Revisiting the effects of Cas9 on p53-inactivating mutations reveals sex-biased genome editing by CRISPR-Cas9

Mengbiao Guo[1,*], Yuanyan Xiong[1,*]

[1] Key Laboratory of Gene Engineering of the Ministry of Education, Institute of Healthy Aging Research, School of Life Sciences, Sun Yat-sen University, Guangzhou 510006, China.

[*] To whom correspondence should be addressed.

guomb3@mail.sysu.edu.cn, Tel: +86-20-39943419, Fax: +86-20-39943778

xyyan@mail.sysu.edu.cn, Tel: +86-20-39943531, Fax: +86-20-39943778

Abstract

Enache *et al.* report that Cas9 alone can activate the p53 pathway and select for p53-inactivating mutations. We reanalyzed *TP53* mutations in Parental-cells (no introduction of Cas9) and Cas9-cells (Cas9-expressing derivatives) from the Enache *et al.* study and found significantly shrinking inactivating subclonal mutations of *TP53* in Cas9-cells, which means Cas9 also selects against *TP53*-inactivating mutations. We also examined the sex-biased effect of Cas9, which was implied in their results but was not noticed and discussed by Enache *et al.* We further highlighted the sex bias in large-scale CRISPR-Cas9 screening experiments in various cell lineages. We suggest the sex bias effect may need to be considered in future clinical applications using CRISPR-Cas9.

Introduction

Enache *et al.*[1] have recently presented an interesting and important study demonstrating that the protein Cas9 (CRISPR-associated protein 9, commonly used for genome editing in the CRISPR-Cas9 system) alone can activate the p53 pathway and specifically select for p53-inactivated mutants. The CRISPR-Cas9 system was known to interact with p53[2-5]. In their study, they profiled 165 pairs of human cancer cell lines without treatment (termed Parental-cells) or with Cas9 expression (termed Cas9-cells) and found non-silent *TP53* mutations emerged only in Cas9-cells but not in Parental-cells (Fig. 3c in ref.[1]). They further showed that

ten inactivating subclonal mutations of *TP53* in eight cell lines showed significant expansion (Fig. 3d in ref.[1]), but without mentioning inactivating mutations shrinking in Cas9-cells. They emphasized that new non-silent *TP53* mutations only emerged in Cas9-cells and *TP53*-inactivating subclonal mutations tended to expand in Cas9-cells.

We think this message is questionable after reanalyzing *TP53* somatic mutations from their exome sequencing experiments (see **Methods**). We observed that four inactivating mutations from four cell lines were significantly shrinking (**Fig. 1a**, P= 0.039), including one that was instead shown as expanding by Enache *et al* (786O-I, Fig. 3d in ref.[1]). Therefore, we think Cas9 not only selected for some p53-inactivating mutations but also against other inactivating mutations. Based on our observation and given the small number of cell lines with emerging non-silent *TP53* mutations in Cas9-cells, it seems possible that some *TP53*-inactivating mutations disappeared in Cas9-cells but were not detected. Moreover, other possibilities of *TP53* aberrations cannot be ruled out, for example, changes in DNA methylation or transcriptional regulators binding in the *TP53* promoter[6-8]. Therefore, even for cell lines without p53 activation after Cas9 introduction, *TP53* status may also need to be monitored after Cas9 introduction (workflow shown in Extended Data Fig. 5 in ref.[1]).

