivity on Cas9 activity. We isolated the variant tsRC9 which displayed robust activity at 29°C but negligible one at 37°C.

While it is desirable to ramp up Cas9 activity by a certain signal, it is equally attractive to decrease activity at will once Cas9 has performed its duty, which could for example be accomplished with tsRC9 [33•]. An interesting alternative tactic is the targeting of Cas9 for destruction after successful cleavage events in a cell-cycle dependent manner [35]. A logical extension to this approach would

be the fusion of Cas9 with degrons that are inducible by small ligands, e.g., auxin [36], or by light [37,38].

Controlling sgRNA Availability to Regulate Cas9 Activity

As Cas9 must associate with its RNA ligands to exert its function, activity can also be regulated by modulating the availability, conformation, or interaction with other factors of the sgRNA (or, of the dual tracrRNA:crRNA). These approaches offer advantages in terms of orthogonal regulation of different on-target activities (as differently modified sgRNAs can be used in multiplexing scenarios) and potentially also in terms of better regulation dynamics, because the sgRNA usually shows much faster cellular turnover than the Cas9 protein itself [39]. Liu *et al.* [40••] embedded riboswitches, i.e. RNA aptamers that are responsive to binding of (usually) small-molecule ligands, into the sgRNA at its 3' end to render the accessibility of the crRNA targeting region and the ability of Cas9 to bind its DNA target dependent on the presence of ligands such as theophylline. In a different approach [41••], the sgRNA was incapacitated by hybridization with a protector DNA oligonucleotide; photolabile linkers embedded in the protector DNA allowed its destruction by UV irradiation, resulting in release of the sgRNA and turning on of Cas9 activity.

Transcriptional and Epigenetic Control with dCas9

In addition to the above approaches that afford control over Cas9 binding and cleavage activities, a number of strategies for regulating the activity of systems based on the cleavage-deficient dCas9 have been developed. All these strategies have in common that dCas9 is employed as an inert, RNA-guided, sequence-specific DNA-binding protein to which effector components are recruited via signal-dependent interactions with the Cas9 protein or the RNA ligands. In two related studies [42,43•], the photoreceptor cryptochrome 2 from *Arabidopsis thaliana* (Cry2) which interacts with

the CIB1 protein under blue light [44] was combined with dCas9. CIB1 (or, its N-terminal part CIBN) and Cry2 were fused to dCas9 and strong trans-activating effectors, e.g., VP64 or p65, respectively. Blue light promoted the dimerization of Cry2:CIB1 thereby recruiting the transactivators to desired sites in the genome targeted by dCas9. In this manner, expression from endogenous genetic loci could be upregulated in light-dependent manner. Instead of Cry2:CIB1, different protein modules that heterodimerize in the presence of rapalogs or the plant hormones abscisic acid and gibberellin, respectively, have also been employed [45•,46]. Again, effector modules could be recruited to desired loci in signal-controlled fashion. Remarkably, logic gates and genetic circuits were constructed that made the output dependent on the presence or absence of either or both of the hormones, although this approach required parallel expression of two orthogonal Cas9 variants outfitted with the respective interaction domains. As previously demonstrated [9], none of these approaches [42,43•,45•,46] is restricted to activators but in principle they extend to other transcriptional and epigenetic effectors, e.g., repressors and histone deacetylases, as well as fluorescent reporters.

While the above approaches require covalent fusion of dCas9 with suitable interaction domains, effectors can also be recruited via interactions with the RNA components. To this end, short RNA aptamer sequences were embedded into or appended to the 3' end of the sgRNA [47–50]. The aptamers specifically interact with cognate RNA-binding proteins, e.g., the coat protein of the bacteriophage MS2, and can therefore be used to recruit desired effectors to the dCas9:sgRNA complex. Strikingly, aptamer-based recruitment [47,48,50] afforded markedly improved upregulation of endogenous loci compared to the above approaches. A recent investigation demonstrates how control by external signals can be added to the aptamer-recruitment platform [51•]. Here, the transactivating effectors were not only fused to RNA-binding domains but also to destabilized protein domains that promote rapid proteasomal degradation. Addition of suitable compounds stabilizes these domains and the attached RNA-binding effector complexes, thus

yielding a ligand-inducible dCas9 system. Notably, multiplexed regulation of several genes is readily possible by parallel use of multiple sgRNAs and transactivating effectors, rather than requiring expression of orthogonal Cas9 variants, cf. above [45•,46]. In a variation of the approach, the ligand-sensitive destabilized domains were fused to the N and C termini of Cas9 to achieve ligand-induced cleavage activity [51•].

