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☐ Initial submission ☐ Revised version ☒ Final submission

Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

Experimental design

1. Sample size

Describe how sample size was determined.

We routinely do 3 biological replicate experiments and all experiments shown in this manuscript contain at least 3 replicates. No power analyses were done a priori.

2. Data exclusions

Describe any data exclusions.

There was only one data exclusion (experiment in Figure 4b). It was determined in the experiment that the i53 expression was unusually low due to low viral titer so one set of datapoint was excluded.

3. Replication

Describe whether the experimental findings were reliably reproduced.

The core assertion of this manuscript is that i53 is a robust and versatile tool for HDR-based genome editing. In the revised manuscript, we now have 3 independent groups confirming this finding.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

There was no randomization in this study.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

There was no data blinding.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☒	☐	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	A statement indicating how many times each experiment was replicated
☒	☐	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☒	☐	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☒	☐	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	GraphPad Prism v7, Origin 5.0 software (Microcal), ImageQuant, FlowJo v10, Phenix Suite.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	All relevant plasmids are already deposited at Addgene. Accession numbers are indicated in the Methods section.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	We detail the source of the antibodies in the Methods section. In all cases, the antibodies have a long history of being used either by our group (e.g. 53BP1, Flag, H2AX, HA and GFP antibodies).

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	All human cell lines original from ATCC or are derivatives of ATCC.
b. Describe the method of cell line authentication used.	We routinely authenticate our cell lines with STR analysis. Genetic knockouts were validated by genomic sequencing and using antibodies
c. Report whether the cell lines were tested for mycoplasma contamination.	Cell lines are routinely tested for mycoplasma contamination.
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	N/A

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	N/A

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	N/A

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Flow Cytometry Reporting Summary

Form fields will expand as needed. Please do not leave fields blank.

Data presentation

For all flow cytometry data, confirm that:

☒ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ 3. All plots are contour plots with outliers or pseudocolor plots.

☒ 4. A numerical value for number of cells or percentage (with statistics) is provided.

Methodological details

5. Describe the sample preparation.	For all DR-GFP, BFP-to-GFP, and H2B-tagging assays, cells were collected from tissue culture plates by trypsinzation, diluted into a larger volume of PBS with 10% FBS, then sorted immediately. For the Hsp90a1 targeting assays, cells were trypsinized and was with cold PBS once. Then cells were resuspended in cold PBS with Ca, Mg and 3% FBS.
6. Identify the instrument used for data collection.	BD Biosciences FACSCalibur, BD Biosciences Fortessa, and BD Biosciences Fortessa X20-HTS. For the Hsp90a1 targeting assays, BD™ LSRII HTS cell analyzer
7. Describe the software used to collect and analyze the flow cytometry data.	BD FACSDiva for data collection and FlowJo version 10 for analysis
8. Describe the abundance of the relevant cell populations within post-sort fractions.	N/A
9. Describe the gating strategy used.	For all DR-GFP, BFP-to-GFP, and H2B-tagging assays, live cells were first gated from the FSC/SSC plots, with events of very low or very high FSC and/or SSC being excluded. The live cell fraction constituted >80% of total events. For the DR-GFP assay, the boundary between GFP-negative and GFP-positive was drawn from the plots of control samples where cells hadn't been transfected with IsceI. For examples of how boundaries between "positive" and "negative" cells were drawn in the H2B-tagging assay, please see Supplemental Figure 5c. For examples of how boundaries between "positive" and "negative" cells were drawn in the BFP-to-GFP assay, please see Supplemental Figures 7c and 8a. For the ZsGreen Hsp90a1 targeting assay, live cells were gated from the FSC/SSC plots to exclude events of very low or very high FSC and/or SSC. The fraction constituted >80% of total events. The boundary between ZsGreen-negative and ZsGreen-positive was drawn according to control samples that were not transfected. FITC voltage was established in order

to confine the negative population below 10*3 and positive population no further than 10*5

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☐