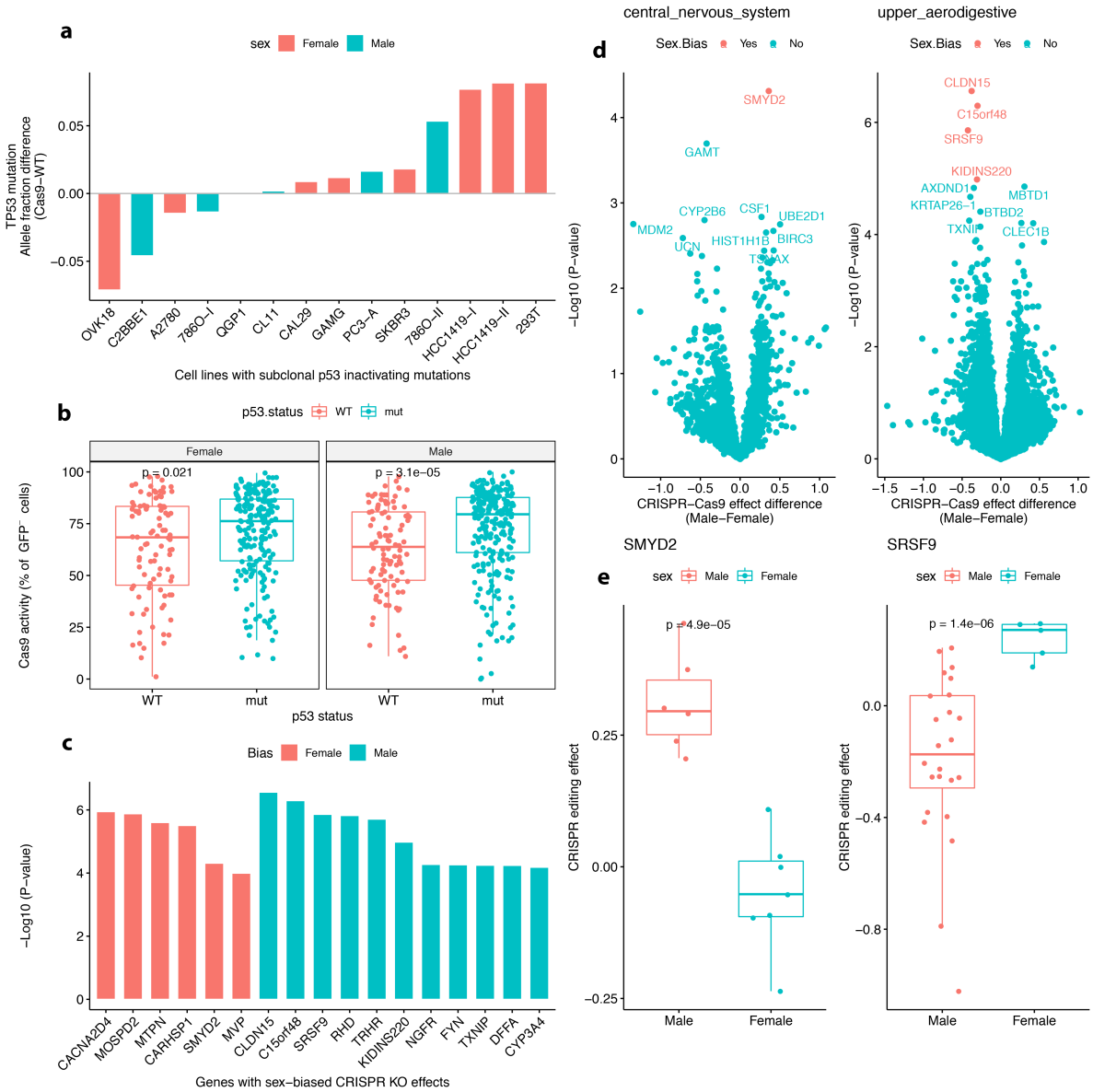

Fig.1 Bidirectional changes of *TP53*-inactivating subclonal mutations in Cas9-cells and sex-biased KO effects of CRISPR-Cas9. (**a**) Cas9 both selects for (*P*= 0.0050) and against (*P*= 0.039) *TP53*-inactivating subclonal mutations, with the largest changes found in females. Two-sided paired t-test. (**b**) The signal of Cas9 activity difference between p53-WT and p53-MUT was mainly from male cell lines (right, *P*=3.1E-5) and barely from female cell lines (left, *P*=0.021). In females, the difference was only marginally significant. This is related to Fig. 5a in the study by Enache *et al.* Bar, median; box, 25th and 75th percentile; whiskers, 1.5 times the interquartile range of the lower and upper quartiles; points, individual cell lines. Two-sided Wilcoxon-test. (**c**) Genes from various cell lineages showing sex-biased KO effects in