Conclusions and Future Perspectives

Advances in the development of switchable (d)Cas9 variants have been nothing but tremendous. Over the past two years, several options have arrived by which (d)Cas9 availability and activity can be controlled at the post-translational stage (as opposed to regulating the expression of Cas9) in its overall levels and spatiotemporal timing, thus affording enhanced control over the analysis, control and engineering of genomes. The unrelenting progress in this field has to large extent benefited from the adaption of established and previously successful concepts, e.g., in the design of split Cas9 variants and the recurring, creative deployment of the ERT:4-HT switch. Given the recent developments, we expect additional Cas9 variants to become available that afford control by different signals and with enhanced performance. Variants which could be activated by red light appear of particular interest as they would facilitate optogenetic applications in living animals.

The choice of one particular variant among the various switchable Cas9 proteins will be governed by the needs and constraints of a given application. One consideration is the nature and compatibility of the trigger signal which at present can be either small ligands, light or temperature changes. The optogenetic approach, i.e. using light as the trigger, offers the advantage of better spatial control. Another aspect that may be required is reversibility of the response in at least two regards. First, can the trigger signal be readily removed or shut off? Changes in illumination and temperature appear to have the edge over small-molecule ligands which would need to be washed

out. Second, is the molecular mechanism leading to Cas9 activation reversible? Strategies that involve the breaking or formation of covalent bonds, e.g., photo- and ligand-induced uncaging or the intein-mediated methods, are not, whereas the others usually are. A subsidiary consideration is the kinetics of the response; how fast can Cas9 activation be effected, how rapidly can activity be shut off (if at all)? Specifically, Cas9 has been shown to have long DNA residence times, even post-cleavage which might interfere with desired double-strand break repair outcomes [52]. It remains to be investigated whether any of the methods presented here can, upon signal removal or addition, increase dissociation rates of DNA-bound Cas9 (instead of decreasing association rates of the free Cas9:RNA complex). Furthermore, the degree of regulation by signal and the leakiness of the system, i.e. activity in the absence of signal, play important roles. Whereas many dCas9 applications can arguably tolerate low leak activities, applications that employ Cas9 to effect irreversible genome rearrangements usually cannot.

Finally, researchers interested in the generation of new and the improvement of existing Cas9 variants also need to consider how transferable the engineering strategies are between different CRISPR systems [53]. For example, Cas9 from *Staphylococcus aureus* [54] is smaller in size, has different requirements for the protospacer adjacent motif (PAM) sequence, and may be advantageous in certain applications [45•], while Cpf1 [55] which cleaves its own gRNA has advantages in multiplexing applications [55,56]. Moreover, additional CRISPR systems are being discovered and mechanistically characterized at amazing pace [2,3], including such that act on RNA rather than DNA [57]. We anticipate that modes of control that rely on appending interaction domains to a CRISPR effector protein or aptamers to the sgRNA should be easily transferable, while approaches that modify the core of the CRISPR effector itself (such as split constructs or insertions) will be harder to engineer. Given the rapid development of diverse variants of *S. pyogenes* Cas9 which can be triggered by external cues and which we review here, we expect switchable variants of other CRISPR systems to become available shortly. Lastly, the existence of natural CRISPR-

inhibitory proteins [12•,13•] hints at the possibility of developing small-molecule inhibitors of Cas9, which would offer additional, complementary ways of regulating activity.

Acknowledgements

Research has been funded through the Deutsche Forschungsgemeinschaft [grant RI2468/1-1 to F.R.]; the Alexander-von-Humboldt Foundation [Sofja-Kovalevskaya Award to A.M.; AvH Professorship to E.C.]; the Einstein Foundation Berlin [grant IPF-2012-148 to F.R. & A.M.]; the German Federal Ministry for Education and Research [to E.C.]; the Helmholtz Association [to E.C.]; the Max Planck Society [to E.C.]; the Royal Swedish Academy of Sciences [Göran Gustafsson Prize to E.C.]; the Swedish Research Council [to E.C.]; and Umeå University [to E.C.].

References

Certain papers have been highlighted as

- • of special interest
- •• of outstanding interest

1. Doudna JA, Charpentier E: **The new frontier of genome engineering with CRISPR-Cas9**. *Science* 2014, **346**:1258096.

2. Makarova KS, Wolf YI, Alkhnbashi OS, Costa F, Shah SA, Saunders SJ, Barrangou R, Brouns SJJ, Charpentier E, Haft DH, et al.: **An updated evolutionary classification of CRISPR-Cas systems**. *Nat Rev Micro* 2015, **13**:722–736.

3. Shmakov S, Smargon A, Scott D, Cox D, Pyzocha N, Yan W, Abudayyeh OO, Gootenberg JS, Makarova KS, Wolf YI, et al.: **Diversity and evolution of class 2 CRISPR-Cas systems [Internet]**. *Nat Rev Micro* 2017, **advance online publication**.

4. Gaj T, Gersbach CA, Barbas CF: **ZFN, TALEN and CRISPR/Cas-based methods for genome engineering**. *Trends Biotechnol* 2013, **31**:397–405.

5.• Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**:816–821.

