# A Split Cas9 Architecture for Inducible Genome Editing and Transcription Modulation

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## To the Editor

The RNA-guided endonuclease Cas9 has been harnessed as a tool for genome editing in mammalian cells[1, 2]. In addition, strategies employing catalytic inactive Cas9 can direct effector proteins to genomic targets[3-5] to achieve transcriptional modulation. Here, we demonstrate that Cas9 can be split into two fragments and rendered chemically inducible by rapamycin sensitive dimerization domains for controlled reassembly to mediate genome editing and transcription modulation.

To develop a split Cas9 system, we identified eleven potential split sites based on a crystal structure of Cas9 in complex with sgRNA and complementary target DNA[6] (**Fig. 1a** and **Supplementary Fig. 1a**). The resulting C- and N-term Cas9 fragments, Cas9(N) and Cas9(C) respectively, were fused to FK506 binding protein 12 (FKBP), and FKBP rapamycin binding (FRB) domains[7] of the mammalian target of rapamycin (mTOR) (**Supplementary Fig. 1b** and **Fig. 1b**), respectively. We tested all split-Cas9 sets by targeting the *EMX1* locus in human embryonic kidney 293FT (HEK293FT) cells. Using the SURVEYOR nuclease assay, we detected insertion/deletion (indels) mutations mediated by all split-Cas9 sets in cells treated with rapamycin. In addition, moderate levels of indels could also be detected in the absence of rapamycin (**Supplementary Fig. 1c-d**). The observed background activity was not due to residual nuclease activity of individual split pieces (data not shown). Using a small set of split-Cas9 lacking dimerization domains we found that Cas9 split fragments can auto-assemble in cells (**Supplementary Fig. 1e-g**), which explained our observed background activity.

After establishing that background activity in the split-Cas9 system is due to spontaneous auto-assembly of Cas9, we hypothesized that keeping each Cas9 fragment spatially separated may reduce background activity[8]. To sequester the Cas9(N)-FRB fragment in the cytoplasm, where it is less likely to dimerize with the nuclear-localized Cas9(C)-FKBP fragment, we replaced the two nuclear localization sequences (NLSs) on Cas9(N)-FRB with a single nuclear export sequence (NES) (Cas9(N)-FRB-NES). In the presence of rapamycin, Cas9(N)-FRB-NES dimerizes with Cas9(C)-FKBP-2xNLS to reconstitute a complete Cas9 protein, which shifts the balance of nuclear trafficking toward nuclear import and allows DNA targeting (**Fig. 1c-d**). We tested our strategy with split-4 and split-5 (**Fig. 1a**) and found that a single NES is sufficient to reduce background activity below the detection limit of the SURVEYOR assay (**Fig. 1e**). Our data show that spatial sequestration of Cas9-FRB/FKBP split fragments inside the cell, combined with rapamycin activated dimerization, allows for inducible activation of the Cas9 nuclease.

High dosage of Cas9 can exacerbate indel frequencies at off-target (OT) sequences[9]. We speculated that induction of low levels of Cas9 activity could be used to reduced off-target indels compared to constitutively active Cas9, which may exhibit high on-target activity but also elevated levels of off-target activity. Therefore we generated a lentivirus construct for split-5 (LSC-5 for lenti split-Cas9 split-5) (**Fig. 1f**) and transduced HEK293FT cells with an MOI of   0.3 followed by puromycin selection for 5 days.

DNA from wtCas9 transduced HEK293FT cells were analyzed by deep sequencing 4 weeks after transduction, whereas those from LSC-5 transduced were analyzed after 6 weeks, to account for 12 days of consecutive treatment with 200 nM rapamycin (**Fig. 1g**). In cells transduced with a lentivirus carrying both wt-Cas9 and a *EMX1*-targeting sgRNA, we detected ~95% indels at the on-target site as well as mutations at four validated off-target sites (OT-1 to 4). In comparison, on-target indel frequency in cells transduced with LSC-5 was ~43% after 12 days of rapamycin treatment. In untreated cells, no significant difference in *EMX1* on-target indels could be detected between LSC-5 and control samples. Furthermore, no significant increase in OT indels could be detected in cells transduced with LSC-5 regardless of rapamycin treatment (one-way ANOVA, p>0.9999). Transient transfection experiments using the same guide and wt-Cas9 showed that at 32-50% on-target mutation rate, indels at OT-4 were between 10-20% (**Supplementary Table 1**). Taken together, these data demonstrate that stable, low copy expression of split Cas9 can be used to induce substantial indels at a targeted locus without high mutation at off-target sites.