CRISPR-Cas9 screening. (**d**) Volcano plots showing significantly biased KO effects in both sexes in the central nervous system (left, p53-WT) and upper areodigestive cells (right, p53-MUT). The ten most promising genes to show sex bias, ranked by nominal P-values, were labeled. (**e**) Boxplots showing two gene examples of significantly sex-biased KO effects in (**d**). *SMYD2* in the central nervous system (*P*=4.9E-5, left, p53-WT) and *SRSF9* in the upper areodigestive cells (*P*=1.4E-6, right, p53-MUT). Two-sided t-test.

Results and Discussion

Interestingly, we found that the largest effects of many results shown by Enache *et al.* were from female cells, including the largest fold-change of p53 activation (BT159: female, Fig. 1e in ref.[1]), more DNA damage foci (MCF7: female, Fig. 2 in ref.[1]), and the largest *TP53*-inactivating subclonal mutations expanding or shrinking (293T, HCC1419, and OVK18: female, **Fig. 1a**). On the other hand, both emerging non-silent *TP53* mutations in Cas9-cells were from male cells (JHH7 and SNU1, Fig. 3c in ref.[1]). More importantly, the signal of Cas9 activity difference between p53-WT and p53-MUT cells (Fig. 5a in ref.[1]) was largely from males (*P*=3.1E-5) and barely from females (*P*=0.021) (**Fig. 1b**).

This may not be a coincidence, and possibly reflects the sex-biased effects of Cas9 due to the differential functions and regulations of p53 between sexes. According to two recently published studies, p53 plays a key role in chromosome X inactivation by enhancing Xist transcription during neural tube development in females[9], and is differentially regulated in both healthy and cancer samples between females and males[10]. Our recent work also shows that p53 regulates the active X chromosome dosage compensation, but with different effect sizes between sexes[11]. Importantly, some vital genomic regulators interacting with p53 are encoded by genes on chromosome X[10].

Due to the potentially large-scale clinical usage of CRISPR-Cas9 in the future in patients with various diseases, sex-specific effects should be studied carefully and considered beforehand. However, we have found no report discussing sex-biased CRISPR-Cas9 knockout (KO) effects to date. We thus briefly investigated the potential sex bias in the CRISPR screening data from the DepMap[12] project (20Q2, see **Methods**). We obtained a list of 17 genes (seven with protein-protein interaction (PPI) with *TP53*[13], **Fig. 1c, Supplementary Fig. S1-S4, Supplementary Table S1**) showing significant sex-biased effects by comparing cell lines from males and