This study deciphered the DNA targeting mechanism of type II-A CRISPR from *Streptococcus pyogenes*. It demonstrated for the first time that Cas9 can be easily programmed using a dual tracrRNA:crRNA or sgRNA to target DNA sites specifically.

6. Nishimasu H, Ran FA, Hsu PD, Konermann S, Shehata SI, Dohmae N, Ishitani R, Zhang F, Nureki O: **Crystal structure of Cas9 in complex with guide RNA and target DNA**. *Cell* 2014, **156**:935–949.

7. Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA: **Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression**. *Cell* 2013, **152**:1173–1183.

8. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, et al.: **CRISPR-Mediated Modular RNA-Guided Regulation of Transcription in Eukaryotes**. *Cell* 2013, **154**:442–451.

9. Konermann S, Brigham MD, Trevino AE, Hsu PD, Heidenreich M, Cong L, Platt RJ, Scott DA, Church GM, Zhang F: **Optical control of mammalian endogenous transcription and epigenetic states**. *Nature* 2013, **500**:472–476.

10. Chen B, Gilbert LA, Cimini BA, Schnitzbauer J, Zhang W, Li G-W, Park J, Blackburn EH, Weissman JS, Qi LS, et al.: **Dynamic Imaging of Genomic Loci in Living Human Cells by an Optimized CRISPR/Cas System**. *Cell* 2013, **155**:1479–1491.

11. Nuñez JK, Harrington LB, Doudna JA: **Chemical and Biophysical Modulation of Cas9 for Tunable Genome Engineering**. *ACS Chem. Biol.* 2016, **11**:681–688.

12.• Pawluk A, Amrani N, Zhang Y, Garcia B, Hidalgo-Reyes Y, Lee J, Edraki A, Shah M, Sontheimer EJ, Maxwell KL, et al.: **Naturally Occurring Off-Switches for CRISPR-Cas9**. *Cell* 2016, **167**:1829–1838.e9.

Prophage proteins were discovered that inhibit the function of Cas9 from *Neisseria meningitidis*.

13.• Rauch BJ, Silvis MR, Hultquist JF, Waters CS, McGregor MJ, Krogan NJ, Bondy-Denomy J: **Inhibition of CRISPR-Cas9 with Bacteriophage Proteins**. *Cell* 2017, **168**:150–158.e10.

Prophage proteins were discovered that inhibit the function of Cas9 from *S. pyogenes*.

14. Bondy-Denomy J, Garcia B, Strum S, Du M, Rollins MF, Hidalgo-Reyes Y, Wiedenheft B, Maxwell KL, Davidson AR: **Multiple mechanisms for CRISPR-Cas inhibition by anti-CRISPR proteins**. *Nature* 2015, **526**:136–139.

15. Didovyk A, Borek B, Tsimring L, Hasty J: **Transcriptional regulation with CRISPR-Cas9: principles, advances, and applications**. *Current Opinion in Biotechnology* 2016, **40**:177–184.

16. Pollock R, Clackson T: **Dimerizer-regulated gene expression**. *Curr. Opin. Biotechnol.* 2002, **13**:459–467.

17. Truong D-JJ, Kühner K, Kühn R, Werfel S, Engelhardt S, Wurst W, Ortiz O: **Development of an intein-mediated split–Cas9 system for gene therapy**. *Nucl. Acids Res.* 2015, **43**:6450–6458.

18.• Chew WL, Tabebordbar M, Cheng JKW, Mali P, Wu EY, Ng AHM, Zhu K, Wagers AJ, Church GM: **A multifunctional AAV-CRISPR-Cas9 and its host response**. *Nat Meth* 2016, **13**:868–874.

Protein construct size can be a limiting in delivery with adeno-associated viruses. This problem was overcome by utilizing a split-intein Cas9 approach, which also allowed to deliver Cas9-effector fusions.

19. Wright AV, Sternberg SH, Taylor DW, Staahl BT, Bardales JA, Kornfeld JE, Doudna JA: **Rational design of a split-Cas9 enzyme complex**. *PNAS* 2015, **112**:2984–2989.

20.• Zetsche B, Volz SE, Zhang F: **A split-Cas9 architecture for inducible genome editing and transcription modulation**. *Nat. Biotechnol.* 2015, **33**:139–142.

Description of split Cas9 that dimerized dependent on the presence of rapalogs. By fusing nuclear localization and export signals to the two halves of Cas9, an additional layer of control was provided.

21.• Nguyen DP, Miyaoka Y, Gilbert LA, Mayerl SJ, Lee BH, Weissman JS, Conklin BR, Wells JA: **Ligand-binding domains of nuclear receptors facilitate tight control of split CRISPR activity**. *Nature Communications* 2016, **7**:12009.

Addition of ligand-binding domains to split Cas9 achieves signal-dependent control of Cas9 localization and cellular activity.