Next we sought to explore whether the split-Cas9 architecture can be applied to catalytically inactive Cas9 (dCas9) to mediate inducible transcription activation. We cloned split-4 fragments harboring a D10A point mutation in the FRB fusion (dCas9(N)-FRB-2xNES) and a N863A point mutation in the FKBP fusion and added a VP64 transactivation domain to Cas9(C)-FKBP-2xNLS (dCas9(C)-FKBP-2xNES-VP64) (**Fig. 2a**). These fragments reconstitute a catalytically inactive Cas9-VP64 fusion (dCas9-VP64).

We tested split dCas9-VP64 by activating *ASCL1*, *MYOD1* or *IL1RN* transcription in HEK293FT cells, using four previously validated sgRNAs[10] per gene. Cells were treated with rapamycin 24 hours after transfection and maintained in 200nM rapamycin until

harvested for RNA at 48 hours after transfection. Significant increase in mRNA levels, compared to untransfected HEK293FT, could be detected using quantitative real-time PCR (qPCR) for all three genes (one-way ANOVA, *ASCL1* $p<0.0001$, *MYOD1* $p<0.0001$, *IL1RN* $p<0.0001$) (**Fig. 2b** and **Supplement figure 2a**). Background activity was low compared to rapamycin induced cells (+rapamycin/−rapamycin ratio, *ASCL1* = 57, *MYOD1* = 27, *IL1RN* = 552) and not significant compared to untransfected cells (one-way ANOVA, $p>0.99$).

To test whether transcriptional activation is reversible upon withdrawal of rapamycin, we activated *Neurog2* expression in N2A cells and *ASCL1* in HEK293FT cells (**Fig. 2c and Supplement figure 2b**). Cells were treated with rapamycin 24 hours after transfection. Rapamycin was either withdrawn after 2 hours or replaced every 24 hours for continual induction. Cells were harvested at 2, 6, 12, 24 and 72 hours post rapamycin treatment and mRNA levels were analyzed by qPCR. *Neurog2* and *ASCL1* levels increased during the entire study with no significant difference between continual rapamycin treatment and a 2-hour treatment (correlation coefficient, *Neurog2* = 1, *ASCL1* = 1). Given the persistent activation after rapamycin withdrawal, this system will be useful for experiments where synchronized activation is beneficial, such as cellular differentiation or development, or modulation of genes that adversely affect the health of growth of the cell.