114 females (see **Methods**). These genes include *SMYD2* (brain cells, *P*=4.9E-5), a protein
115 methyltransferase of *TP53*, and *SRSF9* (upper aerodigestive cells, *P*=1.4E-6), a critical splicing
116 factor for mRNA (**Fig. 1d,e**). Only *MOSPD2* is located on the chromosome X. We found sex-
117 biased KO effects in both p53-WT and p53-MUT cells, but not for *TP53* (however, note that
118 its negative regulator *MDM2* in brain cells had a *P*-value of 0.0018, which may help explain
119 their p53 activation results in brain tumor BT159, **Fig. 1d**), possibly due to its complex
120 regulatory network, or due to the difference between introduction of Cas9 alone and together
121 with guide RNAs. We also noticed that many genes had good nominal significance, although
122 they did not pass the multiple-test correction due to possibly small effect sizes and the heavy
123 multiple-test burden (**Supplementary Fig. S1-S4**).
124
125 We think the possible sex-biased effects of Cas9 may provide a possible reason for their failure
126 to detect p53 activation in Cas9-expressing HCT116 (male) cells. For the cell competition
127 (HCT116, male) and drug response (MCF7, female) assays in the study by Enache *et al.*, it
128 would be interesting to know if the effects would be stronger or weaker by using cell lines from
129 the other sex.
130
131 Collectively, we do not question the importance of the study by Enache *et al.*, but they may
132 consider further improving their results related to our observations. Our analysis may also
133 provide advices for future CRISPR screening experiments.
134
135 **Methods**
136
137 <u>Reanalysis of exome sequencing data</u>
138
139 First, by following their variant filtering strategy, two subclonal inactivating mutations from
140 two cell lines (including 786O-I or p.P278A showing expansion in Fig. 3d in ref.1 by Enache
141 *et al.*) were shrinking in Cas9-cells. Second, we examined the allele frequency (AF)
142 requirement of 'AF<0.48 or AF>0.52' in Parental-cells, which should be meant to exclude the
143 possibility that an observed somatic mutation in a cell line may simply be the same
144 heterozygous SNV in all Parental-cells. We retained these somatic mutations if 'AF<0.48 or
145 AF>0.52' was met in either Parental-cells or Cas9-cells, with the same other requirements.
146 Three inactivating mutations from three cell lines were shrinking. Third, we examined the
147 coverages of inactivating somatic mutations of *TP53*, which showed a minimal coverage of 63

148 reads (since Enache *et al*. used 2% as the minimal AF threshold, 2% means one read minimal)
149 and that most mutations (86%) were covered by >=100 reads and about half (46%) by >=200
150 reads (**Supplementary Fig. S5**). It means lowering the 2% cutoff to 1% probably will also
151 work for most mutations. After using 1% as cutoff, we observed that four inactivating
152 mutations from four cell lines were shrinking (P=0.039).
153
154 Analysis of sex-biased KO effects for CRISPR-Cas9 screening
155
156 We obtained the gene editing effects data for CRISPR screening, gene mutations from Cancer
157 Cell Line Encyclopedia (CCLE), and all cell line information from the DepMap project (20Q2,
158 https://depmap.org/portal/). Cells were separated into two groups by p53 status, p53-WT and
159 p53-MUT (with p53 non-silent mutations as defined in the Enache *et al* study). For both groups,
160 cell lineages with >=10 samples (at least 3 samples for both males and females) were retained
161 for downstream analysis. First, we tested the sex-biased KO effects in each cell lineage for a
162 list of 2,007 genes interacting with *TP53*, obtained from the PPI database STRING
163 (https://string-db.org/) by requiring interaction confidence scores larger than 400. Then, we
164 extended the test to all genes screened by DepMap. Differential KO effects were compared by
165 two-sided t-test and adjusted P-values smaller than 0.2 were considered significant. All analysis
166 and visualizations were performed using R language (http://www.r-project.org/). Number of
167 *TP53*-WT cell lines available: lung (n=18), central_nervous_system (n=17), skin (n=34),
168 colorectal (n=12), soft_tissue (n=20), kidney (n=14), peripheral_nervous_system (n=13).
169 Number of *TP53*-MUT cell lines available: lung (n=88), central_nervous_system (n=43), skin
170 (n=20), blood (n=29), colorectal (n=24), soft_tissue (n=16), pancreas (n=30),
171 upper_aerodigestive (n=30), urinary_tract (n=24), bile_duct (n=22), bone (n=15), gastric
172 (n=20), esophagus (n=25), plasma_cell (n=14).
173
174 **Data availability**
175 All data sets are available within the article or its Supplementary Information. DepMap data
176 were downloaded from https://depmap.org/. Protein-protein interactions with *TP53* were
177 downloaded from STRING (http://string-db.org/).
178
179 **Code availability**
180 All of the code used to generate and/or analyze the data is publicly available.
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Author contributions

MBG conceived the idea, performed the analysis, and drafted the manuscript. YYX provided writing input. Both authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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