22. Kawano F, Suzuki H, Furuya A, Sato M: **Engineered pairs of distinct photoswitches for optogenetic control of cellular proteins**. *Nature Communications* 2015, **6**:6256.

23.•• Nihongaki Y, Kawano F, Nakajima T, Sato M: **Photoactivatable CRISPR-Cas9 for optogenetic genome editing**. *Nat Biotech* 2015, **33**:755–760.

Fusion of split Cas9 to photoassociating dimerization domains afforded spatiotemporally resolved and reversible optogenetic control.

24. Ziegler T, Möglich A: **Photoreceptor engineering**. *Front. Mol. Biosci* 2015, **2**:30.

25.• Hemphill J, Borchardt EK, Brown K, Asokan A, Deiters A: **Optical Control of CRISPR/Cas9 Gene Editing**. *J. Am. Chem. Soc.* 2015, **137**:5642–5645.

Photocaging of a lysine residue essential for Cas9 activity resulted in a non-functional protein. Upon UV irradiation, the caged amino acid was liberated and Cas9 activity fully restored. Requires incorporation of unnatural amino acids and is irreversible.

26.• Luo J, Liu Q, Morihiro K, Deiters A: **Small-molecule control of protein function through Staudinger reduction**. *Nat Chem* 2016, **8**:1027–1034.

Modification of the same lysine residue as in [25•] with a small-molecule cleavable protecting group led to an inactive Cas9 that can be restored to native, fully active enzyme by small-molecule addition. Requires incorporation of unnatural amino acids.

27. Liu KI, Ramli MNB, Woo CWA, Wang Y, Zhao T, Zhang X, Yim GRD, Chong BY, Gowher A, Chua MZH, et al.: **A chemical-inducible CRISPR-Cas9 system for rapid control of genome editing**. *Nat Chem Biol* 2016, **12**:980–987.

28. **iGEM Freiburg 2013**. http://2013.igem.org/Team:Freiburg

29. Niopek D, Wehler P, Roensch J, Eils R, Di Ventura B: **Optogenetic control of nuclear protein export**. *Nat. Commun.* 2016, **7**:10624.

30. Yumerefendi H, Lerner AM, Zimmerman SP, Hahn K, Bear JE, Strahl BD, Kuhlman B: **Light-induced nuclear export reveals rapid dynamics of epigenetic modifications**. *Nat. Chem. Biol.* 2016, **12**:399–401.

31. Davis KM, Pattanayak V, Thompson DB, Zuris JA, Liu DR: **Small molecule–triggered Cas9 protein with improved genome-editing specificity**. *Nat Chem Biol* 2015, **11**:316–318.

32.•• Oakes BL, Nadler DC, Flamholz A, Fellmann C, Staahl BT, Doudna JA, Savage DF: **Profiling of engineering hotspots identifies an allosteric CRISPR-Cas9 switch**. *Nat Biotech* 2016, **34**:646–651.

Positions in Cas9 were determined that tolerate insertions of unrelated protein domains. Insertion at one such site of the ligand-binding domain of the estrogen receptor yielded a Cas9 variant that was allosterically regulated by a small-molecule ligand yet retained maximum activity close to wild-type Cas9.

33.• Richter F, Fonfara I, Bouazza B, Schumacher CH, Bratovič M, Charpentier E, Möglich A: **Engineering of temperature- and light-switchable Cas9 variants**. *Nucl. Acids Res.* 2016, doi:10.1093/nar/gkw930.

Insertion of a photodissociating dimerization domain at surface-exposed positions of Cas9 yielded a moderately light-sensitive, single-chain variant. In addition, the first temperature-sensitive Cas9 variant was discovered: robust activity at a permissive temperature of 29°C was eliminated at 37°C.

34. Conrad KS, Bilwes AM, Crane BR: **Light-induced subunit dissociation by a light-oxygen-voltage domain photoreceptor from Rhodobacter sphaeroides**. *Biochemistry* 2013, **52**:378–391.

35. Gutschner T, Haemmerle M, Genovese G, Draetta GF, Chin L: **Post-translational Regulation of Cas9 during G1 Enhances Homology-Directed Repair**. *Cell Reports* 2016, **14**:1555–1566.

36. Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M: **An auxin-based degron system for the rapid depletion of proteins in nonplant cells**. *Nat Meth* 2009, **6**:917–922.

37. Renicke C, Schuster D, Usherenko S, Essen L-O, Taxis C: **A LOV2 domain-based optogenetic tool to control protein degradation and cellular function**. *Chem. Biol.* 2013, **20**:619–626.

38. Bonger KM, Rakhit R, Payumo AY, Chen JK, Wandless TJ: **General method for regulating protein stability with light**. *ACS Chem. Biol.* 2014, **9**:111–115.

39. Ma H, Tu L-C, Naseri A, Huisman M, Zhang S, Grunwald D, Pederson T: **CRISPR-Cas9 nuclear dynamics and target recognition in living cells**. *J Cell Biol* 2016, **214**:529–537.

