Supplementary Information

Cas9 activates the p53 pathway and selects for p53-inactivating mutations

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Supplementary Note 1

CRISPR-Cas9 editing is often performed in two steps: first, a stable Cas9-expressing cell line is generated; then, single guide RNA (sgRNA) is introduced. Both of these steps involve several events that could potentially lead to genomic evolution, including the transduction, passaging and antibiotic selection of cells. As the Cas9-expressing cell line is often used as a control to its gene-knockout counterpart, genetic or transcriptional alterations that accumulated in the Cas9-expressing cell line prior to sgRNA introduction would likely go unnoticed.

Supplementary Note 2

Of note, in the top 10% of the most transcriptionally-different cell line pairs (17 of 165), a median of 735 genes were differentially expressed by at least two-fold, out of a median of 6,134 expressed genes per cell line (considering only the 10,147 genes included in our transcriptional analysis). Therefore, ~12% of the transcriptome was altered by at least two-fold following Cas9 introduction in these cell lines (**Fig. 1a** and **Supplementary Table 1**).

Supplementary Note 3

The observed p53 pathway upregulation was not associated with Cas9 activity or with the strength of the global effect of Cas9 expression on the transcriptome (**Extended Data Fig. 1g**), indicating that its detection is not merely a byproduct of a stronger overall transcriptional response.

Supplementary Note 4

It was recently reported that p53 inhibits CRISPR-Cas9 genome editing[10-13], but the p53-mediated DNA damage response was detected in the presence of DNA-cutting sgRNAs. Here, we observed that p53 activation could be induced by the expression of Cas9 alone. Moreover, the inhibitory effect of p53 was suggested to be linked to the increased sensitivity of non-transformed cells to double strand breaks[10,11], whereas here we observed p53 activation in cancer cells.

Supplementary Note 5

We note that these differences may have nothing to do with Cas9 *per se*, but may result from cell line diversification due to culture bottlenecks[2]. We repeated the analysis using only the subset of mutations listed as recurrent somatic mutations in the COSMIC database[30], and found that they followed the same trend, with an average of 0.62 mutations appearing, and 0.38 mutations disappearing, in the Cas9 vs. the WT lines (p=0.038; **Extended Data Fig. 4c**).

Supplementary Note 6

We confirmed by visual inspection that the appearance of these mutations was not an artifact of low sequencing coverage or misalignment issues (**Extended Data Fig. 4d**), and found that in SNU1, the mutation pre-existed in the WT line and expanded beyond the calling threshold (2%) in the Cas9 line, whereas in JHH7 no evidence for the mutation was found in the WT line.

Supplementary Note 7

We note that we cannot rule out the possibility that functional mutations in additional genes that are rarely mutated (and therefore not sufficiently represented in our dataset) could also be similarly selected for on Cas9 introduction. However, our results indicate that acquisition of p53-inactivating mutations is the most common selection event in Cas9-expressing cultures.

Supplementary Note 8

We selected these because mutations in these genes were also observed as emerging in Cas9 cell lines (**Fig. 2b**), and because knockout HCT116 cells could be obtained from the same source as the *TP53* knockout cells (**online Methods**).

Supplementary Note 9

Future studies should determine whether nuclease-dead Cas9 and/or other nucleases such as Cas12a are similarly susceptible to p53 activation and mutation.

Supplementary Fig. 1: Example of the gating strategy used in the cell competition experiments.