**LETTER**

*In vivo* tunable CRISPR mediates efficient somatic mutagenesis to generate tumor models

Dear Editor,

CRISPR/Cas9 has revolutionized genome editing technology due to its simplicity and robustness (Mali et al., 2013). Several inducible CRISPR/Cas9 systems recently developed make spatiotemporal genome editing possible (Kernemann et al., 2013; Balboa et al., 2015; Dow et al., 2015; Zetsche et al., 2015; Liu et al., 2016; Kleinjan et al., 2017; Maji et al., 2017; Senturk et al., 2017; Lu et al., 2018). However, whether these inducible CRISPR/Cas9 systems can mediate efficient *in vivo* somatic mutagenesis for tumor modeling remains to be tested. Destabilizing domain (DD), a member of the post-translationally inducible elements, can be fused to Cas9 protein to construct an inducible CRISPR/Cas9 system (Senturk et al., 2017). The DD tag guides newly synthesized DD-Cas9 protein to the proteasome for degradation, which can be abrogated by ligand trimethoprim (TMP) (Fig. 1A) (Iwamoto et al., 2010; Sando et al., 2013). Because of the convenience of TMP delivery and no endogenous targets found for TMP in mammals, TMP-inducible Cas9 stabilization would be extremely attractive for achieving post-translationally tunable genome editing *in vivo*.

To obtain an efficient and tightly controlled DD-Cas9, we created five variants with distinct configurations, all on a *piggyBac* transposon plasmid backbone (Fig. 1B). We next determined the activities of these Cas9 variants by targeting three human genes (*TET1*, *TET2* and *TET3*) by T7 Endonuclease I (T7EI) assay (Figs. 1C, 1D and S1). In the presence or absence of TMP, no insertion/deletion (indel) was detected in the non-transfected (NT) control group (Fig. 1D). Among these five variants, F5D (DD fused to N-terminal of Cas9 protein) displayed significantly higher activity than other variants when TMP was added, but demonstrated no activity in the absence of TMP (Fig. 1D). Hence, the F5D construct was used in all subsequent experiments. To optimize the working concentration of TMP, we examined Cas9 protein expression from F5D under eight TMP concentrations. At 0.01 μmol/L TMP, stabilized Cas9 protein started to show up. As TMP concentration increased, Cas9 protein level increased gradually as well and reached a plateau at 1 μmol/L TMP (Fig. 1E). Therefore, 1 μmol/L TMP was used in all subsequent *in vitro* studies. Finally, we assayed the kinetics of DD mediated protein degradation. Cells were treated with TMP for 1, 2, 6, 12, 24, 48, 72 h and harvested for Cas9 detection by Western blot. As soon as 2 h after TMP treatment, rapid Cas9 stabilization was detected and became undetectable 12 h after TMP withdrawal (Fig. 1F). In summary, these results demonstrated that F5D is highly efficient for *in vitro* genome editing and the DD-Cas9 is tightly controlled by TMP at the protein level.

Our previous work demonstrated that tail vein injection of sgRNAs for *Cdkn2a* and *Trp53*, an hNRAS<sup>G12V</sup> overexpression plasmid, along with Cas9 expression plasmid achieved almost 100% liver tumorigenesis in mice (Xu et al., 2017). To determine whether DD-Cas9 works *in vivo* in a tunable manner, 26 mice were randomly separated into four groups and injected through tail vein with different plasmid combination (Table 1 and Fig. S2) to induce tumors (Fig. 2A). When examined six weeks after injection, mice in group A (no F5D, no TMP), group B (no F5D, +TMP) and group C (F5D, no TMP) were all tumor-free (Fig. 2B). In group D (F5D, +TMP), liver tumors of various sizes with strong GFP fluorescence were found in eight of ten injected mice (Fig. 2B). These data suggested that F5D could induce tumors in a tunable manner as anticipated. Next, we performed T7EI assay and confirmed the successful gene targeting of both *Cdkn2a* and *Trp53* in tumor tissues (Figs. 2C and S2B). In addition, TA-cloning and Sanger sequencing confirmed indel mutations at the two genomic loci (Fig. 2D). Finally, we carried out histological analysis and proliferation detection via H&E and Ki67 staining respectively. Compared with normal liver sections, tumors induced showed a tubular growth morphology and were highly proliferative (Fig. 2E). Taken together, these results indicated that DD-Cas9 can inducibly mediate efficient liver tumorigenesis in adult mice, and DD-Cas9 can be exploited *in vivo* to engineer endogenous genes in an inducible manner.