40.•• Liu Y, Zhan Y, Chen Z, He A, Li J, Wu H, Liu L, Zhuang C, Lin J, Guo X, et al.: **Directing cellular information flow via CRISPR signal conductors**. *Nat Meth* 2016, **13**:938–944.

sgRNAs were modified with ligand-sensitive riboswitch aptamers such that the active Cas9:RNA complex only formed in presence of cognate ligands. Modification of the sgRNA instead of Cas9 facilitates differential regulation of separate gene targets in multiplexing scenarios.

41.•• Jain PK, Ramanan V, Schepers AG, Dalvie NS, Panda A, Fleming HE, Bhatia SN: **Development of Light-Activated CRISPR Using Guide RNAs with Photocleavable Protectors**. *Angew. Chem. Int. Ed.* 2016, **55**:12440–12444.

The sgRNA was incapacitated by heteroduplex formation with reverse-complementary protector DNA oligonucleotides. UV radiation destroyed photosensitive linkers within the protector leading to its fragmentation and liberation of the sgRNA. Facilitates multiplexing, as in [40•], but requires chemical preparation of protector DNA and is irreversible.

42. Nihongaki Y, Yamamoto S, Kawano F, Suzuki H, Sato M: **CRISPR-Cas9-based Photoactivatable Transcription System**. *Chem. Biol.* 2015, **22**:169–174.

43.• Polstein LR, Gersbach CA: **A light-inducible CRISPR-Cas9 system for control of endogenous gene activation**. *Nat Chem Biol* 2015, **11**:198–200.

Light-dependent activation of transcription was achieved by linking dCas9 and the strong transactivator VP64 to photoassociating dimerization domains. One reversibly switchable system had minimal background activity but light-induced activity on par with direct fusions between dCas9 and VP64.

44. Kennedy MJ, Hughes RM, Peteya LA, Schwartz JW, Ehlers MD, Tucker CL: **Rapid blue-light-mediated induction of protein interactions in living cells**. *Nat. Methods* 2010, **7**:973–975.

45.• Gao Y, Xiong X, Wong S, Charles EJ, Lim WA, Qi LS: **Complex transcriptional modulation with orthogonal and inducible dCas9 regulators**. *Nat Meth* 2016, **13**:1043–1049.

Two dCas9 homologues were fused to ligand-binding domains to allow ligand-induced recruitment of effector units. Use of the homologues enabled orthogonal, tunable and reversible regulation of different target genes as well as the construction of simple logic gates with predictable and defined outcomes depending on the presence of two different ligands.

46. Bao Z, Jain S, Jaroenpuntaruk V, Zhao H: **Orthogonal Genetic Regulation in Human Cells Using Chemically Induced CRISPR/Cas9 Activators [Internet]**. *ACS Synth. Biol.* 2017, doi:10.1021/acssynbio.6b00313.

47. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, Hsu PD, Habib N, Gootenberg JS, Nishimasu H, et al.: **Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex**. *Nature* 2015, **517**:583–588.

48. Zalatan JG, Lee ME, Almeida R, Gilbert LA, Whitehead EH, La Russa M, Tsai JC, Weissman JS, Dueber JE, Qi LS, et al.: **Engineering Complex Synthetic Transcriptional Programs with CRISPR RNA Scaffolds**. *Cell* 2015, **160**:339–350.

49. Shechner DM, Hacisuleyman E, Younger ST, Rinn JL: **Multiplexable, locus-specific targeting of long RNAs with CRISPR-Display**. *Nat Meth* 2015, **12**:664–670.

50. Mali P, Aach J, Stranges PB, Esvelt KM, Moosburner M, Kosuri S, Yang L, Church GM: **CAS9 transcriptional activators for target specificity screening and paired nickases for cooperative genome engineering**. *Nat Biotech* 2013, **31**:833–838.

51.• Maji B, Moore CL, Zetsche B, Volz SE, Zhang F, Shoulders MD, Choudhary A: **Multidimensional chemical control of CRISPR-Cas9**. *Nat. Chem. Biol.* 2017, **13**:9–11.

Ligand-sensitive degrons were fused to aptamer-binding domains and transcriptional effectors. In the absence of ligand, proteasomal degradation occurred, but in presence of the ligand, the complex was stabilized, could hence bind to specific aptamers embedded in the sgRNA of Cas9 and exert desired effects at specific genomic target sites.

52. Richardson CD, Ray GJ, DeWitt MA, Curie GL, Corn JE: **Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA**. *Nat Biotech* 2016, **34**:339–344.

53. Wright AV, Nuñez JK, Doudna JA: **Biology and Applications of CRISPR Systems: Harnessing Nature's Toolbox for Genome Engineering**. *Cell* 2016, **164**:29–44.