Taken together, we have demonstrated that Cas9 can be split into two distinct fragments, which reconstitute a functional full-length Cas9 nuclease when brought back together by chemical induction. The split Cas9 architecture will be useful for a variety of applications. For example, split Cas9 may enable genetic strategies for restricting Cas9 activity to intersections of cell populations by putting each fragment under a different tissue specific promoter. Additionally, different chemically inducible dimerization domains, such as abscisic acid or gibberalin sensing domains, may also be employed to generate an array of inducible Cas9 molecules fused to different modulatory domains to construct synthetic transcriptional networks.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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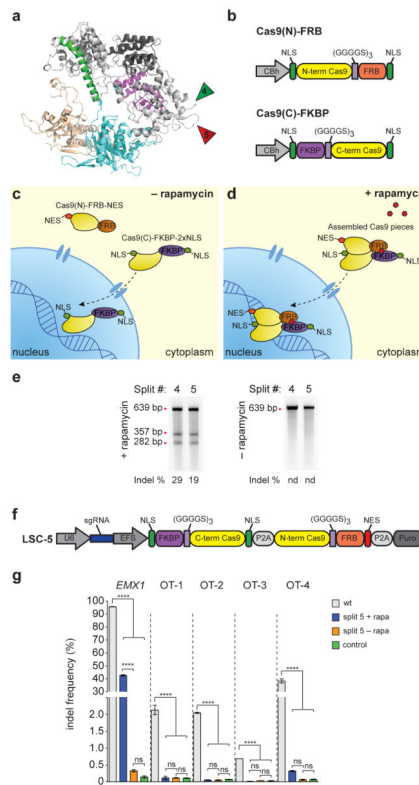
**Figure 1. Generation and optimization of inducible split-Cas9**
(**a**) Ribbon representation of Cas9. Triangles indicate split sites for split-4 (green) and split-5 (red) (**b**) Diagram of inducible split Cas9 fusions. N- and C-term pieces of human codon-optimized *S.pyogenes* Cas9 are fused to FRB and FKBP dimerization domains, respectively. (**c** and **d**) Strategy for optimizing the split Cas9 system. In the absence of rapamycin (**c**), the Cas9(N)-FRB-NES piece is sequestered in the cytoplasm due to the addition of an NES. The Cas9(C)-FKBP piece contains two NLSs and is actively imported into the nucleus. In the presence of rapamycin (**d**), Cas9(N)-FRB-NES binds to Cas9(C)-FKBP. NLSs of the resulting reassembled Cas9 mediate nuclear importation and subsequent binding to the targeted locus. (**e**) Representative SURVEYOR assay for split-4 and -5 mediated indels at the human *EMX1* locus, with (left) and without (right) rapamycin. Arrowheads indicate expected SURVEYOR fragments. Nd = not detected (**f**) Schematic of lentiviral split Cas9 plasmid containing U6 promoter-driven sgRNA, EFS promoter-driven split Cas9 pieces and puromycin resistance gene (puro). 2A self-cleaving peptides (P2A) separate both split Cas9 pieces and puro. (**g**) Indel frequencies measured by deep sequencing at the *EMX1* locus and four annotated OT. Indels were measured 4 weeks (wt-Cas9; n=2 biological replicates) or 6 weeks (split Cas9; n=3 biological replicates) after transduction (****p<0.0001). Rapamycin treatments lasted 12 days. Mean ± s.e.m. in all panels.
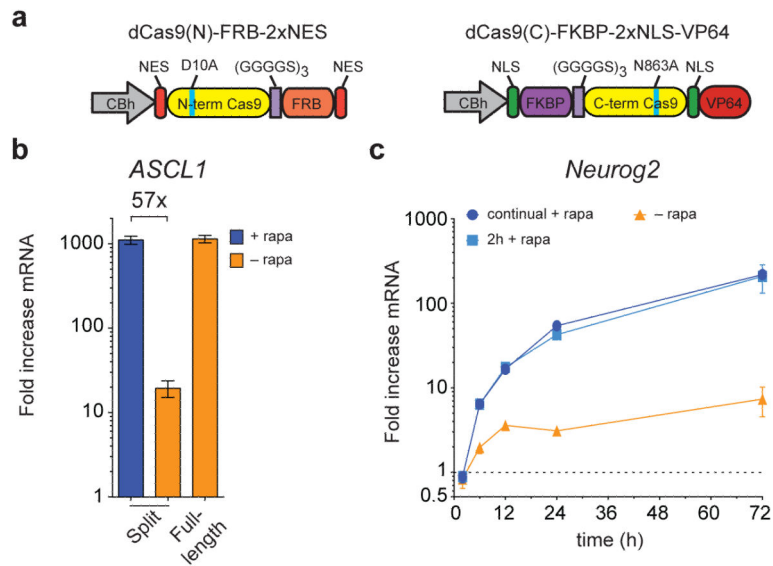
**Figure 2. Inducible transcriptional activation using split dCas9-VP64 fusions**
(**a**) Schematic of dCas9(N)-FRB-2xNES and dCas9(C)-FKBP-2xNLS-VP64 fusions used for transcriptional activation. Each piece harbors an annotated point mutation (D10A or N863A), which reconstitutes a dead Cas9 upon rapamycin-induced assembly. A VP64 transcriptional activator domain is fused to the C-term end of the dCas9(C)-FKBP-2xNLS-VP64 piece. (**b**) *ASCL1* expression measured by qPCR in HEK293FT cells transfected with split-4 (Split) and four sgRNAs per gene. Expression was measured in cells with and without rapamycin (n=4 biological replicates), compared to full-length dead Cas9-VP64 (full-length) (n=3 biological replicates). Untransfected cells were used as baseline. (**c**) *Neurog2* expression in N2A cells measured by qPCR 2, 6, 12, 24 and 72 hours after rapamycin treatment (n=3 biological replicates for each time point). Cells were treated continual with rapamycin (dark blue circles), only treated for 2 hours (light blue squares) or untreated (orange triangles). Untransfected cell were used as baseline. Mean ± s.e.m. in all panels.