TMP is inexpensive, non-toxic and can penetrate the placental barrier and the blood-brain barrier. All these advantages make TMP ideal to stabilize DD tagged proteins *in vivo* (Iwamoto et al., 2010). In the current study, we fused...
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**A**

![Diagram showing the tunability of CRISPR by adding or removing TMP.]

**B**

PB3

PB5

S9

3D

F5D

5FD

F53D

5F3D

**C**

**TET1**

| Condition | Indel (%) |
|-----------|-----------|
| L         | 0         |
| NT        | 0         |
| S9        | 0         |
| 3D        | 0         |
| F5D       | 0         |
| 5FD       | 0         |
| F53D      | 0         |
| 5F3D      | 0         |

**D**

**TET1**

| Condition | Indel (%) |
|-----------|-----------|
| NT        | 0         |
| S9        | 0         |
| 3D        | 0         |
| F5D       | 0         |
| 5FD       | 0         |
| F53D      | 0         |
| 5F3D      | 0         |

**TET2**

| Condition | Indel (%) |
|-----------|-----------|
| NT        | 0         |
| S9        | 0         |
| 3D        | 0         |
| F5D       | 0         |
| 5FD       | 0         |
| F53D      | 0         |
| 5F3D      | 0         |

**TET3**

| Condition | Indel (%) |
|-----------|-----------|
| NT        | 0         |
| S9        | 0         |
| 3D        | 0         |
| F5D       | 0         |
| 5FD       | 0         |
| F53D      | 0         |
| 5F3D      | 0         |

**E**

**F**

| Condition | Anti-Flag | Anti-β-Tubulin |
|-----------|-----------|----------------|
| NT        | 0         | 0              |
| 0.01      | 0         | 0              |
| 0.1       | 0         | 0              |
| 0.5       | 0         | 0              |
| 1         | 0         | 0              |
| 10        | 0         | 0              |
| 100       | 0         | 0              |
| 500       | 0         | 0              |

| Condition | Relative protein levels (β-Tubulin) |
|-----------|-------------------------------------|
| NT        | 0.0                                 |
| 0         | 0.0                                 |
| 1         | 0.0                                 |
| 6         | 1.0                                 |
| 12        | 1.5                                 |
| 24        | 1.5                                 |
| 48        | 1.5                                 |
| 72        | 1.0                                 |
| -2        | 0.5                                 |
| -6        | 0.0                                 |
| -12       | 0.0                                 |
| -24       | 0.0                                 |

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DD to different domains of Cas9 to form five variants with the purpose of tuning Cas9 expression at the protein level. Although F5D met our demands well both in vitro and in vivo, more configurations could be tested for other purposes. In conclusion, our successful in vivo delivery of an inducible CRISPR-Cas9 may provide a foundation for future safer gene therapy.

**FOOTNOTES**

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A

Trp53-sgRNA
Cdkn2a-sgRNA-hNRAS\textsuperscript{G12V}
CAG-PBase

Tumor induction
No F5D, No TMP
No F5D, +TMP
F5D, No TMP
F5D, +TMP

Analysis

B

No F5D, No TMP
No F5D, +TMP
F5D, No TMP
F5D, +TMP

BF

GFP

C

| Cdkn2a | Trp53 |
|--------|-------|
| wt     | wt    |
| tu     | tu    |

500 bp

200 bp

Indel (%)

0 41 0 25

D

Cdkn2a

wt : GCGCTGCCTGCTGACCCGGCAGAGG
mut1 : GCGCTGCCTGCTGACCCGGCAGAGG -1 bp
mut2 : GCGCTGCCTGCTGACCCGGCAGAGG -5 bp

Trp53

wt : GCTCGATGGTGGTGGGATGGCTACAC
mut1 : GCTCGATGGTGGTGGGATGGCTACAC -1 bp

E

No F5D, No TMP
No F5D, +TMP
F5D, No TMP
F5D, +TMP

H&E

Ki67
Sen Wu, Xiaomeng An and Linlin Li conceived and designed the experiments. Xiaomeng An performed the experiments. Xiaomeng An, Linlin Li and Sen Wu wrote the paper. Xiaomeng An and Linlin Li contributed equally to this article.

Xiaomeng An, Linlin Li, and Sen Wu declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

Xiaomeng An¹, Linlin Li², Sen Wu²*

¹ State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, China
² State Key Laboratory of Veterinary Etiological Biology, National Foot and Mouth Diseases Reference Laboratory, Key Laboratory of Animal Virology of Ministry of Agriculture, Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou 730000, China

Correspondence: swu@cau.edu.cn (S. Wu)

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