54. Nishimasu H, Cong L, Yan WX, Ran FA, Zetsche B, Li Y, Kurabayashi A, Ishitani R, Zhang F, Nureki O: **Crystal Structure of Staphylococcus aureus Cas9**. *Cell* 2015, **162**:1113–1126.

55. Fonfara I, Richter H, Bratovič M, Le Rhun A, Charpentier E: **The CRISPR-associated DNA-cleaving enzyme Cpf1 also processes precursor CRISPR RNA**. *Nature* 2016, **532**:517–521.

56. Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, Winblad N, Choudhury SR, Abudayyeh OO, Gootenberg JS, et al.: **Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array**. *Nat Biotech* 2017, **35**:31–34.

57. Abudayyeh OO, Gootenberg JS, Konermann S, Joung J, Slaymaker IM, Cox DBT, Shmakov S, Makarova KS, Semenova E, Minakhin L, et al.: **C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector**. *Science* 2016, **353**:aaf5573.

Figures

Figure 1

Principal applications of RNA-guided Cas9 and leverage points for controlling its cellular activity.

Cleavage-competent Cas9 is used to introduce single-strand nicks or double-strand breaks at

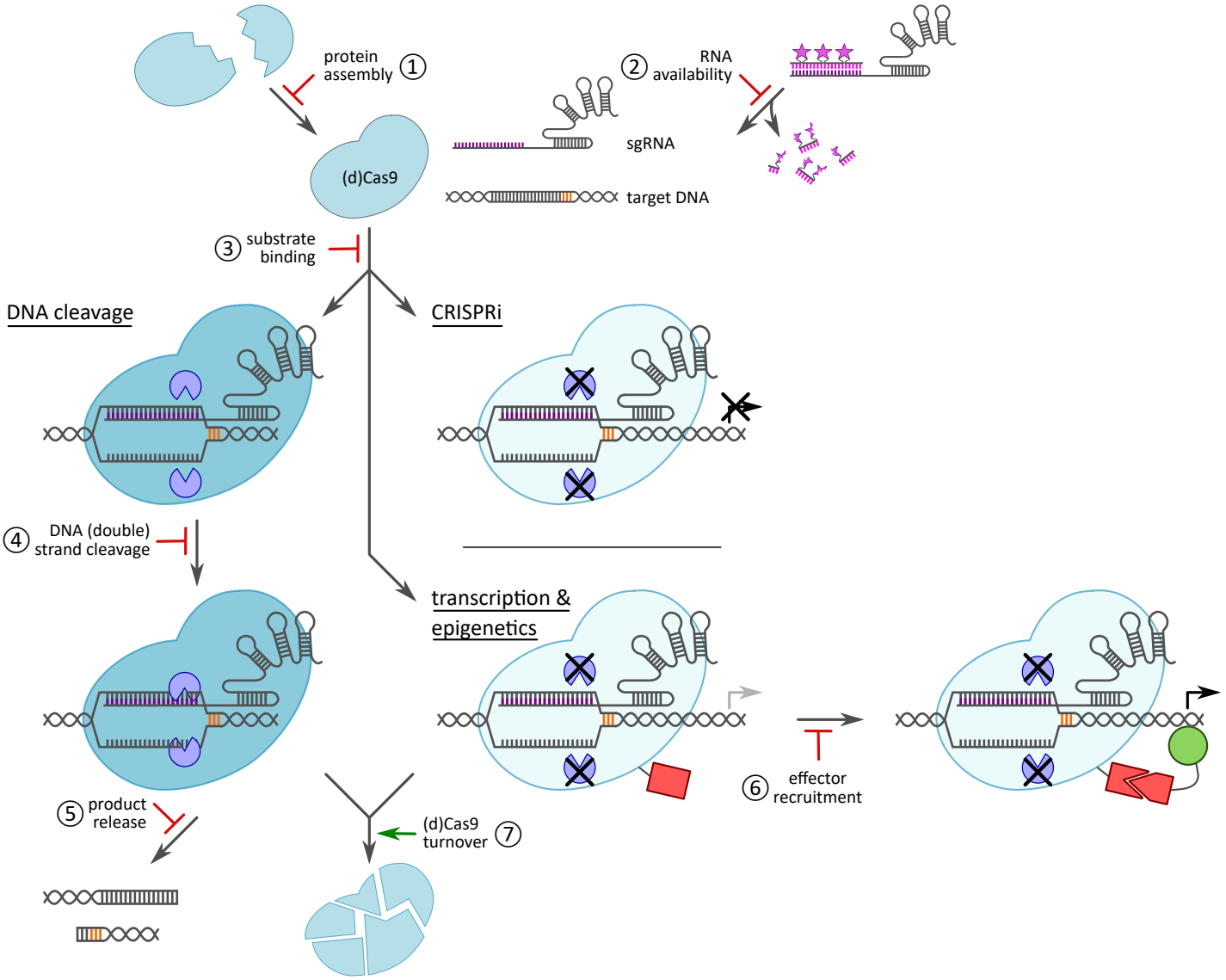
specific, unique sites in genomes. The cleavage-deficient dCas9 serves as a sequence-specific DNA-binding protein and can be connected to effector modules that exert desired biological output, e.g., up- or down-regulation of transcription. To fulfill its intended function, (d)Cas9 has to mediate several steps, all of which potentially offer toeholds for interfering with (red symbols). Control can be exerted by regulating the availability of (d)Cas9 (①) and the sgRNA (②). (d)Cas9 then has to bind a sgRNA (or, tracrRNA:crRNA) and find its PAM sequence and the adjacent, cognate DNA target (③). Cas9 additionally has to cleave one or both DNA strands (④) and release the reaction products afterwards (⑤). In case of dCas9, DNA effector units must be recruited (⑥). To turn off (d)Cas9 activity, its cellular turnover can be regulated (⑦).

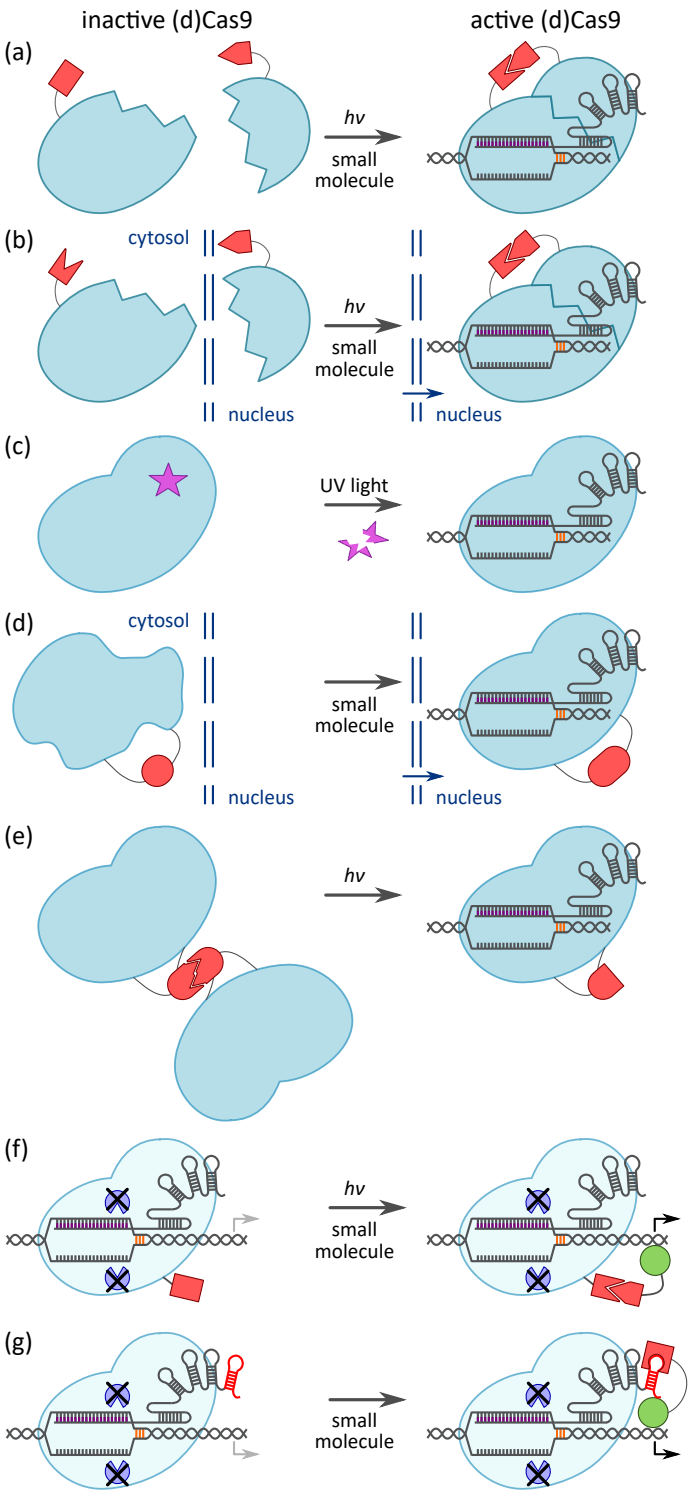
Figure 2

Select case studies of (d)Cas9 variants that are switchable by external trigger signals. (a, b) Split Cas9 can be reconstituted and activity thus regained via fragment dimerization induced by light or small-molecule ligands [20•,21•]. Spontaneous dimerization and leak activity can be minimized by directing one fragment to the cytosol (panel b). (c) Modification of a lysine residue with a photolabile caging group renders Cas9 inactive, presumably because of interference with RNA and DNA binding [25•]. UV light liberates the caging group and restores activity. (d) Terminal fusion with the ligand-binding domain (LBD) of the estrogen receptor ETR leads to sequestration of Cas9 in the cytosol [27]; addition of 4-hydroxytamoxifen (4-HT) induces nuclear translocation. Insertion of the ETR-LBD sketched here, rather than terminal fusion, additionally results in allosteric activation by 4-HT [32••]. (e) Insertion of a dimeric light-oxygen-voltage (LOV) photosensor into Cas9 leads to formation of a sterically hindered, inactive complex [33•]. Blue light raises Cas9 activity, presumably via dissociation of the LOV sensor. (f, g) dCas9 is used as a sequence-specific DNA-binding protein to which effector modules are recruited in a signal-dependent manner [42,43•,48,51•]. Recruitment

491 is either achieved via covalent fusion of interaction domains to Cas9 (panel f) or via RNA aptamers

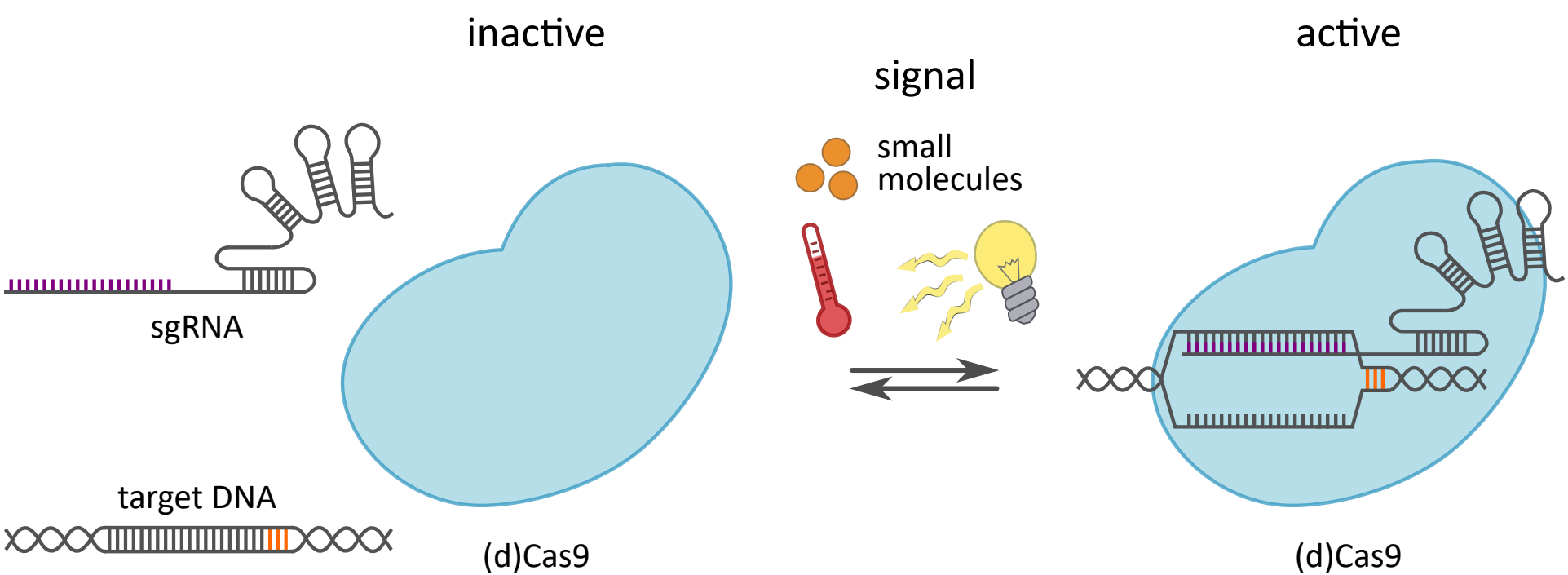
492 inserted into or appended to the sgRNA (panel g).


protein assembly ①
(d)Cas9
② RNA availability
sgRNA
target DNA
③ substrate binding
DNA cleavage
CRISPRi
④ DNA (double) strand cleavage
transcription & epigenetics
⑤ product release
(d)Cas9 turnover ⑦
⑥ effector recruitment


inactive (d)Cas9
active (d)Cas9
(a)
hv
small molecule
(b)
cytosol
nucleus
hv
small molecule
nucleus
(c)
UV light
(d)
cytosol
nucleus
small molecule
nucleus
(e)
hv
(f)
hv
small molecule
(g)
small molecule

Highlights

- diverse Cas9 variants have been engineered that are switchable by external signals
- suitable signals include addition of small molecules, light and temperature changes
- to achieve control, the Cas9 protein itself or its programmable RNAs are modified
- switchable Cas9 variants are engineered as split or single-chain proteins
- switchable variants mitigate effects caused by excessive and untimely Cas9 activity


inactive
signal
active
small
molecules
sgRNA
target DNA
(d)Cas9
(d)Cas9